Predicting Toxicities of Reactive Metabolite–Positive Drug Candidates

Amit S. Kalgutkar[1] and Deepak Dalvie[2]

Pharmacokinetics, Dynamics and Metabolism Department, Pfizer Worldwide Research and Development, [1]Cambridge, Massachusetts 02139 and [2]San Diego, California 92121; email: amit.kalgutkar@pfizer.com, deepak.dalvie@pfizer.com

Annu. Rev. Pharmacol. Toxicol. 2015. 55:35–54

First published online as a Review in Advance on October 6, 2014

The *Annual Review of Pharmacology and Toxicology* is online at pharmtox.annualreviews.org

This article's doi: 10.1146/annurev-pharmtox-010814-124720

Keywords

adverse drug reactions, ADRs, bioactivation, covalent binding, cytochrome P450, electrophile, glutathione, hepatotoxicity, idiosyncratic, liver microsomes, structural alert, precision medicine, prediction in pharmacology

Abstract

Because of the inability to predict and quantify the risk of idiosyncratic adverse drug reactions (IADRs) and because reactive metabolites (RMs) are thought to be responsible for the pathogenesis of some IADRs, the potential for RM formation within new chemical entities is routinely examined with the ultimate goal of eliminating or reducing the liability through iterative design. Likewise, avoidance of structural alerts is almost a standard practice in drug design. However, the perceived safety concerns associated with the use of structural alerts and/or RM screening tools as standalone predictors of toxicity risks may be overexaggerated. Numerous marketed drugs form RMs but do not cause idiosyncratic toxicity. In this review article, we present a critique of the structural alert/RM concept as applied in drug discovery and evaluate the evidence linking structural alerts and RMs to observed toxic effects. Pragmatic risk mitigation strategies to aid the advancement of drug candidates that carry a RM liability are also discussed.

INTRODUCTION

The formation of electrophilic reactive metabolites (RMs) is considered to be an undesirable feature in drug candidates. This notion arises from evidence linking RM liability with mechanism-based inactivation of cytochrome P450 (CYP) isoforms, which can result in clinical drug-drug interactions (DDIs) (1) and/or covalent modification of DNA that results in mutagenicity (2). Furthermore, it is now widely accepted that RMs, as opposed to the parent molecules from which they are derived, can also be responsible for the etiology of some idiosyncratic adverse drug reactions (IADRs) (3–6). The term idiosyncratic simply implies that little is known about the underlying mechanism and that the toxicity is unpredictable. IADRs can manifest in drug-treated patients as rare and sometimes life-threatening reactions that cannot be explained by the primary pharmacology of the drug. For instance, felbamate is used to treat convulsions but can cause aplastic anemia and hepatotoxicity. Many IADRs are immune mediated and occur in very low frequency in a small subset of patients either acutely or as a delayed response. The observations that certain IADRs (e.g., hypersensitivity associated with the antiretroviral agent abacavir, hepatotoxicity associated with the nonsteroidal anti-inflammatory drug lumiracoxib and the antiretroviral drug nevirapine) are linked to specific human leukocyte antigen (HLA) genes (7–9) provide compelling evidence for the immune-mediated nature of these toxicities. The precise mechanisms of IADRs remain unclear; however, the vast majority may be caused by immunogenic conjugates formed via the covalent interaction of a RM with cellular proteins, resulting in cellular dysfunction or an immune response via the formation of a hapten (10). The link between RM formation and drug toxicity first became evident from studies on the hepatotoxic anti-inflammatory agent acetaminophen. Mechanistic studies, which have served as a gold standard for drug toxicity assessment over the decades (11), established the CYP-mediated oxidation of acetaminophen to a reactive quinone-imine species that could deplete levels of the endogenous antioxidant glutathione (GSH) and/or bind covalently to liver biomacromolecules, leading to hepatotoxicity. Idiosyncratic toxicities are, by definition, difficult to reproduce in the human population, and there are few, if any, generally applicable animal models for examining them (12). Consequently, reliably predicting the occurrence of IADRs with new drug candidates represents a significant challenge in preclinical drug discovery and development. Under the basic premise that a molecule devoid of RM formation could mitigate idiosyncratic toxicity risks, in vitro screens [e.g., reactive metabolite trapping with GSH and/or protein covalent binding in NADPH-supplemented human liver microsomes (HLM)] have been implemented to assess CYP-mediated RM formation for new molecular entities with the ultimate goal of minimizing or eliminating this liability through iterative medicinal chemistry (13, 14).

THE STRUCTURAL ALERT/REACTIVE METABOLITE CONCEPT IN DRUG DESIGN: WHAT HAS RETROSPECTIVE STRUCTURE TOXICITY ANALYSIS TAUGHT US?

Because the link between RM formation and idiosyncratic toxicity is not well understood, one tactic frequently adopted in drug discovery is that of avoidance. The term avoidance refers to a philosophical argument wherein certain functional groups (known as structural alerts or toxicophores) must be excluded from drug design, irrespective of whether these substituents would offer pharmacologic (e.g., improved intrinsic potency), pharmacokinetic (e.g., low plasma clearance), and/or biopharmaceutical (e.g., improved aqueous solubility) advantages. The concept of structural alerts (extensively reviewed in Reference 15) originated from studies that characterized the mechanism of RM formation within numerous drugs associated with idiosyncratic toxicity. An analysis of 68 drugs recalled or associated with a black box warning (BBW) for

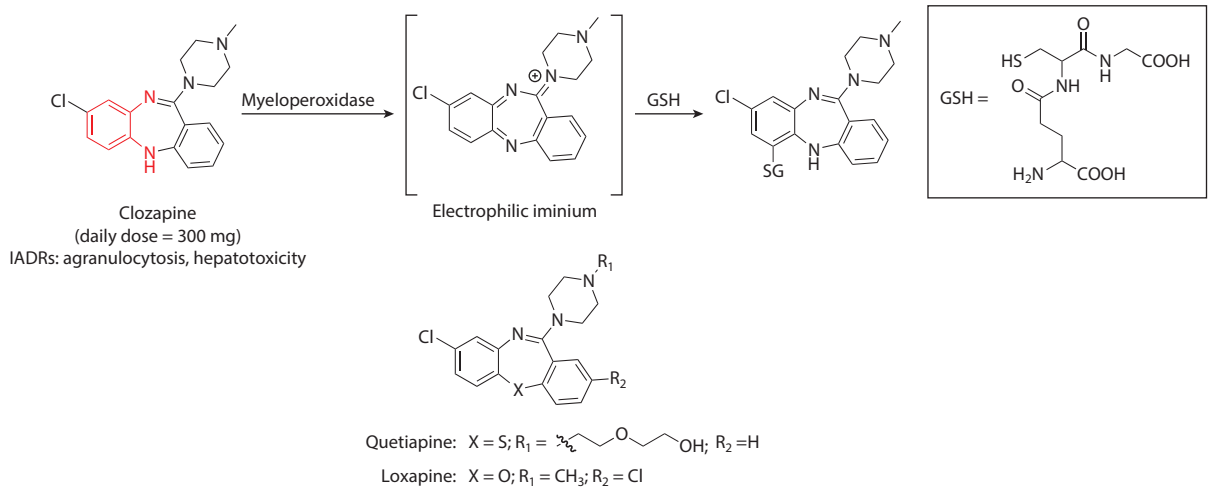


Figure 1

Structure-toxicity relationships for the dibenzodiazepine derivatives: clozapine versus loxapine and quetiapine. Abbreviations: GSH, glutathione; IADR, idiosyncratic adverse drug reaction.

idiosyncratic toxicity indicated that 55 (80.8%) contained one or more structural alerts, and evidence for RM formation [characterization of adducts with biological nucleophiles such as GSH and/or covalent binding to target organ tissue (e.g., liver microsomes)] was provided for 36 out of the 55 drugs (65%) (16). A prominent structural alert in the analysis was the aniline/anilide motif, which was present in ~30 out of the 68 (44%) toxic drugs. Among all known structural alerts, the aniline/anilide motif is perhaps most notorious for its association with mutagenicity, direct organ toxicity, methemoglobinemia, and immunogenic allergenic toxicity (17).

A compelling argument for chemotype-specific toxicity is also evident from structure-activity relationship (SAR) studies, wherein the absence of RM liability is consistent with the improved safety profile of successor drugs. For instance, whereas clozapine use is limited by a high incidence of agranulocytosis and hepatotoxicity, quetiapine and loxapine are not associated with these adverse events. Clozapine exhibits covalent binding to human neutrophils in vitro via the myeloperoxidase-catalyzed oxidation of the dibenzodiazepine ring to a reactive iminium ion, which covalently binds to target tissue and GSH (**Figure 1**) (18, 19). Proteins covalently modified with clozapine have been detected in neutrophils of patients being treated with clozapine; this finding reaffirms the relevance of the in vitro studies (19). In the cases of quetiapine and loxapine, the bridging nitrogen atom is replaced with a sulfur or oxygen atom (**Figure 1**); consequently, these drugs cannot form a reactive iminium species (20). Although anecdotal for the most part, the structure-toxicity relationships suggest that avoiding structural alerts in drug design would lead to therapeutic agents that do not cause IADRs. In fact, knowledge-based systems such as Derek for Windows that are used to predict the toxicity of a chemical from its structure have evolved from such findings. Predictions from knowledge-based systems, however, can be misleading at times. For example, 2-aminopyridine and 2-aminopyrimidine are not predicted to be structural alerts in Derek and are commonly utilized in drug discovery as aniline replacements. Certainly, the 2-aminopyrimidine scaffold found in the anxiolytic agent buspirone is not metabolized to a RM, unlike the aniline derivative and RM-positive hepatotoxin nefazodone (**Figure 2**) (21).

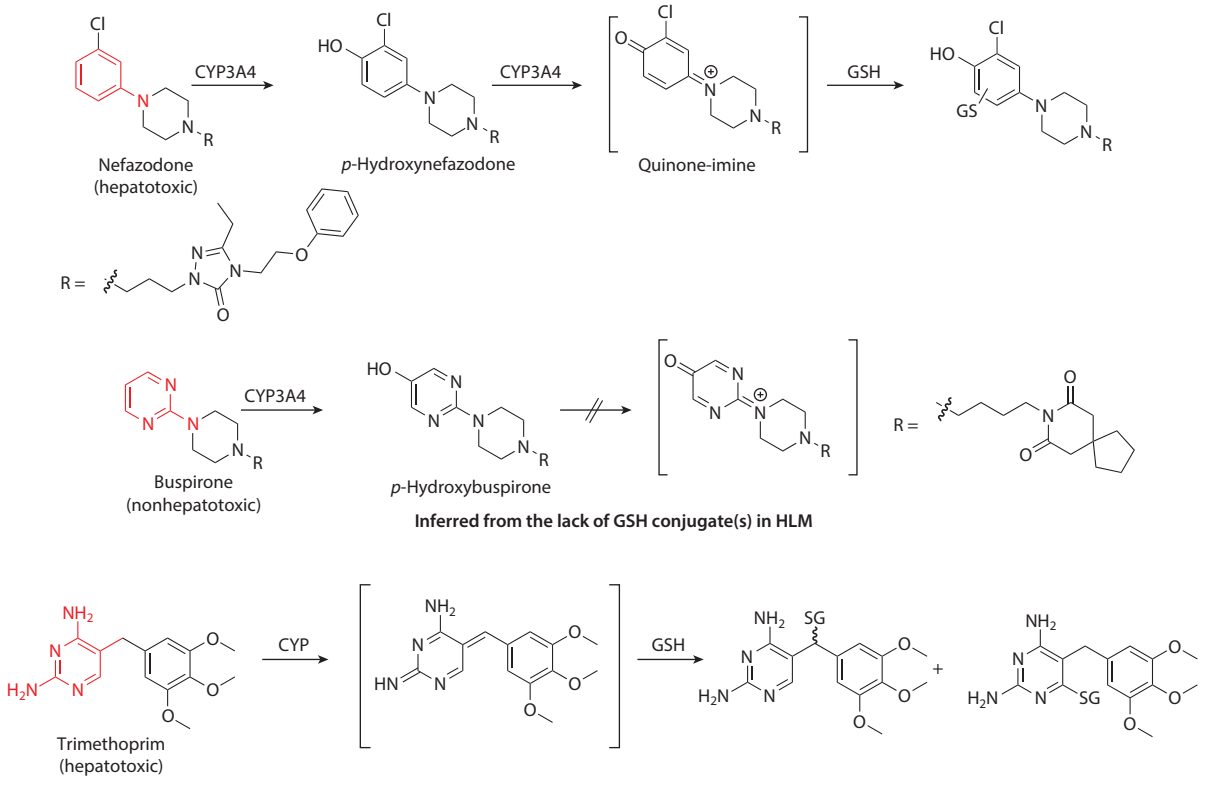


Figure 2

RM formation within aniline and aminopyrimidine structural alerts. The aniline (nefazodone) and aminopyrimidine (buspirone and trimethoprim) alerts are highlighted in red. Abbreviations: CYP, cytochrome P450; GSH, glutathione; HLM, human liver microsomes; RM, reactive metabolite.

However, an exception to the rule is the two-electron oxidation of the 2-aminopyridine group in trimethoprim to an electrophilic imine-methide species, which is a causative factor in the idiosyncratic toxicity associated with this antibacterial drug (**Figure 2**) (22). As novel (and proprietary) functional groups are continuously sought in drug design, unanticipated bioactivated pathways leading to RMs may emerge and thus expand the existing knowledge on structural alerts.

As such, the application of the structural alert concept in drug design has several shortcomings. First, not all compounds possessing structural alerts are metabolized to RMs. The likelihood of RM formation depends on the binding pose of the compound in the catalytic site of the drug-metabolizing enzyme (e.g., CYP) and the subsequent positioning of the structural alert toward metabolism to a RM. Metabolism may occur at a site other than the structural alert and lead to nonreactive products. For example, both sudoxicam and meloxicam (**Figure 3**) contain the 2-aminothiazole structural alert, but only sudoxicam forms the reactive acylthiourea, which appears to be responsible for its hepatotoxicity (23). Although the introduction of a methyl group at the C-5 position on the thiazole ring in meloxicam is the only structural difference, the change dramatically alters the metabolic profile. Oxidation of the C-5 methyl group to the alcohol (and carboxylic acid) metabolites constitutes the major metabolic fate of meloxicam in humans (24). Additionally, alternative routes of drug clearance could also influence the metabolism of structural alerts, as in the cases of ranitidine and pramipexole (**Figure 3**). Both drugs are eliminated by urinary

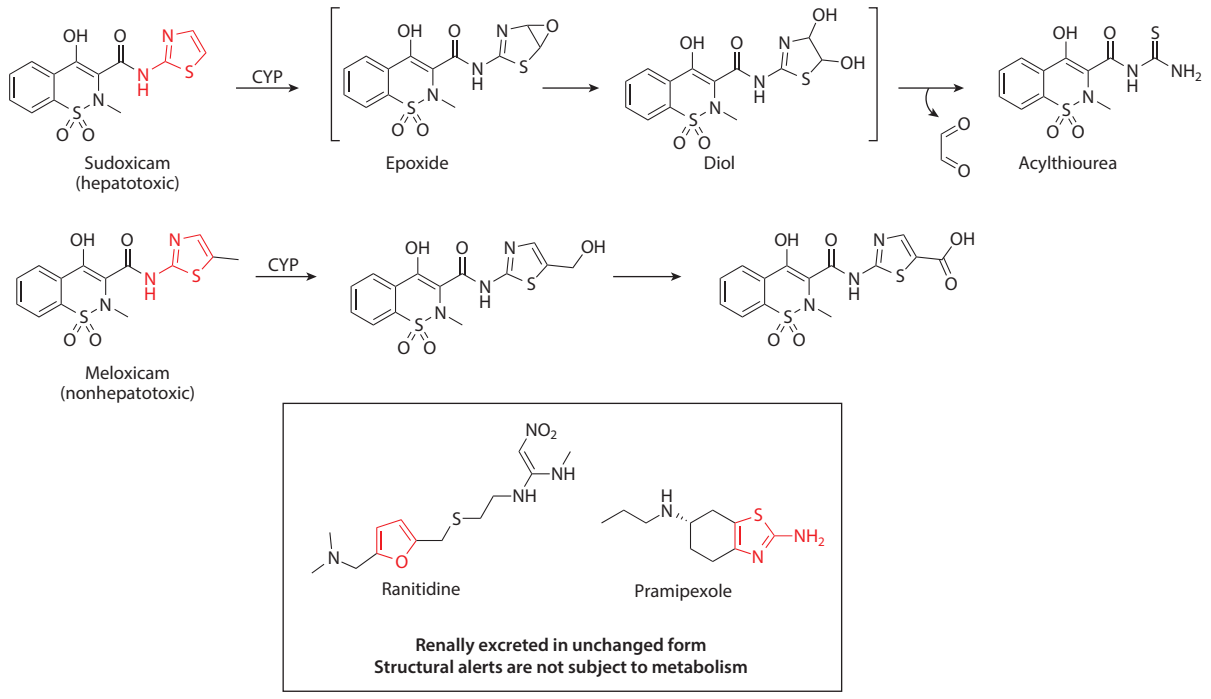


Figure 3

Illustrations of drugs that contain structural alerts but do not form RMs. Structural alerts are highlighted in red. Abbreviations: CYP, cytochrome P450; RM, reactive metabolite.

excretion primarily in unchanged parent form (25, 26), and no RM formation is seen on the furan and 2-aminothiazole structural alerts. Notably, ranitidine and pramipexole are marketed as agents for the treatment of peptic ulcers and Parkinson's disease and generally do not cause IADRs.

Second, because the categorization of structural alerts is knowledge based, avoiding as-yet-unknown structures that can form RMs is not possible. For example, the mechanism for RM formation within the anticonvulsant felbamate, which is associated with aplastic anemia and hepatotoxicity, involves the formation of the electrophilic α,β-unsaturated 2-phenylpropenal via an uncharacteristic multistep process (**Figure 4**) (27). Evidence for the occurrence of this pathway in vivo has arisen from the characterization of urinary mercapturic acid conjugates following felbamate administration to humans (28). As seen in **Figure 4**, felbamate is devoid of prototypical structural alerts.

Third, and more importantly, structural alerts fall into one of two categories: ones that form RMs versus all others. There is no clear distinction as to when a particular functional group is viewed as a structural alert. The vast majority of marketed drugs possess a phenyl ring, which is a structural alert because its biotransformation to the corresponding phenol metabolite proceeds through a reactive epoxide intermediate, which can be trapped with GSH in some cases (15, 16). Removing simple phenyl rings from the repertoire of substituents in drug design is practically impossible, and mankind would be deprived of countless useful therapies if phenyl-containing drugs had not been developed because of the phenyl ring's status as a structural alert.

Finally, the simplistic notion that the absence of a structural alert and/or RM liability in a drug candidate serves as a guarantee of its safety is not necessarily true. There is no evidence

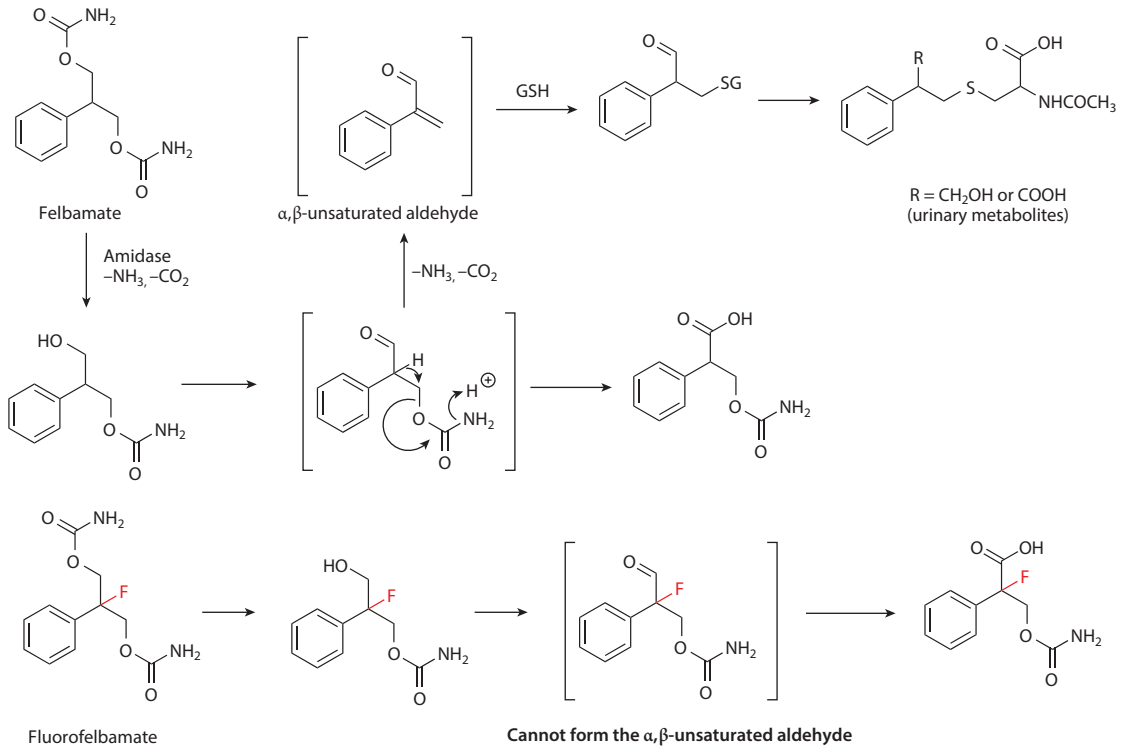


Figure 4

Mechanism of RM formation within the anticonvulsant felbamate, which led to the design of fluorofelbamate. Abbreviations: GSH, glutathione; RM, reactive metabolite.

that the idiosyncratic hepatotoxicity associated with the recalled thrombin inhibitor ximelagatran (**Figure 5**) is associated with RM formation, and the drug does not exhibit any alerts in its chemical structure (29). Likewise, there are no structural alerts or evidence for RM formation within drugs such as chlormezanone, isoxicam, pemoline, and flecainide (**Figure 5**), which have been withdrawn owing to idiosyncratic toxicity (16). The widely prescribed antihyperlipidemic agent niacin (**Figure 5**) does not contain conventional structural alerts but possesses the highest potential for hepatotoxicity when administered in the sustained-release form. The hepatotoxic effects of niacin are related to a high-affinity, low-capacity metabolic pathway that affords nicotinamide and *N*-methyl-2- and *N*-methyl-4-pyridone-5-carboxamide metabolites; thus, the sustained-release formulation can lead to higher levels of toxic metabolites (30). The alternative competing metabolic pathway is a low-affinity, high-capacity conjugation pathway (involving the formation of a glycine amide metabolite) that leads to prostaglandin-mediated vasodilation and subsequent cutaneous flushing (31). The immediate-release formulation overwhelms the higher-affinity oxidation pathway, and the majority of the niacin dose is metabolized via the high-capacity glycine conjugation pathway, leading to a much lower rate of hepatotoxicity (30).

Although structural alerts must be used with caution, particularly at the lead optimization/candidate selection stage in drug discovery, it is imperative to demonstrate experimentally whether structural alerts, if present in a molecule of interest, actually are prone to RM formation. In the case of RM-positive drugs, identifying the biochemical mechanism and the enzymes responsible

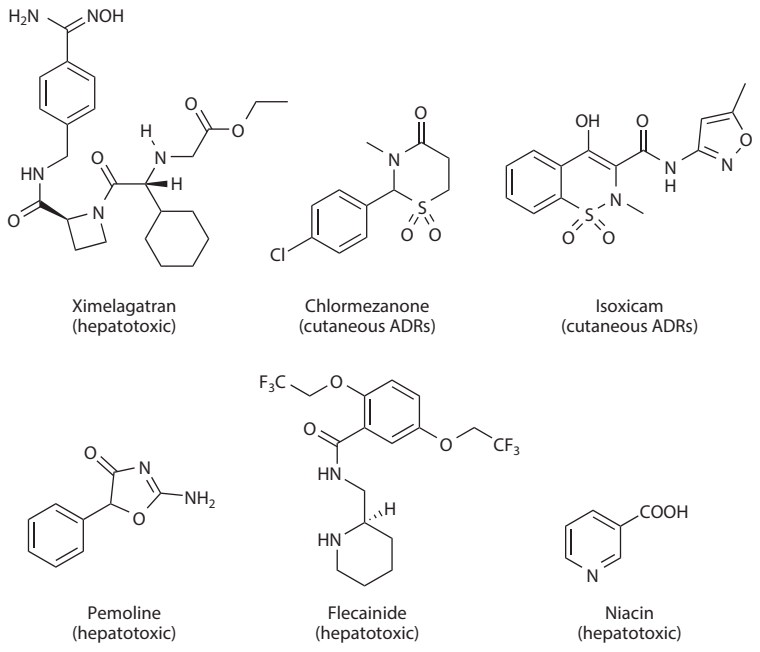


Figure 5

Illustrations of drugs associated with IADRs that do not contain structural alerts and do not form RMs. Abbreviations: ADR, adverse drug reaction; IADR, idiosyncratic adverse drug reaction; RM, reactive metabolite.

for RM formation is necessary. The information can then be used, as appropriate, to modify the structure of the RM-positive drugs in order to eliminate the liability. For instance, fluorofelbamate was specifically designed to eliminate the RM liability of felbamate on the basis of the bioactivation mechanism depicted in **Figure 4**. The strategic placement of the fluorine atom on the benzylic position prevents the β-elimination process that affords the α,β-unsaturated aldehyde (32). Successful case studies involving metabolism-guided design to circumvent RM formation in drug discovery are abundant in the medicinal chemistry/chemical toxicology literature (33–36). For instance, in the course of efforts leading to the discovery of taranabant, a selective and potent inhibitor of the cannabinoid-1 receptor and a Phase III clinical candidate for the treatment of obesity, the lead compound **1** depicted in **Figure 6** revealed a high level of covalent binding to HLM in a NADPH-dependent fashion, consistent with RM formation. Elucidation of the structure of the GSH conjugate suggested that the RM was an arene oxide intermediate derived from epoxidation of the electron-rich phenoxy ring (37). Replacement of the phenoxy ring with the trifluoromethylpyridyl ring afforded taranabant, which was devoid of RM formation yet retained potency and selectivity against the cannabinoid-1 receptor.

Another example pertains to the discovery of the first glucokinase activator, piragliatin (**Figure 6**), which has shown efficacy (e.g., lowering of pre- and postprandial glucose levels, improvements in insulin secretory profile) in Phase II clinical trials in patients with type 2 diabetes (38). The prototype candidate RO0281675 (**Figure 6**) was withdrawn from Phase I clinical trials owing to its narrow safety margin in preclinical toxicology studies. In chronic toxicology studies in rats and dogs, RO0281675 caused reversible hepatic lipidosis, which was believed to occur via the metabolism of the 2-aminothiazole motif to a thiourea metabolite. The hypothesis

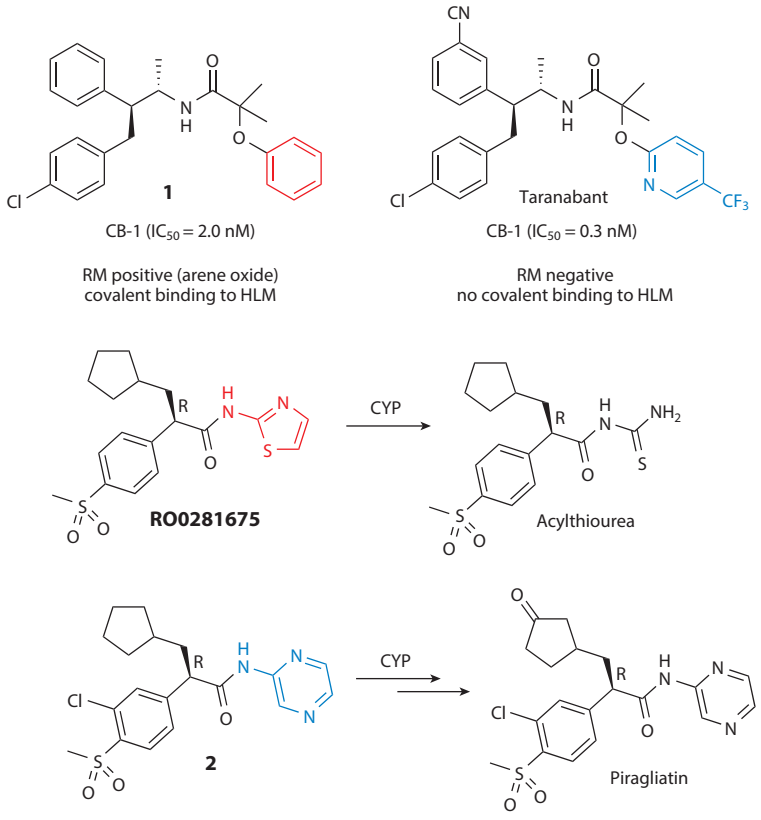


Figure 6

Medicinal chemistry tactics to eliminate RM liability on the basis of established pathways of bioactivation. Structural alerts are highlighted in red, and functional groups that are not structural alerts are highlighted in blue. Abbreviations: CB-1, cannabinoid-1; CYP, cytochrome P450; HLM, human liver microsome; RM, reactive metabolite.

was further substantiated on the basis of two observations: (*a*) the thiourea derivative was formed as a metabolite upon incubating RO0281675 in NADPH-supplemented liver microsomes from preclinical species and humans, and (*b*) five-day toxicity studies in rats with an authentic standard of the thiourea metabolite led to hepatic lipidosis in a manner similar to that noted with RO0281675. Subsequent SAR studies seeking thiazole ring replacements led to the identification of a pyrazine-based lead analog, labeled compound **2** in **Figure 6**. In vitro metabolite identification revealed several oxidative metabolites on the cyclopentyl ring of compound **2**, which were synthesized and shown to possess pharmacological activity comparable with that of the compound itself. Additional profiling of in vitro and in vivo safety and efficacy of the oxidative metabolites led to the selection of piragliatin as the clinical candidate. Subchronic and chronic toxicology studies with piragliatin in rats and dogs revealed no evidence of hepatic lipidosis. Furthermore, piragliatin is relatively less lipophilic than compound **2** (clog P of compound **2** = 2.69 versus clog P of piragliatin = 0.47) and exhibits superior oral absorption (lower plasma clearance leading to increased oral absorption) in preclinical species and humans. In practice, however, the exercise of eliminating or reducing RM formation is not trivial; medicinal chemistry tactics to eliminate RM

formation could confer a detrimental effect on pharmacology (e.g., changes in agonist/antagonist behavior, subtype selectivity for target receptor or enzyme) and/or pharmacokinetic attributes. In our experience, if the structural alert can be readily replaced with an alternative moiety without significant loss of desired pharmacology/pharmacokinetic attributes, then doing so is advisable. As a result, the need for additional risk assessment beyond the standard drug safety package as well as further internal debate on this topic for the remainder of the development program could be avoided.

Although the strategy of circumventing IADR risks via removing RM liability represents a pragmatic starting point in drug design, there is growing concern that the perceived safety hazards associated with structural alerts and RM-positive compounds may be overexaggerated. Several blockbuster drugs contain structural alerts and form RMs but do not cause idiosyncratic toxicity, suggesting that the structural alert concept and RM screening tools in drug discovery may be too stringent and thus could halt the advancement of novel medicines. A survey of 108 structurally distinct drugs that were among the most prescribed in 2009 revealed that 58 (53%) contained structural alerts, and evidence for RM formation has been provided in 24 out of the 58 (41%) cases (16). Likewise, 13 out of the 15 small-molecule drugs, which constitute the top drugs based on total sales in 2009, possess structural alerts. In vitro and/or in vivo experimental evidence for RM formation has been presented for 10 out of the 13 drugs (16). Overall, the analysis indicates that the percentage of structural alert–positive and/or RM-positive drugs in the most-prescribed or total-sales drug category is largely similar to that noted for drugs recalled or associated with a BBW. The alerts are fairly diverse in nature and include aniline/anilide, thiophene, olefin, and quinone precursors found in the toxic drugs. In the case of the top-ranked drug (on the basis of dispensed prescriptions and sales) atorvastatin (Lipitor®) (**Figure 7**), CYP3A4-catalyzed monohydroxylation on the acetanilide structural alert leads to the formation of the active *ortho*- and *para*-hydroxyacetanilide metabolites (39), which can be oxidized to reactive quinone-imine species in a manner similar to that noted for acetaminophen. The observation that atorvastatin covalently binds to HLM in a NADPH-dependent fashion partially validates the hypothesis (40). Interestingly, atorvastatin was ranked number one in terms of dispensed prescriptions and total sales for 2009.

In some cases, RM formation is essential to the pharmacological activity of a drug. In the case of the blockbuster cardiovascular drug and $P2Y_{12}$ purinoreceptor antagonist clopidogrel, the thiophene structural alert is metabolized by a CYP enzyme or enzymes to a pharmacologically active RM (speculated to be an electrophilic sulfenic acid). This RM forms a covalent disulfide linkage with a cysteinyl residue on the $P2Y_{12}$ receptor in platelets, leading to inhibition of platelet aggregation (**Figure 7**) (41, 42). Similar to clopidogrel, the antidepressant duloxetine contains a pendant thiophene ring, which can be oxidized by CYP to RMs. Indeed, incubation of duloxetine in NADPH- and GSH-supplemented HLM indicated the presence of several GSH conjugates (43). Interestingly, structural characterization of these conjugates reveals that GSH adduction occurs on the naphthalene ring rather than on the thiophene ring and likely proceeds via a reactive epoxide intermediate (**Figure 7**).

In humans, the antidepressant paroxetine is metabolized by CYP2D6 on the 1,3-benzodioxole structural alert to a catechol intermediate (44). The process also leads to the mechanism-based inactivation of the CYP isozyme and DDIs with CYP2D6 substrates in the clinic (45). In vitro studies with [^{3}H]-paroxetine have demonstrated the NADPH-dependent covalent binding to human liver microsomal and S9 proteins and have also demonstrated the characterization of GSH conjugates of reactive quinone metabolites (**Figure 8**) (46). The selective estrogen receptor modulator raloxifene is metabolized by CYP3A4 on the phenolic structural alerts to yield reactive quinone species that can be trapped with GSH (47). The process is also accompanied by the

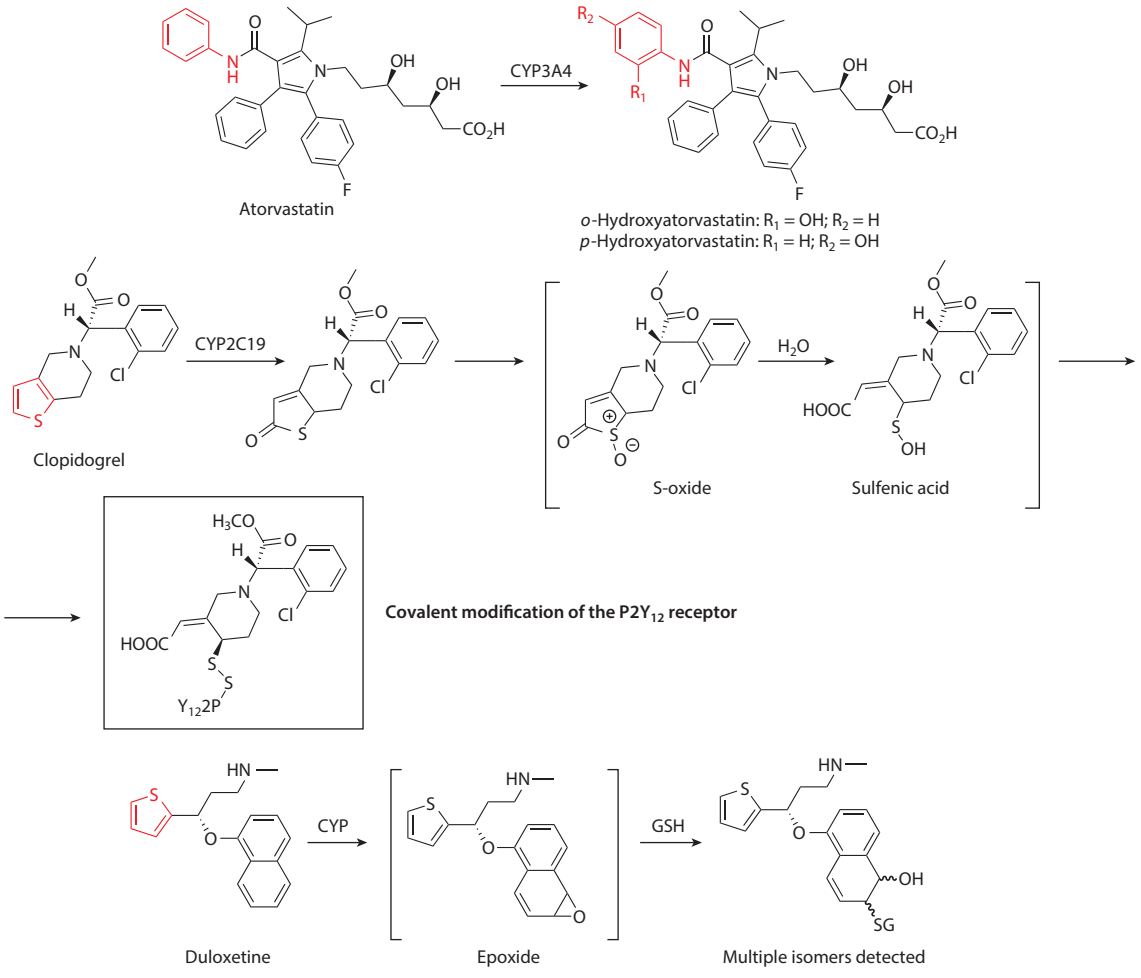


Figure 7

Examples of commercial blockbuster drugs that form RMs. Structural alerts are highlighted in red. Abbreviations: CYP, cytochrome P450; GSH, glutathione; RM, reactive metabolite.

mechanism-based inactivation of CYP3A4. Cyclobenzaprine is a skeletal muscle relaxant, which is metabolized on the olefin structural alert to yield the corresponding dihydrodiol metabolite in significant quantities in human urine (**Figure 8**) (48). The formation of the dihydrodiol metabolite is consistent with olefin epoxidation as a rate-limiting step.

In addition to the above analysis, examination of the structural trends for recently approved drugs (2009–present) revealed the presence of structural alerts in several cases (**Figure 9**). Foremost among these is the thiophene structural alert in the sodium glucose cotransporter 2 inhibitor and antidiabetic agent canagliflozin. However, its principal elimination mechanism in humans involves glucuronidation (on the sugar moiety) by UGT (uridine diphosphate glucose glucuronosyltransferase) enzymes (49). The lack of GSH conjugate formation in canagliflozin incubations in HLM suggests that the thiophene ring is latent to CYP metabolism (A.S. Kalgutkar, unpublished observations). The selective direct factor Xa inhibitors apixaban and rivaroxaban are new oral anticoagulants that contain structural alerts (*para*-methoxyaniline and *bis*-anilide motifs in

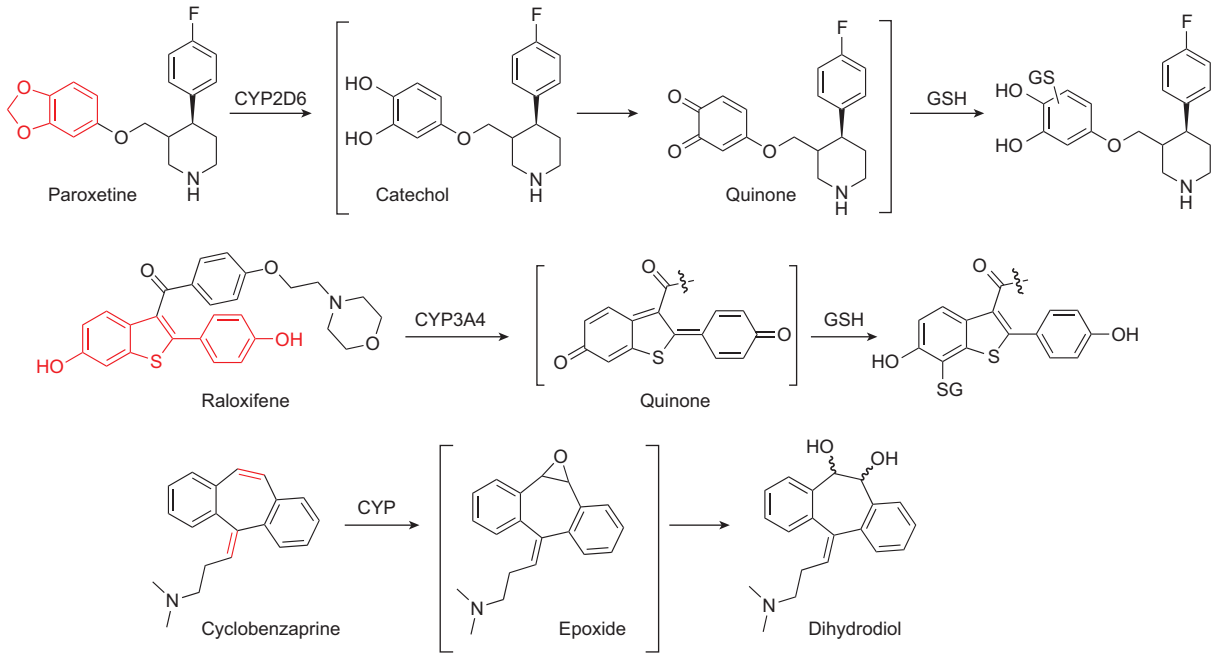


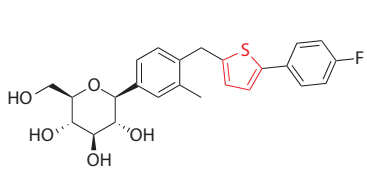
Figure 8

Additional examples of successfully marketed drugs that form RMs. Structural alerts are highlighted in red. Abbreviations: CYP, cytochrome P450; GSH, glutathione; RM, reactive metabolite.

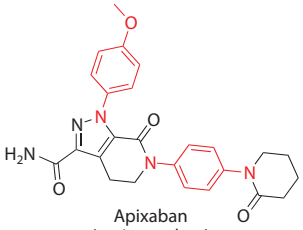
apixaban; chlorothiophene and *bis*-anilide motifs in rivaroxaban). Human mass balance studies using [^{14}C]-apixaban and rivaroxaban indicate that the alerts in those compounds are not subject to metabolism and/or RM formation (50, 51). In the case of rivaroxaban, the pendant chlorothiophene motif is essential for pharmacology and cannot be replaced. The aniline structural alert is also present in the oral direct thrombin inhibitor dabigatran. However, dabigatran is not subject to oxidative metabolism by CYP enzymes in humans (52).

The remainder of drugs flagged for structural alert presence (depicted in **Figure 9**) have been approved for various oncology indications. Enzalutamide is an androgen receptor antagonist used in the treatment of metastatic castration-resistant prostate cancer. Radiolabeled mass balance studies in humans with [^{14}C]-enzalutamide revealed *N*-dealkylation and amide bond hydrolysis as the principal routes of metabolism (53). The thiourea structural alert in enzalutamide is not subject to metabolism. Pomalidomide is a thalidomide derivative and has been approved for the treatment of relapsed and refractory multiple myeloma. In humans, a significant proportion of the metabolism occurs on the aniline structural alert (via the catalytic action of CYP1A2 and CYP3A4) to yield the corresponding *ortho*- and *para*-hydroxyaniline derivatives as stable metabolites (54). However, GSH conjugates of the quinone-imine species (the two-electron oxidation product of the hydroxyaniline metabolites of pomalidomide) have not been observed in HLM incubations (55). The *ortho*-hydroxyaniline metabolite of pomalidomide is subject to glucuronidation and is one of the major metabolites in human excreta.

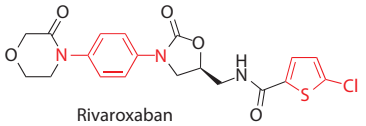
Bosutinib, cabozantinib, pazopanib, ponatinib, crizotinib, and vandetanib are tyrosine kinase inhibitors. In humans, bosutinib is metabolized primarily by CYP3A4 to yield *N*-desmethyl bosutinib and oxydechlorinated bosutinib as major circulating metabolites. The oxydechlorinated



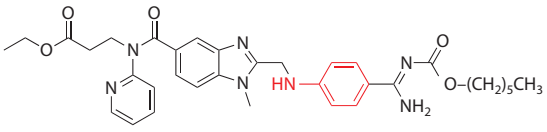
Canagliflozin
(antidiabetic)
Daily dose = 300 mg



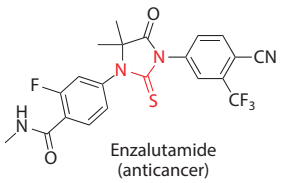
Apixaban
(anticoagulant)
Daily dose = 10 mg



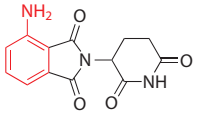
Rivaroxaban
(anticoagulant)
Daily dose = 20 mg



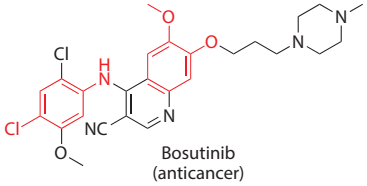
Dabigatran
(anticoagulant)
Daily dose = 150 mg



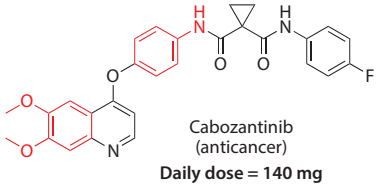
Enzalutamide
(anticancer)
Daily dose = 160 mg



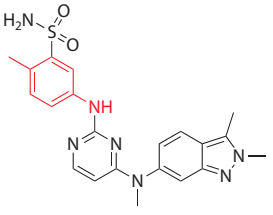
Pomalidomide
(anticancer)
Daily dose = 4.0 mg



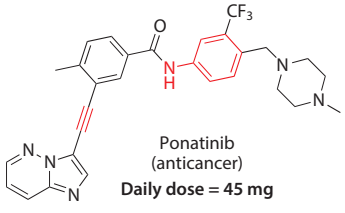
Bosutinib
(anticancer)
Daily dose = 500 mg



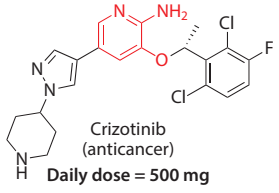
Cabozantinib
(anticancer)
Daily dose = 140 mg



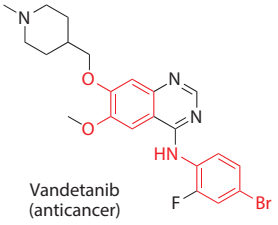
Pazopanib
(anticancer)
Daily dose = 800 mg



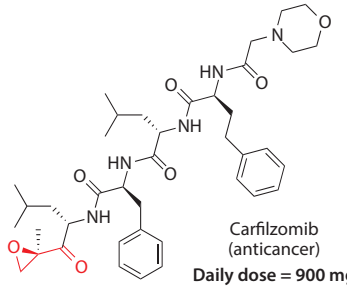
Ponatinib
(anticancer)
Daily dose = 45 mg



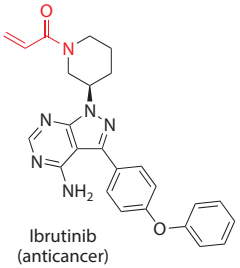
Crizotinib
(anticancer)
Daily dose = 500 mg



Vandetanib
(anticancer)
Daily dose = 300 mg



Carfilzomib
(anticancer)
Daily dose = 900 mg



Ibrutinib
(anticancer)
Daily dose = 560 mg

Figure 9

Recently approved drugs (2009–present) that contain structural alerts. Structural alerts are highlighted in red.

bosutinib metabolite is an *ortho*-hydroxyaniline derivative with the potential to form a quinone-imine species. However, there are no literature reports that provide evidence for the formation of electrophilic quinone species in the course of bosutinib metabolism. Cabozantinib was granted orphan drug status by the United States Food and Drug Administration (FDA) in 2011 and approved in 2012 for the treatment of medullary thyroid cancer. Cabozantinib contains two structural alerts: anilide and dialkoxyether. However, the primary pathway of cabozantinib clearance in humans involves *N*-oxidation by CYP3A4 (56) and does not appear to involve metabolism of either of the two structural alerts. Pazopanib and ponatinib have been approved for the treatment of renal cell/soft tissue carcinoma and chronic myeloid leukemia, respectively. The two drugs are associated with severe and sometimes fatal hepatotoxicity in clinical studies, which resulted in BBW labels (57, 58). A GSH conjugate has been detected in HLM incubations of pazopanib (59). Whether the RM is generated from a two-electron oxidation of the *para*-methylaniline structural alert in pazopanib is not clear. The metabolism of ponatinib proceeds via CYP-mediated *N*-demethylation and *N*-oxidation pathways. In addition, amide bond hydrolysis affords the corresponding carboxylic acid and amine (aniline) derivatives as metabolites (60). There are no reports on the oxidation of the aniline metabolite to a RM or RMs as a causative factor to account for the hepatotoxicity associated with ponatinib. Several structural alerts are also found in crizotinib (*ortho*-alkoxyaniline) and vandetanib (aniline, bromobenzene, and dialkoxyaromatic). Oxidative metabolism plays a significant role in the elimination of crizotinib in humans. Metabolic profiling demonstrated that crizotinib and a lactam metabolite (formed via oxidation of the piperidine ring) were the principal circulating components in plasma. Other metabolites, representing <10% of circulating radioactivity individually, included glucuronide and sulfate conjugates of *O*-desalkyl crizotinib and *O*-desalkyl crizotinib lactam. The *O*-desalkyl crizotinib metabolite is a hydroxyaniline derivative and possible precursor of electrophilic quinone-imine species. In the case of vandetanib, there is no evidence for metabolism on any of the structural alerts. Unchanged vandetanib, vandetanib *N*-oxide, and *N*-desmethyl vandetanib are the principal components detected in plasma and excreta following oral administration of vandetanib to humans (61).

The last two illustrations of recently approved drugs that contain structural alerts, carfilzomib and ibrutinib, are intrinsically electrophilic in nature. Carfilzomib is a selective and irreversible proteasome inhibitor that has been approved for the treatment of multiple myeloma. Carfilzomib is intrinsically electrophilic in nature owing to the presence of the epoxyketone group that irreversibly binds to the 20S proteasome (62). Despite its electrophilic nature and a high daily intravenous dose (900 mg), carfilzomib has demonstrated a favorable safety profile and significant antitumor activity in patients with relapsed and refractory multiple myeloma. In humans, carfilzomib has a short half-life ($t_{1/2} \sim 30$ min) and is cleared largely extrahepatically via peptidase cleavage and hydrolysis of the electrophilic epoxide (63). In vitro, carfilzomib demonstrates time- and concentration-dependent inhibition of human CYP3A4, consistent with mechanism-based inactivation (63). Direct alkylation of the enzyme by the epoxyketone moiety can be ruled out because enzyme inhibition requires NADPH cofactor. This observation suggests that carfilzomib is metabolized by CYP3A4 to a reactive species that covalently adducts to the CYP protein. RM trapping studies have not been performed with carfilzomib. In early 2013, the FDA granted ibrutinib breakthrough-therapy designations as a monotherapy for patients with two B cell malignancies: patients with relapsed or refractory mantle cell lymphoma who have received prior therapy and patients with Waldenström's macroglobulinemia. In November 2013, ibrutinib was approved for the treatment of mantle cell lymphoma. Ibrutinib is an orally administered selective covalent inhibitor of Bruton's tyrosine kinase. Like carfilzomib, ibrutinib is intrinsically electrophilic owing

to the presence of the acrylamide substituent that covalently binds to a cysteine residue in the tyrosine kinase (64).

DEALING WITH REACTIVE METABOLITES IN DRUG DISCOVERY—THE PATH FORWARD

The mere presence of structural alerts cannot in itself predict the type, severity, or incidence of IADRs associated with drugs. Likewise, RM screening tools (exogenous trapping with nucleophiles and/or protein covalent binding in HLM) are not intended to predict toxicity; rather, they are meant merely to detect the formation of RMs, some of which may carry a toxic liability. Experiments that unambiguously define a 1:1 relationship between RM formation (e.g., the in vitro and in vivo characterization of GSH conjugates) and toxicity in humans are extremely rare. Although GSH adducts and/or downstream mercapturic acid metabolites that are measured in vivo represent short-term exposure to RMs, protein adducts reflect the internal exposure of cells to RMs in vivo, which is more relevant for risk assessment purposes. Whether covalent binding measures in vivo are likely to be more informative about the in vivo safety risk than covalent binding studies in vitro remains to be established. This uncertainty arises from a paucity of data on absolute levels of in vivo covalent binding that could lead to a toxic outcome versus levels of binding that are safe. At the present time, there is no consensus on a preclinical discovery strategy to investigate safety hazards and risks posed by RM formation for a particular drug candidate in humans. Ultimately, only studies in humans can currently be used to unearth mechanisms of serious IADRs, to determine cause and effect with respect to RM formation in humans, and subsequently to determine clinical outcome. Although reducing exposure to RMs is viewed as a pragmatic approach to minimize IADR risks during drug development, these strategies should not rely solely on structural alert/RM information, as overall metabolic fate and other considerations (discussed below) provide additional valuable information that can be used in a weight-of-evidence approach toward risk assessment and management. For example, when a structural alert prone to RM formation is essential for intrinsic pharmacologic potency and cannot be replaced, additional information needs to be considered.

The fact that certain classes of drugs, such as the thienopyridine antithrombotics (e.g., clopidogrel), rely on RMs for pharmacological action underscores the concept that bioactivation per se need not equate to a toxicological response. Overall, this conundrum raises a fundamental question: Why are some RM-positive drugs safe, whereas others are not? A limitation of the in vitro RM screens is that they are typically conducted in HLM (in the presence and absence of NADPH cofactor); thus, they examine only CYP-catalyzed RM formation. In some instances, RM formation may be observed in microsomes in a CYP-dependent fashion, but in vivo, the compound may undergo a distinctly different and perhaps more facile metabolic fate that circumvents RM formation. For instance, both paroxetine and raloxifene form GSH conjugates and covalently bind to HLM in a CYP-dependent fashion; however, in vivo, the quinone precursors are metabolized principally via competing *O*-methylation and glucuronidation pathways, respectively (44, 65). It is tempting to speculate that in the modern drug discovery paradigm, paroxetine and raloxifene would likely not be considered as candidates for clinical development because of the high degree of microsomal covalent binding and GSH adduct formation (66). Minimizing false positives requires that initial RM assessments in HLM be followed by more detailed studies in fully integrated in vitro biological matrices such as hepatocytes and/or liver S9 fractions from both human and animal species (67). Establishing a clear understanding of the in vivo clearance mechanisms in animals and how they relate to RM formation in in vitro matrices would lead to data-driven decision making with regard to compound selection.

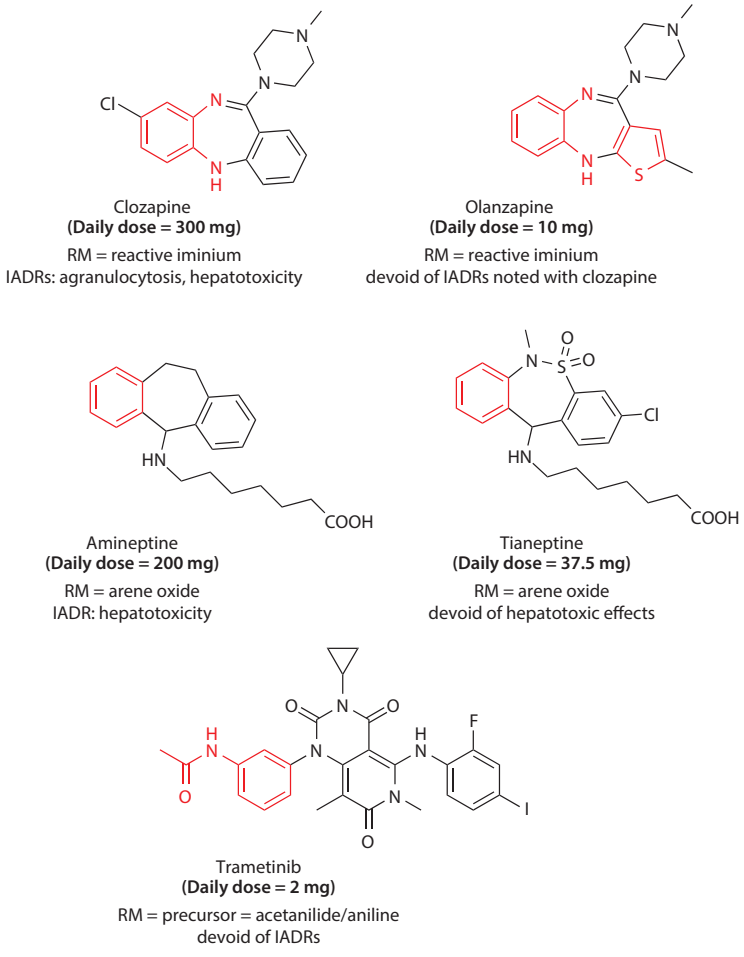


Figure 10

Low-daily-dose drugs do not exhibit the IADR liability associated with high-daily-dose drugs. Structural alerts are highlighted in red. Abbreviations: IADR, idiosyncratic adverse drug reaction; RM, reactive metabolite.

Comparison of the daily dosing regimen of toxic versus nontoxic drugs indicates that high-dose drugs (>100 mg) tend to be the ones that most frequently cause IADRs, whereas low-dose drugs (<50 mg) rarely are problematic in this regard (whether or not these agents are prone to RM formation) (16). The vast majority of structural alert–positive and/or RM-positive drugs in the top 200 list (in terms of dispensed prescriptions and total sales) are low-daily-dose drugs. The improved safety of low-dose drugs could arise from a marked reduction in the total body burden of RM exposure via efficient scavenging by GSH (and other competing metabolic pathways), such that the reactive species are unlikely to exceed the safety threshold needed for toxicity. For example, olanzapine (**Figure 10**) forms a reactive iminium metabolite analogous to the one observed with clozapine, yet olanzapine is not associated with a significant incidence of agranulocytosis (19). One difference between the two drugs is the daily dose; clozapine is given at a dose of >300 mg/day, whereas the maximum recommended daily dose of olanzapine is 10 mg/day. Another example becomes evident upon comparison of the tricyclic antidepressants amineptine

and tianeptine (**Figure 10**). Both drugs form reactive arene oxide species, but only amineptine is hepatotoxic (16, 68, 69). The improved tolerance of tianeptine in the clinic likely arises from the ~5–6-fold lower recommended dose relative to that of amineptine (daily doses of amineptine and tianeptine are 200 mg and 37.5 mg, respectively). Likewise, in the case of clopidogrel, the majority (>70%) of its daily dose of 75 mg is rapidly hydrolyzed by human carboxylesterases to the inactive carboxylic acid metabolite (~80–85% of circulating metabolites) (70), which means that only a small percentage of the parent drug (20 mg or less) is theoretically available for conversion to the active RM. Indeed, covalent binding to platelets accounts for only 2% of radiolabeled clopidogrel in human mass balance studies (71). Finally, trametinib (**Figure 10**), a kinase inhibitor recently approved for the treatment of patients with unresectable or metastatic melanoma, also falls into the category of a structural alert–positive agent but a low-daily-dose drug (72). Despite hydrolysis on the acetanilide alert to the corresponding aniline derivative as a primary metabolic pathway, no idiosyncratic hepatotoxicity has been noted thus far. This absence of hepatotoxicity could be ascribed to the very low recommended dosage (2 mg daily). Recent advances in risk assessment methodologies—such as the estimate of total daily body burden of covalent binding in hepatocytes and the zone classification, which takes the clinical dose into consideration—are positive steps toward quantitative prediction of IADR risks with drug candidates (40, 73, 74). Given this general trend in which low daily dose is a key factor in reducing IADR risks, optimization of lead compounds in drug discovery programs should focus on improving intrinsic pharmacologic potency and optimizing pharmacokinetics as a means of decreasing the projected clinically efficacious plasma concentrations (and hence the dose) and the associated body burden of a parent drug and its metabolites. However, there will be classes of drugs (e.g., antibacterials, antiretrovirals) for which this goal will be difficult to achieve.

Numerous drugs form RMs and cause idiosyncratic toxicity, yet they remain on the market and are widely prescribed because of favorable benefit-risk considerations; for example, Stepan et al. (16) found that aniline sulfonamide antibacterial agents such as sulfamethoxazole and sulfadoxine, which carry a BBW for IADRs including skin rashes and hepatotoxicity, are linked to RM formation. As noted in this work, the aniline/anilide motif is widely used in de novo kinase inhibitor design, as is evident with the recently approved tyrosine kinase inhibitors (see **Figure 9**). Some of them (e.g., pazopanib, ponatinib, and sunitinib) are even associated with cases of idiosyncratic hepatotoxicity, and available circumstantial evidence points toward RM liability as a potential causative factor in some instances. Lapatinib presents an interesting example that also illustrates the weight of unmet medical need over the risk of hepatotoxicity. Lapatinib is used in combination with capecitabine for the treatment of advanced or metastatic breast cancer and is associated with several cases of hepatotoxicity (some resulting in fatalities). Not only is the drug bioactivated to a quinone-imine species, resulting in covalent modification of the CYP3A4 isozyme (75), but its recommended daily dose is 1.25 grams. These observations suggest that the level of risk (e.g., idiosyncratic toxicity, DDI risk due to CYP inhibition) that would be deemed acceptable for drug candidates intended to treat major unmet medical needs, life-threatening diseases, and/or orphan diseases is significantly higher than the acceptable risk level associated with the treatment of chronic nondebilitating conditions for which alternate treatment options are already available. This issue also raises for debate a philosophical question regarding medicinal chemistry investments in removing structural alerts such as the aniline motif, which is widely utilized in kinase inhibitor programs and is challenging to mimic by isosteric replacement. The argument also applies to unprecedented pharmacologic targets or molecules that carry a significant risk with regard to predicted human pharmacokinetics, whose primary goal is to first demonstrate early signs of efficacy in the clinic and/or adequate systemic exposure. For such programs, RM-positive molecules can be advanced into first-in-human studies as probes to address pharmacokinetics

and proof of mechanism, provided they are deemed safe in standard preclinical toxicology studies. While proof of mechanism is being obtained, additional efforts can be invested in the identification of backup molecules that are devoid of RM formation. An illustration of such tactics is evident in our work on the RM-positive 5-trifluoromethylpyrido[4,3-*d*]pyrimidin-4(3*H*)-one short-acting series of calcium receptor antagonists. In these efforts, achieving a narrow window of human pharmacokinetics for safety and efficacy was a primary driver of success (76, 77).

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

LITERATURE CITED

1. Orr ST, Ripp SL, Ballard TE, Henderson JL, Scott DO, et al. 2012. Mechanism-based inactivation (MBI) of cytochrome P450 enzymes: structure-activity relationships and discovery strategies to mitigate drug-drug interaction risks. *J. Med. Chem.* 55:4896–933
2. Dobo KL, Obach RS, Luffer-Atlas D, Bercu JP. 2009. A strategy for the risk assessment of human genotoxic metabolites. *Chem. Res. Toxicol.* 22:348–56
3. Evans DC, Baillie TA. 2005. Minimizing the potential for metabolic activation as an integral part of drug design. *Curr. Opin. Drug Discov. Dev.* 8:44–50
4. Guengerich FP, MacDonald JS. 2007. Applying mechanisms of chemical toxicity to predict drug safety. *Chem. Res. Toxicol.* 20:344–69
5. Park BK, Boobis A, Clarke S, Goldring CE, Jones D, et al. 2011. Managing the challenge of chemically reactive metabolites in drug development. *Nat. Rev. Drug Discov.* 10:292–306
6. Stachulski AV, Baillie TA, Park BK, Obach RS, Dalvie DK, et al. 2013. The generation, detection, and effects of reactive drug metabolites. *Med. Res. Rev.* 33:985–1080
7. Lucas A, Nolan D, Mallal S. 2007. HLA-B*5701 screening for susceptibility to abacavir hypersensitivity. *J. Antimicrob. Chemother.* 59:591–93
8. Singer JB, Lewitzky S, Leroy E, Yang F, Zhao X, et al. 2010. A genome-wide study identifies HLA alleles associated with lumiracoxib-related liver injury. *Nat. Genet.* 42:711–14
9. Haas DW, Mootsikapun P, Ruxrungtham K, Podzamczer D, Nevirapine Toxicogenomics Study Team. 2012. Clinical perspectives on human genetic screening to prevent nevirapine toxicity. *Pers. Med.* 9:773–82
10. Uetrecht J, Naisbitt DJ. 2013. Idiosyncratic adverse drug reactions: current concepts. *Pharmacol. Rev.* 65:779–808
11. Park BK, Kitteringham NR, Maggs JL, Pirmohamed M, Williams DP. 2005. The role of metabolic activation in drug-induced hepatotoxicity. *Annu. Rev. Pharmacol. Toxicol.* 45:177–202
12. Ng W, Lobach AR, Zhu X, Chen X, Liu F, et al. 2012. Animal models of idiosyncratic drug reactions. *Adv. Pharmacol.* 63:81–135
13. Evans DC, Watt AP, Nicoll-Griffith DA, Baillie TA. 2004. Drug-protein adducts: an industry perspective on minimizing the potential for drug bioactivation in drug discovery and development. *Chem. Res. Toxicol.* 17:3–16
14. Argikar UA, Mangold JB, Harriman SP. 2011. Strategies and chemical design approaches to reduce the potential for formation of reactive metabolic species. *Curr. Top. Med. Chem.* 11:419–49
15. Kalgutkar AS, Gardner I, Obach RS, Shaffer CL, Callegari E, et al. 2005. A comprehensive listing of bioactivation pathways of organic functional groups. *Curr. Drug Metab.* 6:161–225
16. Stepan AF, Walker DP, Bauman J, Price DA, Baillie TA, et al. 2011. Structural alert/reactive metabolite concept as applied in medicinal chemistry to mitigate the risk of idiosyncratic drug toxicity: a perspective based on the critical examination of trends in the top 200 drugs marketed in the United States. *Chem. Res. Toxicol.* 24:1345–410
17. Shamovsky I, Ripa L, Blomberg N, Eriksson LA, Hansen P, et al. 2012. Theoretical studies of chemical reactivity of metabolically activated forms of aromatic amines toward DNA. *Chem. Res. Toxicol.* 25:2236–52

18. Liu ZC, Uetrecht JP. 1995. Clozapine is oxidized by activated human neutrophils to a reactive nitrenium ion that irreversibly binds to the cells. *J. Pharmacol. Exp. Ther.* 275:1476–83
19. Gardner I, Leeder JS, Chin T, Zahid N, Uetrecht JP. 1998. A comparison of the covalent binding of clozapine and olanzapine to human neutrophils in vitro and in vivo. *Mol. Pharmacol.* 53:999–1008
20. Uetrecht J, Zahid N, Tehim A, Fu JM, Rakhit S. 1997. Structural features associated with reactive metabolite formation in clozapine analogues. *Chem. Biol. Interact.* 104:117–29
21. Kalgutkar AS, Vaz AD, Lame ME, Henne KR, Soglia J, et al. 2005. Bioactivation of the nontricyclic antidepressant nefazodone to a reactive quinone-imine species in human liver microsomes and recombinant cytochrome P450 3A4. *Drug Metab. Dispos.* 33:243–53
22. Damsten MC, de Vlieger JS, Niessen WM, Irth H, Vermeulen NP, Commandeur JN. 2008. Trimethoprim: novel reactive intermediates and bioactivation pathways by cytochrome P450s. *Chem. Res. Toxicol.* 21:2181–87
23. Obach RS, Kalgutkar AS, Ryder TF, Walker GS. 2008. In vitro metabolism and covalent binding of enol-carboxamide derivatives and anti-inflammatory agents sudoxicam and meloxicam: insights into the hepatotoxicity of sudoxicam. *Chem. Res. Toxicol.* 21:1890–99
24. Davies NM, Skjodt NM. 1999. Clinical pharmacokinetics of meloxicam: a cyclo-oxygenase-2 preferential nonsteroidal anti-inflammatory drug. *Clin. Pharmacokinet.* 36:115–26
25. Bell JA, Dallas FA, Jenner WN, Martin LE. 1980. The metabolism of ranitidine in animals and man. *Biochem. Soc. Trans.* 8:93
26. Diao L, Shu Y, Polli JE. 2010. Uptake of pramipexole by human organic cation transporters. *Mol. Pharm.* 7:1342–47
27. Dieckhaus CM, Thompson CD, Roller SG, Macdonald TL. 2002. Mechanisms of idiosyncratic drug reactions: the case of felbamate. *Chem. Biol. Interact.* 142:99–117
28. Thompson CD, Barthen MT, Hopper DW, Miller TA, Quigg M, et al. 1999. Quantification in patient urine samples of felbamate and three metabolites: acid carbamate and two mercapturic acids. *Epilepsia* 40:769–76
29. Testa L, Bhindi R, Agostoni P, Abbate A, Zoccai GG, van Gaal WJ. 2007. The direct thrombin inhibitor ximelagatran/melagatran: a systematic review on clinical applications and an evidence based assessment of risk benefit profile. *Exp. Opin. Drug Saf.* 6:397–406
30. McKenney JM, Proctor JD, Harris S, Chinchili VM. 1994. A comparison of the efficacy and toxic effects of sustained- versus immediate-release niacin in hypercholesterolemic patients. *JAMA* 271:672–77
31. Pieper JA. 2003. Overview of niacin formulations: differences in pharmacokinetics, efficacy, and safety. *Am. J. Health Syst. Pharm.* 60:S9–14
32. Roecklein BA, Sacks HJ, Mortko H, Stables J. 2007. Fluorofelbamate. *Neurotherapeutics* 4:97–101
33. Hartz RA, Ahuja VT, Zhuo X, Mattson RJ, Denhart DJ, et al. 2009. A strategy to minimize reactive metabolite formation: discovery of (*S*)-4-(1-cyclopropyl-2-methoxyethyl)-6-[6-(difluoromethoxy)-2,5-dimethylpyridin-3-ylamino]-5-oxo-4,5-dihydropyrazine-2-carbonitrile as a potent, orally bioavailable corticotropin-releasing factor-1 receptor antagonist. *J. Med. Chem.* 52:7653–68
34. Subramanian R, Lee MR, Allen JG, Bourbeau MP, Fotsch C, et al. 2010. Cytochrome P450–mediated epoxidation of 2-aminothiazole-based AKT inhibitors: identification of novel GSH adducts and reduction of metabolic activation through structural changes guided by in silico and in vitro screening. *Chem. Res. Toxicol.* 23:653–63
35. Kalgutkar AS, Mascitti V, Sharma R, Walker GW, Ryder T, et al. 2011. Intrinsic electrophilicity of a 4-substituted-5-cyano-6-(2-methylpyridin-3-yloxy)pyrimidine derivative: structural characterization of glutathione conjugates in vitro. *Chem. Res. Toxicol.* 24:269–78
36. Kalgutkar AR, Ryder TF, Walker GS, Orr ST, Cabral S, et al. 2013. Reactive metabolite trapping studies on imidazo- and 2-methylimidazo[2,1-*b*]thiazole-based inverse agonists of the ghrelin receptor. *Drug Metab. Dispos.* 41:1375–88
37. Hagmann WK. 2008. The discovery of taranabant, a selective cannabinoid-1 receptor inverse agonist for the treatment of obesity. *Arch. Pharm.* 341:405–11
38. Sarabu R, Bizzarro FT, Corbett WL, Dvorozniak MT, Geng W, et al. 2012. Discovery of piragliatin—first glucokinase activator studied in type 2 diabetic patients. *J. Med. Chem.* 55:7021–36

39. Lennernas H. 2003. Clinical pharmacokinetics of atorvastatin. *Clin. Pharmacokinet.* 42:1141–60
40. Nakayama S, Atsumi R, Takakusa H, Kobayashi Y, Kurihara A, et al. 2009. A zone classification system for risk assessment of idiosyncratic drug toxicity using daily dose and covalent binding. *Drug Metab. Dispos.* 37:1970–77
41. Dansette PM, Libraire J, Bertho G, Mansuy D. 2009. Metabolic oxidative cleavage of thioesters: evidence for the formation of sulfenic acid intermediates in the bioactivation of the antithrombotic prodrugs ticlopidine and clopidogrel. *Chem. Res. Toxicol.* 22:369–73
42. Savi P, Zachayus JL, Delesque-Touchard N, Labouret C, Hervé C, et al. 2006. The active metabolite of clopidogrel disrupts P2Y12 receptor oligomers and partitions them out of lipid rafts. *Proc. Natl. Acad. Sci. USA* 103:11069–74
43. Wu G, Vashishtha SC, Erve JC. 2010. Characterization of glutathione conjugates of duloxetine by mass spectrometry and evaluation of in silico approaches to rationalize the site of conjugation for thiophene containing drugs. *Chem. Res. Toxicol.* 23:1393–404
44. Haddock RE, Johnson AM, Langley PF, Nelson DR, Pope JA, et al. 1989. Metabolic pathways of paroxetine in animals and man and the comparative pharmacological properties of the metabolites. *Acta Psychiatr. Scand. Suppl.* 350:24–26
45. Venkatakrishnan K, Obach RS. 2005. In vitro–in vivo extrapolation of CYP2D6 inactivation by paroxetine: prediction of nonstationary pharmacokinetics and drug interaction magnitude. *Drug Metab. Dispos.* 33:845–52
46. Zhao SX, Dalvie DK, Kelly JM, Soglia JR, Frederick KS, et al. 2007. NADPH-dependent covalent binding of [^{3}H]paroxetine to human liver microsomes and S-9 fractions: identification of an electrophilic quinone metabolite of paroxetine. *Chem. Res. Toxicol.* 20:1649–57
47. Chen Q, Ngui JS, Doss GA, Wang RW, Cai X, et al. 2002. Cytochrome P450 3A4–mediated bioactivation of raloxifene: irreversible enzyme inhibition and thiol adduct formation. *Chem. Res. Toxicol.* 15:907–14
48. Hucker HB, Stauffer SC, Balletto AJ, White SD, Zacchei AG, Arison BH. 1978. Physiological disposition and metabolism of cyclobenzaprine in the rat, dog, rhesus monkey, and man. *Drug Metab. Dispos.* 6:659–72
49. Devineni D, Curtin CR, Polidori D, Gutierrez MJ, Murphy J, et al. 2013. Pharmacokinetics and pharmacodynamics of canagliflozin, a sodium glucose co-transporter 2 inhibitor, in subjects with type 2 diabetes mellitus. *J. Clin. Pharmacol.* 53:601–10
50. Zhang D, He K, Raghavan N, Wang L, Mitroka J, et al. 2009. Comparative metabolism of ^{14}C-labeled apixaban in mice, rats, rabbits, dogs and humans. *Drug Metab. Dispos.* 37:1738–48
51. Weinz C, Schwarz T, Kubiza D, Mueck W, Lang D. 2009. Metabolism and excretion of rivaroxaban, an oral, direct factor Xa inhibitor, in rats, dogs, and humans. *Drug Metab. Dispos.* 37:1056–64
52. Stangier J. 2008. Clinical pharmacokinetics and pharmacodynamics of the oral direct thrombin inhibitor dabigatran etexilate. *Clin. Pharmacokinet.* 47:285–95
53. Xtandi® [package insert]. Northbrook, IL: Astellas Pharma Inc.; 2012
54. Hoffmann M, Kasserra C, Reyes J, Schafer P, Kosek J, et al. 2013. Absorption, metabolism and excretion of [^{14}C]pomalidomide in humans following oral administration. *Cancer Chemother. Pharmacol.* 71:489–501
55. Chowdhury G, Shibata N, Yamazaki H, Guengerich FP. 2014. Human cytochrome P450 oxidation of 5-hydroxythalidomide and pomalidomide, an amino analogue of thalidomide. *Chem. Res. Toxicol.* 27:147–56
56. Cometriq® [package insert]. South San Francisco, CA: Exelixis Inc.; 2012
57. Votrient® [package insert]. Research Triangle Park, NC: GlaxoSmithKline; 2013
58. Iclusig® [package insert]. Cambridge, MA: Ariad Pharmaceuticals Inc.; 2012
59. Kenny JR, Mukadam S, Zhang C, Tay S, Collins C, et al. 2012. Drug-drug interaction potential of marketed oncology drugs: in vitro assessment of time-dependent cytochrome P450 inhibition, reactive metabolite formation and drug-drug interaction. *Pharm. Res.* 29:1960–76
60. Narasimhan NI, Dorer DJ, Niland K, Haluska F, Sonnichsen D. 2013. Effects of ketoconazole on the pharmacokinetics of ponatinib in healthy subjects. *J. Clin. Pharmacol.* 53:974–81
61. Martin P, Oliver S, Kennedy SJ, Partridge E, Hutchison M, et al. 2012. Pharmacokinetics of vandetanib: three phase I studies in healthy subjects. *Clin. Ther.* 34:221–37
62. Zhou HJ, Aujay MA, Bennett MK, Dajee M, Demo SD, et al. 2009. Design and synthesis of an orally bioavailable and selective peptide epoxyketone proteasome inhibitor (PR-047). *J. Med. Chem.* 52:3028–38

63. Wang Z, Yang J, Kirk C, Fang Y, Alsina M, et al. 2013. Clinical pharmacokinetics, metabolism, and drug-drug interaction of carfilzomib. *Drug Metab. Dispos.* 41:230–37
64. Herman SE, Gordon AL, Hertlein E, Ramanunni A, Zhang X, et al. 2011. Bruton tyrosine kinase represents a promising therapeutic target for treatment of chronic lymphocytic leukemia and is effectively targeted by PCI-32765. *Blood* 117:6287–96
65. Dalvie D, Kang P, Zientek M, Xiang C, Zhou S, Obach RS. 2008. Effect of intestinal glucuronidation in limiting hepatic exposure and bioactivation of raloxifene in humans and rats. *Chem. Res. Toxicol.* 21:2260–71
66. Obach RS, Kalgutkar AS, Soglia JR, Zhao SX. 2008. Can in vitro metabolism-dependent covalent binding data in liver microsomes distinguish hepatotoxic from nonhepatotoxic drugs? An analysis of 18 drugs with consideration of intrinsic clearance and daily dose. *Chem. Res. Toxicol.* 21:1814–22
67. Dalvie D, Obach RS, Kang P, Prakash C, Loi CM, et al. 2009. Assessment of three human in vitro systems in the generation of major human excretory and circulating metabolites. *Chem. Res. Toxicol.* 22:357–68
68. Genève J, Degott C, Letteron P, Tinel M, Descatoire V, et al. 1987. Metabolic activation of the tricyclic antidepressant amineptine: II. Protective role of glutathione against in vitro and in vivo covalent binding. *Biochem. Pharmacol.* 36:331–37
69. Fromenty B, Pessayre D. 1995. Inhibition of mitochondrial beta-oxidation as a mechanism of hepatotoxicity. *Pharmacol. Ther.* 67:101–54
70. Farid NA, Kurihara A, Wrighton SA. 2010. Metabolism and disposition of the thienopyridine antiplatelet drugs ticlopidine, clopidogrel, and prasugrel in humans. *J. Clin. Pharmacol.* 50:126–42
71. Plavix® [package insert]. Bridgewater, NJ: Sanofi-Aventis; 2013
72. Mekinist™ [package insert]. Research Triangle Park, NC: GlaxoSmithKline; 2014
73. Bauman JS, Kelly JM, Tripathy S, Zhao SX, Lam WM, et al. 2009. Can in vitro metabolism-dependent covalent binding data distinguish hepatotoxic from nonhepatotoxic drugs? An analysis using human hepatocytes and liver S-9 fraction. *Chem. Res. Toxicol.* 22:332–40
74. Thompson RA, Isin EM, Li Y, Weidolf L, Page K, et al. 2012. In vitro approach to assess the potential for risk of idiosyncratic adverse reactions caused by candidate drugs. *Chem. Res. Toxicol.* 25:1616–32
75. Teng WC, Oh JW, New LS, Wahlin MD, Nelson SD, et al. 2010. Mechanism-based inactivation of cytochrome P450 3A4 by lapatinib. *Mol. Pharmacol.* 78:693–703
76. Didiuk MT, Griffith DA, Benbow JW, Liu KK, Walker DP, et al. 2009. Short-acting 5-(trifluoromethyl)pyrido[4,3-*d*]pyrimidin-4(3*H*)-one derivatives as orally-active calcium-sensing receptor antagonists. *Bioorg. Med. Chem. Lett.* 19:4555–59
77. Kalgutkar AS, Griffith DA, Ryder T, Sun H, Miao Z, et al. 2010. Discovery tactics to mitigate toxicity risks due to reactive metabolite formation with 2-(2-hydroxyaryl)-5-(trifluoromethyl)pyrido[4,3-*d*]pyrimidin-4(3*H*)-one derivatives, potent calcium-sensing receptor antagonists and clinical candidate(s) for the treatment of osteoporosis. *Chem. Res. Toxicol.* 23:1115–26