# Preface

I have always been interested in chemistry and biology. My undergraduate, graduate, and postdoctoral trainings in pharmacy, medicinal chemistry and pharmacology, respectively, have strengthened this interest and led me to realize that significant advances in medicine have frequently been realized because of research at the chemistry–biology interface. I am hoping that this comprehensive volume on recent advances in bioactivation research will stimulate pharmacologists, medicinal chemists, pharmaceutical scientists, and graduate students in these fields and related areas to consider and use bioactivation research when they explore and chart new frontiers in drug design and drug development and when they consider ways to reduce the side effects of existing drugs by making prodrugs. As for the toxicologists and environmental health scientists, I hope this volume will help them generate the knowledge needed to understand better mechanisms of toxicity to improve human risk assessments and intervention methods after occupational or environmental exposure to various hazardous chemicals.

Adnan A. Elfarra, Ph.D.

# 2

# Role of Bioactivation in Idiosyncratic Drug Toxicity: Structure–Toxicity Relationships

Amit S. Kalgutkar
*Pharmacokinetics, Dynamics and Metabolism Department, Pfizer Global Research & Development, Groton, CT06340, USA*
*e-mail: amit.kalgutkar@pfizer.com*

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## 2.1. Adverse Drug Reactions

Adverse drug reactions (ADRs) continue to be a significant contributor to overall attrition statistics in the pharmaceutical industry. ADRs pose a significant health problem since they contribute to patient morbidity and mortality and represent one of the most common causes for pharmaceutical product recalls and black-box warning labels. Of a total of 548 drugs approved in the period from 1975 to 1999, 45 drugs (8.2%) acquired one or more black-box warnings and 16 (2.9%) were withdrawn from the market (Lasser et al. 2002). An estimated 100 000 fatalities annually are attributed to ADRs making it the fourth to sixth leading cause of death in the United States (Lazarou, Pomeranz and Corey 1998). Therefore, increased emphasis has been placed on the identification of indicators of ADRs in preclinical species and humans as early as possible in the overall discovery/development process.

Adverse drug reactions can be classified into two categories namely type A and type B reactions. Type A or augmented reactions account for approximately 80% of all ADRs and are predictable from the known primary or secondary pharmacology of the drug. They show simple dose–response relationships and, therefore, can be usually avoided by dose reduction and are rarely life-threatening. Type A ADRs can be routinely identified in preclinical toxicological investigations. Typical examples of type A ADRs include the risk of hypotension with antihypertensives and hemorrhage with anticoagulants. Currently, risk assessment of overall toxicity in the clinic is usually based on the safety margin of the drug candidate, which is often the ratio of the no observable adverse effect

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level (NOAEL) in the most sensitive preclinical species and the anticipated efficacious dose/systemic exposure in humans. A combination of clinical signs and histopathological evaluations serve as standard paradigm for identification of organ toxicity in animals, and the risk assessment is then extrapolated to humans. If an adverse event is observed in laboratory animals at systemic drug exposures near those anticipated for clinical efficacy, the drug is generally abandoned from further development. This traditional approach for organ toxicity risk assessment has poor predictability for type B reactions (Olson et al. 2000), since these reactions are not related to the known pharmacology of the drug, and although they are dose-dependent in susceptible individuals, they can occur at any dose within the usual therapeutic range. Although less common than type A reactions, type B reactions can be serious and may be life-threatening. Type B reactions are extremely host-dependent, usually rare, and therefore referred to as idiosyncratic ADRs (IADRs). Furthermore, there are no general animal models that can predict the occurrence of IADRs in humans. Idiosyncratic ADRs can be just as idiosyncratic in animals as they are in humans and furthermore it is rare for animals to show all of the biochemical, clinical, and morphological features characteristic of IADRs in humans. This is not surprising considering that many of the manifestations of idiosyncratic reactions to the same drug can be different in different humans. Comprehensive reviews on this subject have recently appeared in the literature (Roth et al. 2003; Shenton, Chen and Uetrecht 2004).

Given the low frequency of occurrence of type B reactions and lack of available animal models, large clinical trials, exposing perhaps up to 10 000 patients to a new therapeutic agent prior to registration, may not suffice in detecting IADRs reliably. An additional complication is that IADRs usually manifest as overt or symptomatic disease and can occur with intermediate (1–8 weeks) or long (1 year) periods of latency. Drugs can adversely affect almost any organ in the body; however, potentially life-threatening IADRs noted for several drugs include hepatotoxicity, severe cutaneous reactions, anaphylaxis, and blood dyscrasias. Among these, drug-induced liver toxicity is the most common cause for the withdrawal of a drug from the market and it accounts for approximately one-half of the cases of acute liver failure and mimics all forms of acute and chronic liver disease (Gunawan and Kaplowitz 2004). An estimated 1400 drugs have been implicated in causing liver damage in greater than one occasion (Biour et al. 2000). Manifestations of liver injury range from mild, asymptomatic changes in serum transaminases, which occur at high frequency with a number of drugs, to fulminant hepatic failure, which although rare is potentially life-threatening and may necessitate a liver transplant.

## 2.2. Link Between Drug Metabolism and Type B ADRs

One of the liver's main physiological roles is the metabolism of drugs into hydrophilic metabolites (via a combination of oxidative, reductive, and hydrolytic phase I and conjugating phase II pathways) in order to facilitate their elimination. Considering that the liver is exposed to high concentrations of drugs/metabolites after oral administration, it is not altogether

surprising that the organ is often a target for drug-induced toxicity. In most cases hepatic metabolism results in the loss of biological activity of the parent drug, and such metabolic reactions are therefore regarded as detoxification pathways. However, depending on the structural features present in some drugs, the same metabolic events on occasion can generate chemically reactive and toxic metabolites. The concept of drug metabolism to chemically reactive species that covalently modify critical protein components leading to some form of toxicity has its basis in the field of carcinogenicity (Fieser 1938; Miller and Miller 1947), which proposed the carcinogenic and hepatotoxic activity of polycyclic aromatic hydrocarbons and aminoazo dyes to arise from their bioactivation to reactive metabolites. Extension of these concepts to human drug-induced hepatotoxicity was provided from studies in the 1970s on the covalent binding to hepatic tissue by structurally diverse drugs including acetaminophen (Cohen et al. 1997). Based on these collective findings gathered over the last 50 years, biotransformation of relatively inert organic compounds to reactive electrophilic intermediates including free radicals, commonly referred to as metabolic activation or bioactivation, has been speculated to contribute toward certain drug-induced toxicities, including hepatotoxicity, cutaneous ADRs, and blood dyscrasias (Kaplowitz 2004). Inadequate detoxification of reactive metabolites is thought to represent a pathogenic mechanism for tissue necrosis, carcinogenicity, teratogenicity, and immune-mediated toxicity (Figure 2.1; Kaplowitz 2004).

Drug-metabolizing enzymes have evolved to process a plethora of structurally diverse xenobiotics, which are encountered by the organism. These enzymes, however, cannot distinguish between xenobiotics that are bioactivated to reactive metabolites and those that are not. Whether bioactivation will occur for any given molecule will depend on two key factors: (1) does the molecule possess a functionality and/or architecture that is susceptible to bioactivation and (2) is there an alternative (higher affinity but innocuous) route of metabolism within the molecule that minimizes the potential bioactivation of the "suspect" chemical motif within that molecule. Most bioactivation reactions in the liver involve either oxidation or reduction and can often

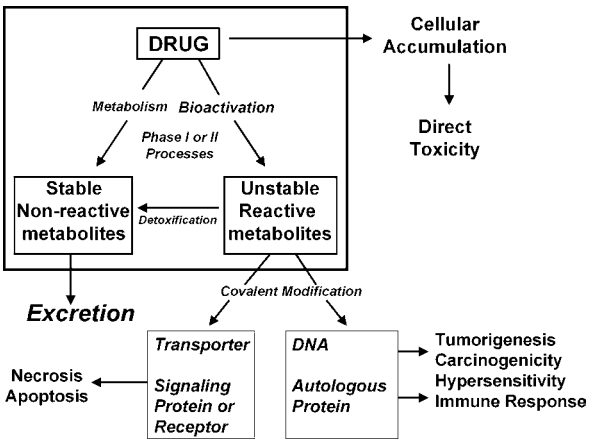


**Figure 2.1** Proposed role of bioactivation in drug toxicity.

be attributed mainly to the action of cytochrome P450 enzymes. In several cases, cytosolic enzymes, such as aldehyde oxidase or alcohol dehydrogenases, have also been noted to participate in the catalysis of bioactivation pathways. Conjugative pathways including glucuronidation and sulfation are also known to transform latent functional groups into electrophilic intermediates. In some cases, bioactivation can involve a single enzymatic reaction, whereas in other cases, multiple enzymatic and/or chemical steps are involved in the production of the ultimate toxin. Besides the liver, neutrophils and monocytes can metabolize drugs to reactive metabolites, especially those drugs that have nitrogen or sulfur in a low oxidation state. The major system involved in this oxidation is the combination of NADPH oxidase and myeloperoxidase (MPO), which generates hypochlorous acid. In such cases, the dominant toxicity will be agranulocytosis and/or aplastic anemia, not hepatotoxicity.

While the detection of a bioactivation process in vitro is relatively straightforward, the downstream consequences of this process as it relates to toxicity remain poorly understood, although, two hypotheses have been proposed to explain the phenomenon. Foremost amongst them is the hapten hypothesis that proposes covalent modification of proteins by a reactive metabolite leading to a "foreign" protein that, in some cases, translates to an immune-mediated adverse reaction. Failure to downregulate potentially deleterious immune responses due to a "foreign" macromolecule may cause ADRs in susceptible patients (Matzinger 2002). For example, sulfonamides such as sulfamethoxazole cause a variety of ADRs including fever, skin rashes, hepatitis, nephritis, and blood dyscrasias, many of which are idiosyncratic in nature. The identification of drug-specific T cells in systemic circulation and blister fluid of susceptible population provides convincing evidence that adverse events associated with sulfamethoxazole require activation of the host's immune system (Hess et al. 1999). In the case of sulfamethoxazole, the immune activation is thought to involve a P450-catalyzed bioactivation of the aniline group to a reactive nitroso metabolite capable of covalently binding to cellular constituents (Hess et al. 1999). The danger hypothesis, which aims to account for the idiosyncratic nature of ADRs in patients, further expands on the hapten hypothesis. In this proposal, drug–macromolecule conjugates lead to cell damage, which in turn generates a "danger signal" that ultimately can result in antibody- or cytotoxic T-cell-mediated responses (Uetrecht 1999). The variability in incidence of such a response in patients is thought to be due to high interindividual differences in the competing processes of cell damage, repair, and host cell defense.

Since the initial hypothesis that covalent binding of acetaminophen to "critical" hepatic proteins may be associated with its hepatotoxic effects, there has been a plethora of publications on the identification of susceptible protein targets (Pumford and Halmes 1997). However, few have attempted to distinguish "critical" from "noncritical" proteins. With acetaminophen, more than 30 hepatic proteins form conjugates with acetaminophen after a toxic overdose of the drug to rodent species (Hinson et al. 2004; Park et al. 2005). The concomitant inactivation of multiple proteins during this process suggests that failure in the cellular machinery is a consequence of multiple parallel events rather than a simple cascade or signaling mechanism. Overall, the complex nature of these events severely

limits our ability to predict whether in vitro bioactivation and accompanying covalent binding of a potential drug candidate to hepatic tissue(s) will or will not ultimately translate in some form of toxicity in animals or for that matter in humans. Even if the drug candidate fails to cause organ toxicity in preclinical species, there is always some concern that the bioactivation observed in human hepatic tissue may have the potential to elicit idiosyncratic immune-mediated ADRs in the susceptible population.

## 2.3. Assays to Monitor Reactive Metabolites in Drug Discovery

Formation of reactive metabolites has received considerable attention and several in vitro assays have been established to monitor and address this phenomenon. Reactive electrophilic metabolites are generally short-lived (with the possible exception of some acyl glucuronides) and are not usually detectable in circulation. Their intracellular formation can be inferred from conjugates derived from reaction with endogenous nucleophiles. Their formation may be modulated by enzyme induction and/or inhibition, and gene deletion in mammals. However, none of these experimental approaches are directly applicable to humans. Consequently, human exposure to chemically reactive metabolites in the liver and in the general circulation is impossible to quantify. Given the inability to predict whether bioactivation phenomenon detected in vitro will ultimately lead to toxicity in the clinic, a general strategy adopted by many within the pharmaceutical community involves the assessment of reactive metabolite formation as early as possible in the selection of drug candidates, with the goal of eliminating or minimizing the formation of reactive species by rational structural modification of lead chemical matter.

### 2.3.1. Covalent Binding

Availability of a radiolabeled compound allows a quantitative assessment of the amount of covalent binding either in vitro (e.g., liver microsomes, cytosol, or S-9 fractions) or in tissue or blood/plasma obtained from preclinical in vivo studies. Some companies have adopted a limit of 50 pM $mg^{-1}$ liver microsomal protein as the cutoff for further development (Evans et al 2004; Evans and Baillie 2005). This limit is based on the commonly observed amount of covalent binding detected in the livers of animals receiving a prototypic hepatotoxin (e.g., acetaminophen, bromobenzene, furosemide, or 4-ipomeanol), which is associated with overt hepatoxicity (about 1000 pM $mg^{-1}$ microsomal protein) and a 20-fold safety margin. However, it should be noted that the 50 pM $mg^{-1}$ microsomal protein is not a hard cutoff. The rigor with which teams adhere to this limit depends on multiple factors such as the therapeutic area, the duration of therapy (acute versus chronic), the target population, first in class or best in class and, of course, the anticipated human pharmacokinetics and dose. Covalent binding studies can be performed in vivo as well. Either tissue or blood/plasma can be examined for the degree of covalent binding. However, covalent binding may require multiple dosing to establish the true impact of the compound. Reactive metabolites formed after the first

dose may be efficiently trapped by nucleophiles such as glutathione (GSH) and eliminated from the body (e.g., via the bile). Once GSH is depleted, the extent of covalent binding with cellular or circulating proteins may increase rapidly, which could result in an ADR. The advantage of covalent binding studies is that they directly measure covalent binding of reactive metabolites to macromolecules, which could cause an adverse immunological response or direct organ toxicity. Nevertheless, no information is available about the nature of the covalently modified proteins. Furthermore, since many drugs display a degree of covalent modification of proteins, but only a fraction thereof cause toxicity, a direct link with a toxicological endpoint is not guaranteed. Indeed, the mechanism of action of some drugs (e.g., aspirin, finasteride, clopidogrel, and omeprazole) involves covalent binding to the target. For finasteride the binding specifically involves 5α-reductase and, therefore, no toxicity due to off-target covalent binding is observed. An added disadvantage of this approach is that covalent binding experiments are laborious. Finally, radiolabeled material is not routinely available in early drug discovery at most pharmaceutical companies.

### 2.3.2. Reactive Metabolite Characterization as Stable Sulfydryl, Amino, and/or Cyano Conjugates

Methodology to elucidate the structure(s) of reactive metabolites typically involves in vitro "trapping" experiments conducted using liver microsomes, cytosol, or S-9 fractions supplemented with NADPH and appropriate nucleophilic trapping agents including thiols (e.g., naturally occurring tripeptide and soft nucleophile GSH and its ethyl ester derivative or *N*-acetylcysteine), amines (e.g., *N*-acetyllysine, semicarbazide, and methoxylamine), or cyanide anion; analysis of the resulting metabolites by liquid chromatography-tandem mass spectrometry (LC-MS/MS) is employed to detect and characterize stable conjugates with the exogenously added nucleophile. Elucidation of the structure of these adducts can provide indirect information on the structure of the electrophilic species, thereby providing insight into the bioactivation mechanism and hence a rationale on which to base subsequent chemical intervention strategies. These trapping agents also serve as surrogate markers of covalent binding of the electrophile to microsomal protein. GSH adducts can be analyzed by LC-MS/MS using either the full-scan mode or constant neutral loss scanning for 129 Da (glutamyl moiety) to detect GSH-related conjugates (Baillie and Davis 1993; Soglia et al. 2004). Hard electrophiles (e.g., electrophilic carbonyl compounds) will preferentially react with hard nucleophiles including amines (e.g., semicarbazide and methoxylamine), amino acids (e.g., lysine), and DNA bases (e.g., guanine and cytosine) (Zhang et al. 1996; Dalvie et al. 2002) affording the corresponding Schiff base, which can be further stabilized by the addition of reducing agents (e.g., sodium borohydride or sodium cyanoborohydride) to generate the more stable amine conjugates. Typical in vitro conditions require the addition of 5 mM of amine trapping agent to the microsomal mixture followed by LC-MS/MS analysis for the formation of stable imine conjugates as indicated in the case of furan ring opening to reactive β-dicarbonyl metabolites (Zhang et al. 1996). The cyanide anion is a "hard" nucleophile

that can be used to trap iminium species. Gorrod et al. (1991) have developed a simple screening method that detects reactive iminium intermediates via reaction with [$^{14}$C]-cyanide. Extent of radiolabeled cyanide incorporation in test compounds that are suspected of forming reactive iminium intermediates is normalized with reference to a standard compound (e.g., S-nicotine). Utilizing this methodology, iminium ion formation has been assessed for several structurally diverse cyclic tertiary amines (Gorrod and Aislaitner 1994).

### 2.3.3. Enzyme Inactivation Studies

In some instances, P450-mediated oxidative bioactivation of drugs to reactive intermediates leads to irreversible inactivation of the P450 enzyme by the reactive species prior to its release from the active site (Murray 1997). Because metabolic activation precludes enzyme inactivation, these drugs fall into the category of mechanism-based inactivators. The mechanism-based inactivation of P450 enzymes may result from irreversible alkylation of an active-site amino acid or the heme prosthetic group or a combination of both. In general, heme alkylation invariably inactivates P450, whereas amino acid alkylation may result in loss of catalytic activity. Inactivation of P450 enzymes often translates into clinically relevant drug–drug interactions as well as immune-mediated IADRs, which can be potentially deleterious. The ability of drug candidates to inactivate P450 function is typically screened for in an industry setting.

### 2.3.4. Metabolite Identification

A thorough understanding of metabolic pathways and the biochemical mechanisms by which metabolites are generated can provide insight to the potential of a compound to yield a reactive species. Both in vitro and in vivo approaches can and should be performed to gain a detailed understanding of the biotransformation pathways that a drug candidate may undergo. Often times, stable downstream metabolites derived from nonenzymatic processing of electrophilic intermediates provide important clues on the generation of reactive metabolites (e.g., the detection of carboxylic acid metabolite in the metabolism of terminal alkynes provides evidence for the generation of a reactive ketene intermediate). The possibility that metabolism occurs at an alternate site and not on the structural alert itself, can also be assessed in these studies. In the absence of an alternate chemical series, this information may be critical toward nominating a candidate containing a structural alert for clinical development.

## 2.4. Strategies to Abrogate Reactive Metabolite Formation: Structure–Activity Relationship Studies

The propensity of a drug candidate to undergo bioactivation to reactive intermediates, usually electrophiles, is a function of the chemistry that ensues following metabolism. Information to qualify certain functional groups as structural alerts (Figure 2.2) has been inferred from myriad

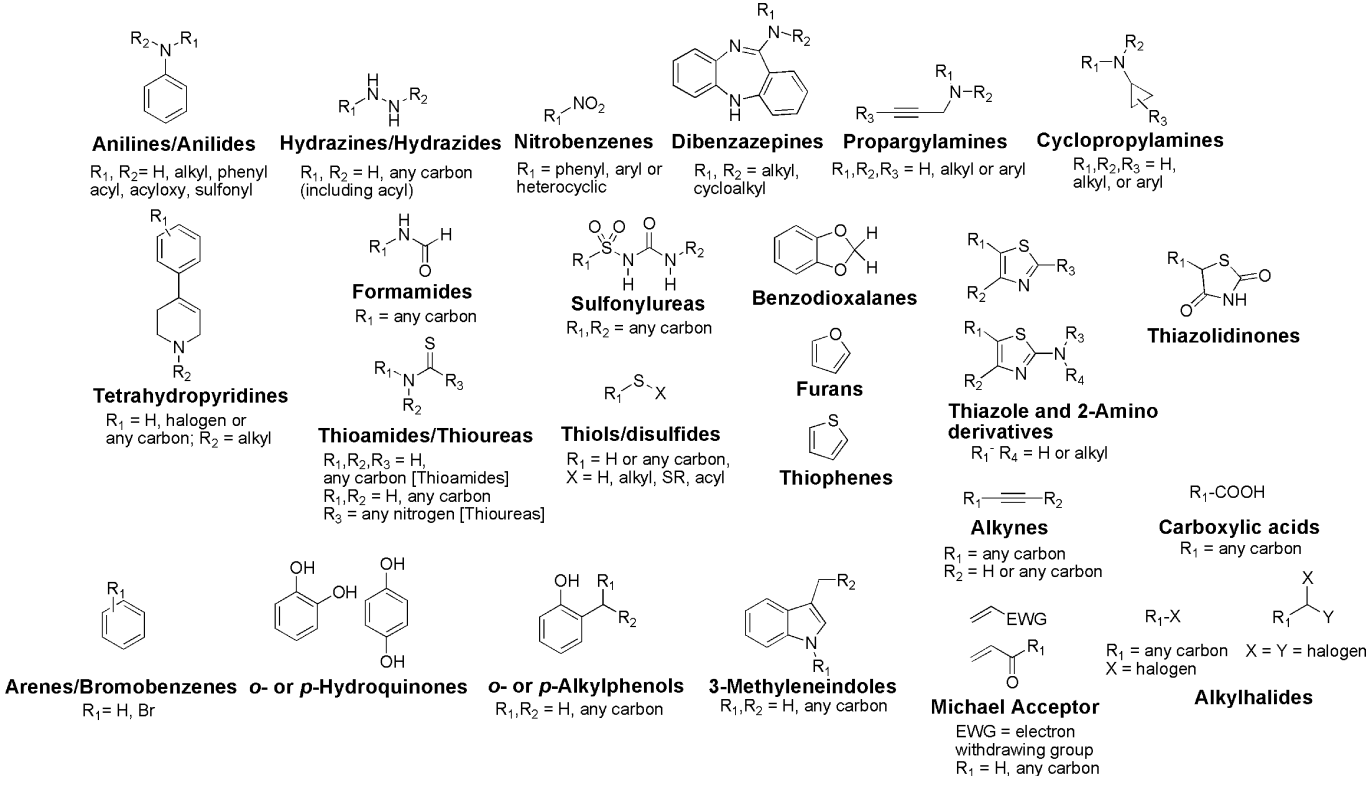


**Figure 2.2** A comprehensive list of organic functional groups susceptible to bioactivation.

examples of protoxins containing these motifs, which upon bioactivation affords reactive metabolites. A comprehensive review that summarizes the existing bioactivation pathways for these functional groups has been recently published (Kalgutkar et al. 2005a). The scope of this concept can be further expanded to include numerous drugs that contain putative structural alerts and are associated with some form of toxicity. Indeed, for many such drugs reactive metabolites formation has been demonstrated and serves to provide a circumstantial link between bioactivation and toxicity. Figure 2.3 provides a glimpse of drugs that have been withdrawn due to toxicity and are also prone to bioactivation. For an exhaustive review on this subject, the reader is advised to refer to the article by Kalgutkar and Soglia (2005).

Numerous examples documenting the elimination of bioactivation liabilities via chemical manipulations have been presented in the literature. In several cases, elimination of bioactivation liability in prototype drugs has also resulted in the elimination of toxicity in the successor agent. It is noteworthy to point out that structural alterations which successfully eliminate the propensity of new chemical leads to undergo bioactivation may also confer a detrimental effect on the desired pharmacological

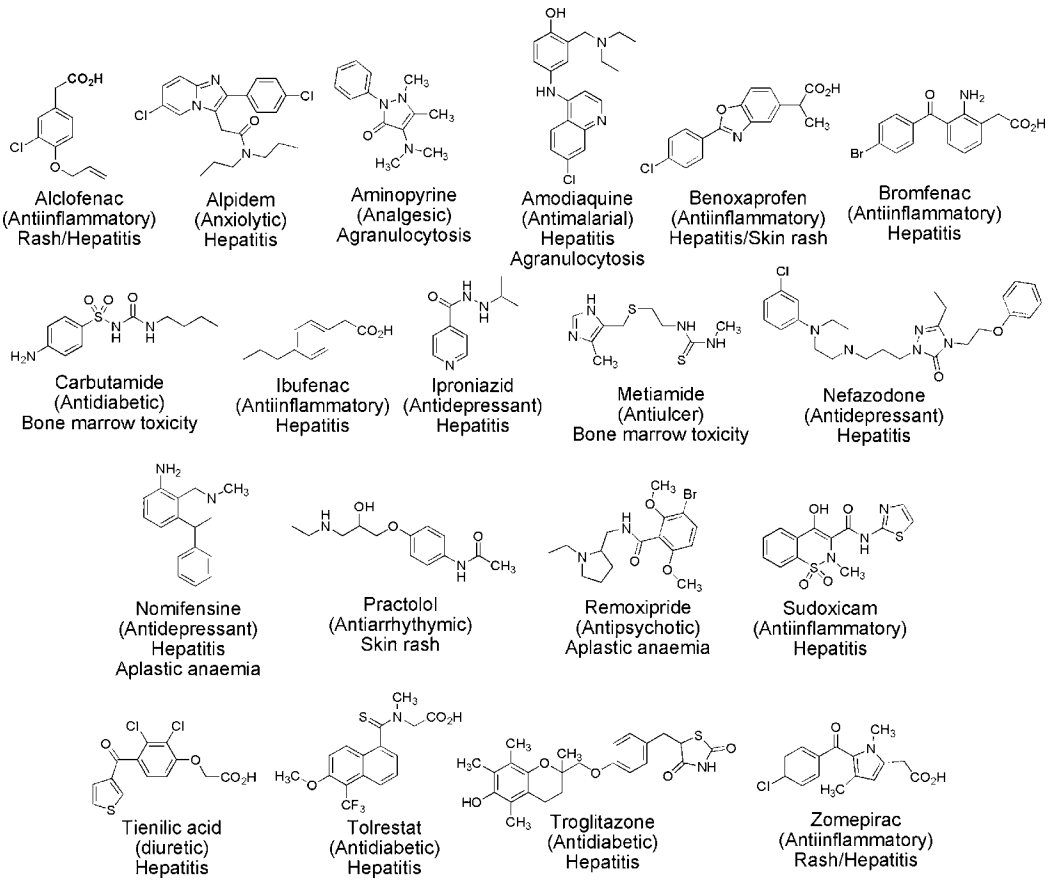


**Figure 2.3** Examples of drugs susceptible to bioactivation, which have been withdrawn due to ADRs.

(possible changes in agonist/antagonist behavior and/or subtype selectivity for target receptor or enzyme) and pharmacokinetic attributes of the chemical matter. Therefore, chemical intervention strategies toward the elimination of bioactivation phenomenon are often an iterative process, the success of which is heavily dependent on a close working relationship between medicinal chemists, pharmacologists, and metabolism scientists.

### 2.4.1. Removal of Structural Alerts

There are several strategies that medicinal chemists can utilize toward elimination of bioactivation potential of lead chemical matter. Foremost amongst which is a strategy involving direct replacement of the potential structural alert with substituents that are generally resistant to metabolism or with groups that undergo biotransformation to nonreactive metabolites. Circumstantial evidence, whereupon removal of structural alerts in prototype therapeutic agents translates into a markedly improved safety profile is illustrated in Figure 2.4. Removal of the aniline substituents in the antiarrhythmic agent procainamide and the antidiabetic agent carbutamide, which are known to be associated with bone marrow toxicity (carbutamide has been withdrawn from the market due to life-threatening bone marrow toxicity), results in flecainide and tolbutamide, respectively, that are devoid of the IADRs observed with the prototype drugs. Likewise, the bone marrow toxicity associated with the prototype H2 receptor antagonist metiamide that led to its withdrawal has not been observed with cimetidine. A key structural difference between the two compounds is the replacement of the thiourea substituent in metiamide that is subject to bioactivation with nonreactive guanidine functionality in cimetidine.

Structure–bioactivation relationships on clozapine and its analogs have been examined (Liegeois et al. 1995, 1999). Replacing the nitrogen that connects the two aryl rings on clozapine with oxygen or sulfur results in marketed drugs loxapine and quetiapine, respectively, that do not undergo peroxidase-mediated bioactivation to the reactive iminium species in neutrophils – a metabolic fate, which has been speculated to contribute toward clozapine-induced agranulocytosis (Figure 2.4) (Uetrecht 1994; Liegeois et al. 1999). Despite administration at doses comparable to clozapine, cases of agranulocytosis with quetiapine and loxapine are extremely rare.

An additional example wherein reactive metabolite formation can be eliminated by direct replacement of the offending motifs is evident in structure–toxicity studies on the antimalarial agent amodiaquine, which has been withdrawn from prophylactic use due to cases of hepatotoxicity and agranulocytosis. Tingle et al. (1995) have shown that exchanging the C'4-phenolic OH group in the antimalarial drug amodiaquine with a fluorine results in a compound that does not undergo the obligatory peroxidase-mediated two-electron oxidation process on the 4-aminophenol motif to the corresponding electrophilic quinone-imine as discernible with the parent compound (see Figure 2.4). It is interesting to note that incorporation of steric bulk around the C'4-phenolic OH group as means to prevent oxidation, however, is not successful in the case of amodiaquine as indicated with the analogous antimalarials pyronaridine and cycloquine that are equally susceptible to bioactivation as the parent drug (Naisbitt et al. 1998).

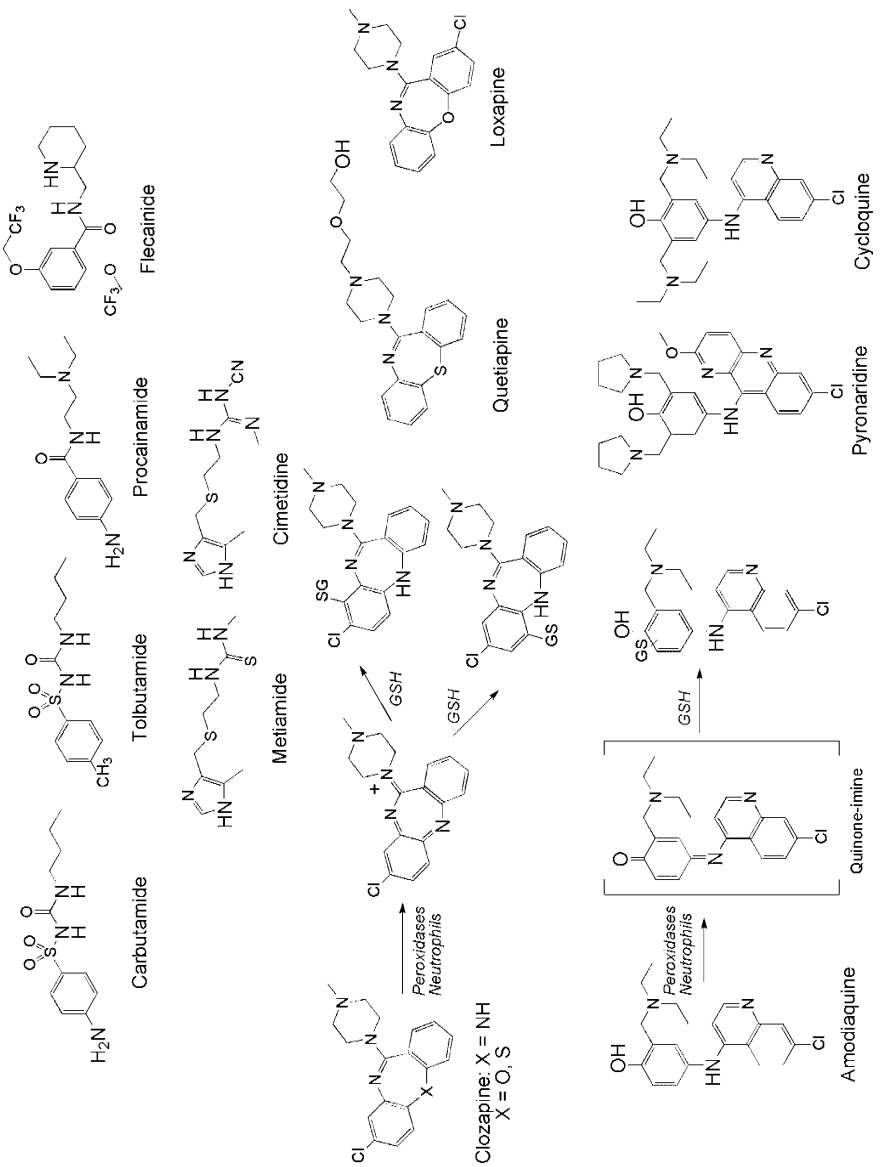


**Figure 2.4** Circumstantial evidence linking chemical structure to IADRs: removal of structural alerts.

### 2.4.2. Blocking Sites of Bioactivation

An alternate approach involves blocking the site of initial metabolism that precludes bioactivation. The strategy is particularly effective in eliminating reactive metabolite formation in instances when bioactivation occurs in a stepwise fashion via an enzymatic or nonenzymatic processing (oxidative, elimination, or rearrangement) of the initially formed latent metabolite(s). In this scenario, neighboring functional groups can participate in the process of sequential metabolic or nonmetabolic steps leading to reactive metabolite formation (e.g., oxidative metabolism of phenol to ortho- or para-hydroquinone followed by further oxidation to the corresponding quinones). An example of successful implementation of this approach to prevent reactive metabolite formation is evident from structure–bioactivation studies conducted on a novel potassium channel opener (Compound **1**) (see Figure 2.5) to elucidate the mechanism of bioactivation resulting in potent mechanism-based inactivation of P4503A4 (Wu et al. 2003). Saturation of the cinnamoyl double bond (compound **2**) or insertion of fluorine on the phenyl ring in the cinnamoyl portion of **1** to afford **3** did not abolish P450 inactivation, whereas replacement of the morpholine ring with a hydrogen atom to afford **4** or substitution of the hydrogen ortho to the morpholine ring with a fluorine atom to yield **5** were successful methods toward eliminating P4503A4 inactivation by **1**. The lack of P450 inactivation by **5** is consistent with a bioactivation pathway involving the initial aromatic hydroxylation ortho to the morpholine ring (or para to the benzylamine methine). Further two-electron oxidation of this initial metabolite by P450 can result in the formation of the reactive quinone-imine or quinone-methide intermediates. The potential formation of either reactive intermediates can be avoided by the introduction of the fluorine atom. In the present example, Compound **5** not only is devoid of P450 inactivation liability but it also retains the pharmacological and pharmacokinetic properties of the prototype Compound **1**.

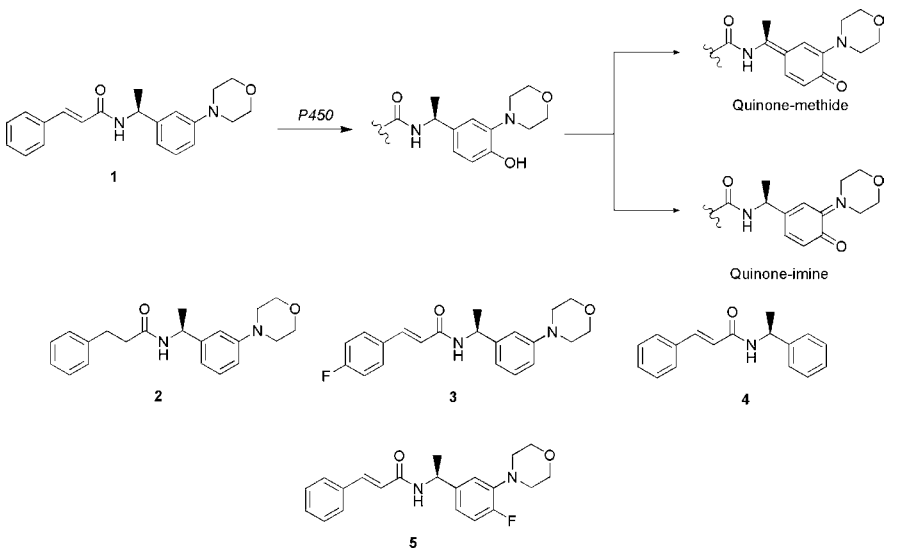


**Figure 2.5** Strategies to abrogate reactive metabolite formation: blocking sites of bioactivation.

### 2.4.3. Introduction of Alternate Metabolic Soft Spots

Apart from strategies that focus on replacement of structural alerts or blocking metabolic sites that preclude bioactivation, chemists can also introduce metabolic soft spots elsewhere on the pharmacophore to divert metabolism. An example of this concept is evident with nifidepine, a well-tolerated drug, despite containing the nitroaromatic structural alert (Figure 2.6). The major metabolic pathways of nifedipine identified in humans are restricted to biotransformations on the available soft spots including oxidation of the 1,4-dihydropyridine ring, hydrolysis of both the methyl ester groups, and/or hydroxylation of the methyl substituent followed by lactonization (Bocker and Guengerich 1986; Guengerich, Peterson and Bocker 1988). There is no evidence for formation of metabolites derived from reductive metabolism of the nitro group in humans.

Additional examples that highlight the success of this strategy are displayed in Figures 2.6 and 2.7. Toxicity associated with platelet aggregation inhibitor ticlopidine has been linked to the P450 or MPO-catalyzed bioactivation of its thiophene ring to reactive *S*-oxides or *S*-chloride intermediates that covalently bind to hepatic proteins and neutrophils, respectively (Figure 2.6; Liu and Uetrecht 2000). In the case of structurally similar drug clopidogrel, introduction of the additional methyl ester group results in a metabolic shift such that ester hydrolysis to the inactive carboxylic acid metabolite constitutes the principal metabolic fate (>85% in circulation) of this drug (Figure 2.6; Reist et al. 2000). Of much interest in this context is the observation that clopidogrel by itself is inactive and requires biotransformation to produce inhibition of platelet aggregation in vivo. Recently, Pereillo et al. (2002) have identified a thiophene ring scission product as the active metabolite of clopidogrel. The formation of this active carboxylic acid metabolite presumably occurs via a P450-catalyzed oxidation of the thiophene ring in clopidogrel to yield a 2-oxothiophene metabolite, hydrolytic cleavage of which generates the active component (see Figure 2.6). Whether the formation of electrophilic intermediates during the oxidative metabolism of the thiophene ring in clopidogrel occurs, remains unknown. Finally, it is important to note that the daily dose of clopidogrel (75 mg) is approximately sixfold lower than that of ticlopidine (500 mg) and this feature may play a key role in the markedly improved safety profile of clopidogrel.

The anxiolytic agent alpidem was withdrawn from the market within the first year of its commercial release due to severe hepatotoxicity that led to fatalities or required immediate liver transplantation. In contrast, the structural analog zolpidem does not possess the hepatotoxic liability associated with its predecessor. Although the reason(s) for this discrepancy remains unclear, it is interesting to note that the chloro-imidazopyridine ring in alpidem is subject to P450 bioactivation leading to the formation of a reactive epoxide that reacts with GSH to yield sulfydryl conjugates (see Figure 2.6), which have been detected in humans (Durand et al. 1992). A key structural difference in the two drugs is the replacement of the two chlorine atoms in alpidem with two metabolically labile methyl groups in zolpidem. Indeed, the primary biotransformation pathways of zolpidem in humans is restricted to the oxidative metabolism of both methyl groups to the corresponding alcohol

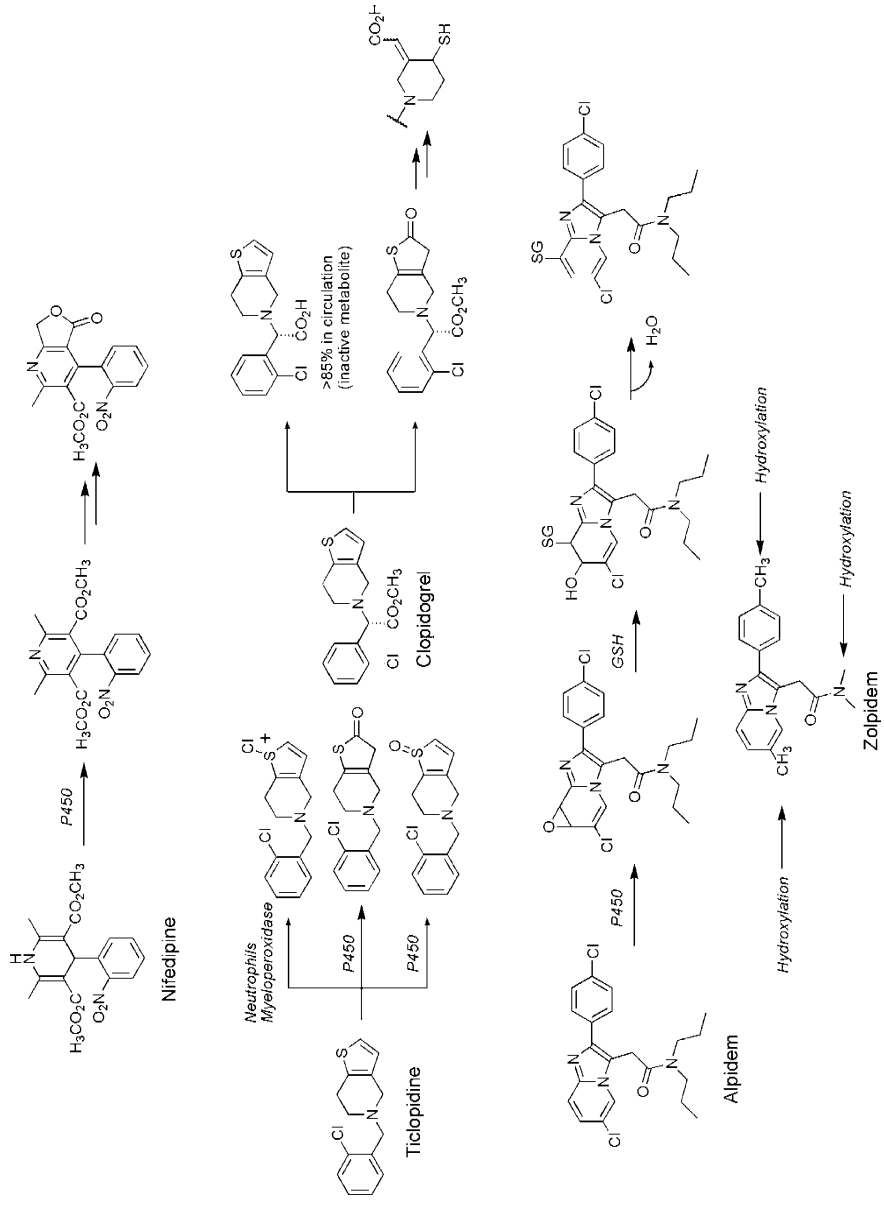


**Figure 2.6** Strategies to abrogate reactive metabolite formation: introducing alternate metabolic soft spots.

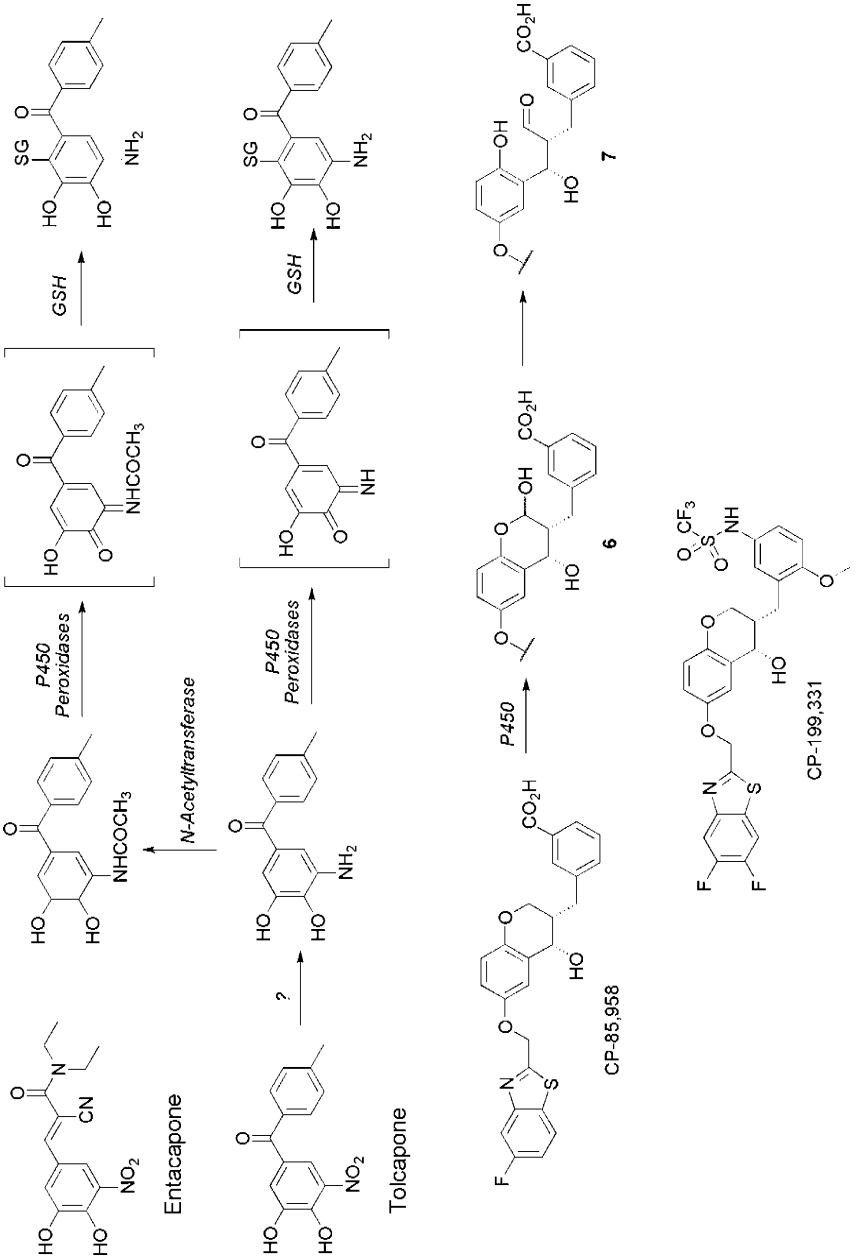


**Figure 2.7** Additional examples where presence of alternate metabolic spots eliminates bioactivation.

and carboxylic acid metabolites and no sulfydryl conjugates of zolpidem have been observed in humans (Durand et al. 1992). Furthermore, alpidem also exhibits potent inhibition of mitochondrial respiration and also depletes GSH in primary hepatocyte cultures, phenomena that are not observed with zolpidem even at high concentrations (Berson et al. 2001). Finally, as observed with clopidogrel and ticlopidine, the daily dose of zolpidem (5–10 mg QD) is significantly lower than that of alpidem (50–200 mg), a feature that may represent an additional and perhaps a more important mitigating factor.

The two catechol-*O*-methyltransferase inhibitors tolcapone and entacapone used for the treatment of Parkinson's disease also provide important illustrations of the effectiveness of this strategy. The use of tolcapone has been associated with a number of problems, including abnormalities in liver function tests and three cases of fatal hepatotoxicity. These problems have led to the drug being withdrawn from the market in some countries and the introduction of a black-box warning and intensive monitoring requirements in the United States. These ADRs, however, do not occur with the use of the structurally related drug entacapone despite administration at doses similar to tolcapone (200–1000 mg QD). Both tolcapone and entacapone are extensively metabolized in humans; a significant portion of tolcapone biotransformation proceeds via reduction of the nitrobenzene group to the aniline derivative, which is then transformed to the corresponding anilide by *N*-acetyltransferase (Jorga et al. 1999). Smith et al. (2003) have shown that both the aniline and the anilide metabolites of tolcapone undergo facile two-electron oxidation to the corresponding quinone-imine metabolites that are trapped with GSH (Figure 2.7). In contrast, no reduction of the nitrobenzene group in entacapone (see Figure 2.7) has been observed in humans and its principal clearance pathway involves N-deethylation of the tertiary amide substituent, isomerization of the active E-isomer to the inactive Z-isomer, followed by glucuronidation of the catechol motif (Wikberg et al. 1993).

A final example is provided with the two cysteinyl leukotriene antagonists CP-85958 and CP-199331 (see Figure 2.7). The clinical development of CP-85958 was discontinued due to unacceptable hepatotoxicity in monkeys (Chambers et al. 1999). Examination of monkey bile samples after dosing with CP-85958 revealed the presence of significant quantities of the corresponding lactol metabolite **6**, presumably generated from a P450-mediated hydroxylation $\alpha$ to the oxygen atom in the chromanol ring. Considering that the toxicity in the monkey may be mediated by the lactol derivative by ring opening to a potentially reactive hydroxyaldehyde intermediate **7**, which can bind to biomacromolecules, an obvious backup strategy was to eliminate this metabolic liability. Numerous structure–activity studies were conducted to prevent hydroxylation of the chromanol ring and efforts included blocking the site of hydroxylation or introducing substituents prone to metabolism at an alternate site in the molecule. Replacement of the benzoic acid portion in CP-85958 that was resistant to oxidative metabolism, with the more labile 4-methoxyphenyl-methanesulfonamide led to the discovery of CP-199331 that not only demonstrated enhanced efficacy against asthma in preclinical models, but also was devoid of hepatotoxic events in the monkey (Chambers et al. 1999). Metabolism studies in primate and human hepatocytes indicated that the

principal route of metabolism of CP-199331 involved O-demethylation of the labile anisole group followed by glucuronidation of the corresponding phenol; no hydroxylation on the chromanol ring in CP-199331 was discernible in the primate and human (Kuperman et al. 2001).

### 2.4.4. Modulation of Biochemical Reactivity via Steric Hindrance

Strategies incorporating substituents either directly on the structural alert or in its immediate vicinity to provide steric hindrance and/or modulate its electronic properties to minimize bioactivation have also been implemented. The success of the former methodology toward the elimination of biochemical reactivity is illustrated with carboxylic acid analogs, which form reactive acyl glucuronides. Idiosyncratic ADRs associated with many carboxylic acid-containing drugs have been attributed to the covalent modification of essential proteins by the corresponding acyl glucuronide metabolites. The covalent binding may occur via two different pathways (Figure 2.8). The first is a transacylation mechanism, where a nucleophilic amino acid on a protein attacks the carbonyl group of the primary acyl glucuronide leading to the formation of an acylated protein and free glucuronic acid. The second is a mechanism of Schiff base formation where condensation occurs between the aldehyde group of a rearranged acyl glucuronide with a lysine residue or an amine group of the N-terminus, leading to the formation of a glycated protein. The formation of the

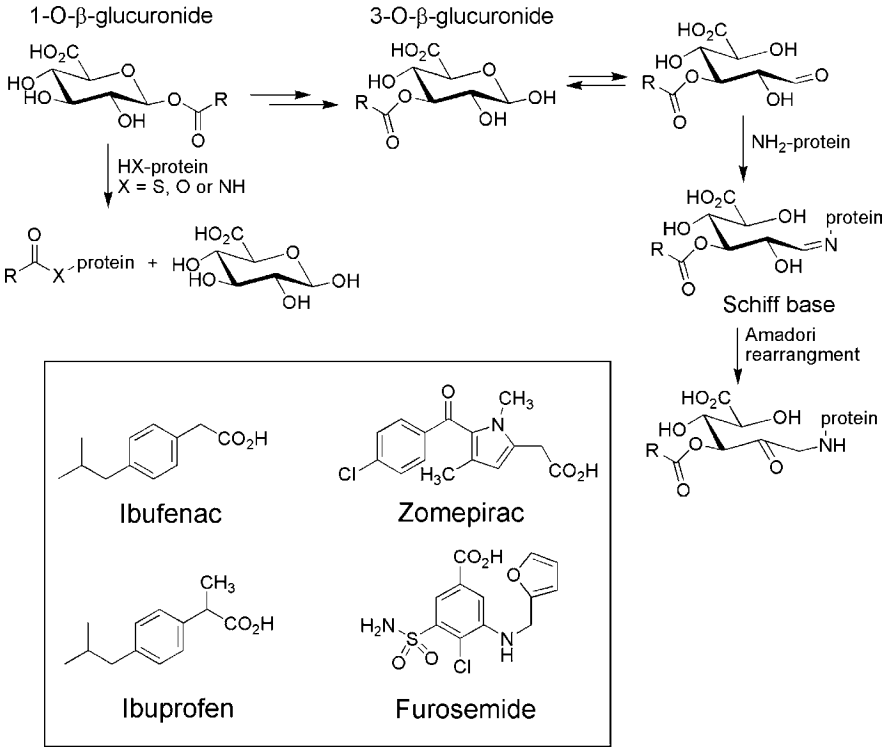


**Figure 2.8** Strategies to diminish biochemical reactivity of electrophiles via introducing steric hindrance.

iminium species is reversible but may be followed by an Amadori rearrangement of the imino sugar to the more stable 1-amino-2-keto product (Ding et al. 1993). A structural relationship between acyl glucuronide degradation to the Schiff base and covalent binding has also been proposed (Benet et al. 1993). Acyl glucuronides of acetic acid derivatives such as the NSAIDs ibufenac, tolmetin, and zomepirac, all of which have been withdrawn due to toxicity, exhibit the highest level of rearrangement and covalent binding, whereas mono-α-substituted acetic acids (2-substituted propionic acids) such as ibuprofen, considered to be the safest nonsteroidal anti-inflammatory drug (NSAID) exhibit intermediate level of acyl glucuronide rearrangement and covalent binding (Bolze et al. 2002; Wang et al. 2004). In contrast, benzoic acid derivatives (e.g., furosemide) indicate the least amount of rearrangement and covalent binding. Overall, these observations imply that inherent electronic and steric properties must modulate the rate of acyl glucuronide rearrangement. For example, resonance stabilization of the carboxylic acid group by the aromatic ring in benzoic acids could be the reason for the lowest extent of acyl glucuronide rearrangement, whereas 2-substituted propionic acid derivatives could display a slower rearrangement rate than the corresponding acetic acid analogs due to the steric hindrance provided by the α-methyl substituent. In this aspect, it is noteworthy to point out that while ibuprofen is considered to be the safest NSAID in the market, its close-in analogue ibufenac was withdrawn due to severe hepatotoxicity. The only structural difference between the two drugs is the presence of the α-methyl substituent in ibuprofen, which presumably slows the rearrangement of the glucuronide (Castillo and Smith 1995).

### 2.4.5. Modulation of Biochemical Reactivity via Changes in the Electronic Properties

Bioactivation of the alkylhalide substituents in inhaled anesthetics to extremely reactive acylating agents is usually due to the availability of an extractable hydrogen atom on the halogenated alkyl carbon. In susceptible patients, halothane, isoflurane, and desflurane can produce severe hepatic injury by an immune response directed against reactive acyl halides covalently bound to hepatic proteins (Satoh et al. 1989). However, the relative incidence of hepatotoxicity due to these agents appears to directly correlate with the extent of their conversion to acyl halides by P450, which in turn may be governed by the leaving group ability of the respective substituents within these drugs. As is seen in Figure 2.9, halothane, which exhibits the greatest incidence of hepatotoxicity in the clinic, undergoes the most conversion to reactive acyl chloride, a feature that can be attributed to the presence of bromide substituent, which is a good leaving group. In contrast, isofluorane and desfluorane also undergo oxidative metabolism resulting in the formation of reactive acyl halides, but the degree to which these anesthetics are bioactivated is significantly lower than halothane (Njoko et al. 1997). Thus, the lower yield of acyl halide formation with isofluorane may be traced back to changes in the electronic environment that reduce overall affinity toward metabolism or to the relatively poor leaving group ability of the difluoromethoxy group compared to the bromide.

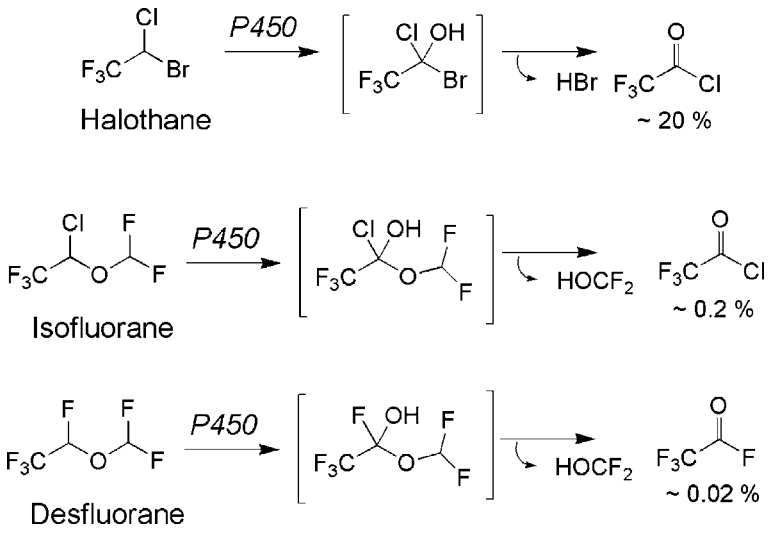


**Figure 2.9** Strategies to diminish biochemical reactivity of electrophiles via modulation of electronic properties.

## 2.5. Factors That Mitigate IADR Risks Associated with Drug Candidates Containing Structural Alerts

There are several other variables that will impact whether the presence of a putative structural alert in a drug candidate will eventually manifest in unanticipated toxicity. First of all, the susceptibility of a functional group to metabolic activation needs to be clearly understood, since there are several examples of commercially successful drugs, which do not exhibit toxicity despite containing structural alerts (Figure 2.10). In many such cases, the structural alert is not involved in metabolism and the primary clearance mechanism normally proceeds via metabolism at an alternate site or by nonmetabolic processes. In other instances, bioactivation may be discernible in standard in vitro systems such as liver microsomes, but the principal clearance mechanism in vivo may involve an altogether different and perhaps more facile metabolic pathway yielding nonreactive metabolites. For example, the two phenolic groups in the selective estrogen receptor modulator raloxifene undergo P4503A4-catalyzed bioactivation in human liver microsomes generating electrophilic quinonoid intermediates (Chen et al. 2002); however, in vivo, glucuronidation of the same phenolic groups in the gut and liver constitutes the principal clearance pathway of raloxifene in humans (Figure 2.10; Kemp, Fan and Stevens 2002). Thus, the likelihood of raloxifene bioactivation in vivo may be in question when compared with the phase II conjugation process, a feature that may provide an explanation for the rare occurrence of ADRs, despite administration at moderately high daily doses of 60 mg QD. When encountered with the challenge of progressing a drug candidate associated with reactive metabolite formation, due consideration also must be given to the intended therapeutic area (e.g., a major unmet medical need or a

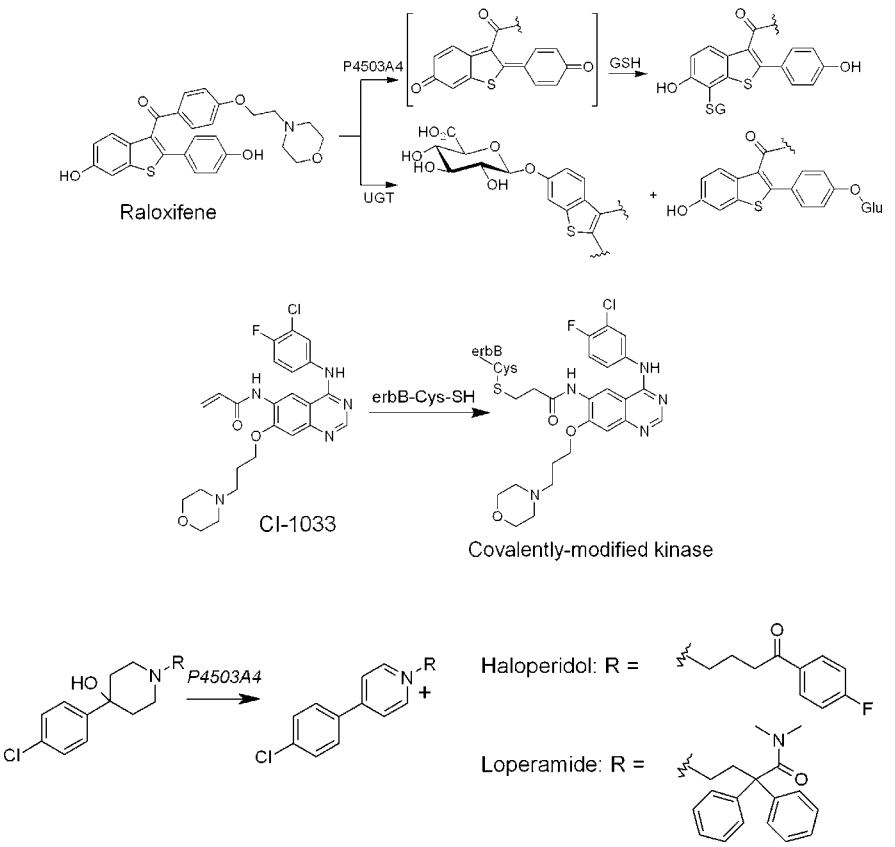


**Figure 2.10** Balancing bioactivation with variables such as therapeutic benefits, regimen, and alternate clearance mechanisms.

life-threatening disease) and usage (acute versus chronic therapy). For example, CI-1033 is an intrinsically electrophilic compound but has shown significant promise in the treatment of breast and other cancers. CI-1033 irreversibly inhibits all four members of the erbB receptor tyrosine kinase family via a chemical reaction of its α,β-unsaturated acrylamide group with a cysteine residue (Figure 2.10) in the ATP-binding pocket in these enzymes (Allen et al. 2003).

Drugs used in a chronic setting appear to be more prone to ADRs than those used in an acute setting. Because toxicity is evident only after few weeks of administration, agents that are administered for 2 weeks or less are rarely associated with bioactivation-related toxicities. This is illustrated with the widely used antidiarrheal agent loperamide that is rarely associated with ADRs, especially, tardive dyskinesia and Parkinsonism, despite its structural similarity to haloperidol and despite its P4503A4-catalyzed metabolism to a potentially neurotoxic pyridinium intermediate (Figure 2.10; Kalgutkar and Nguyen 2004). Plausible reason(s) for the lack of neurotoxic complications associated with loperamide use relative to haloperidol include (1) opiate activity that is restricted to the gastrointestinal tract, (2) therapy that usually last for a few days versus haloperidol use

in a chronic setting, and (3) the findings that loperamide and its positively charged pyridinium metabolite, but not haloperidol, are p-glycoprotein substrates and are denied access to the central nervous system (CNS), where they can potentially damage critical neurons.

Finally, the efficacious dose of the drug candidate may be pivotal as a factor mitigating the risk of toxicity. Type B ADRs are often referred to as dose-independent, but this certainly does not appear to be the case. For instance, the risk of hydralazine-induced lupus is dose-dependent, with a significant increase in frequency at doses >200 mg QD (Cameron and Ramsay 1984). Furthermore, there are no examples of drugs that are dosed below 10 mg $day^{-1}$ that cause IADRs. There are many examples of two structurally related drugs that possess a common structural alert prone to bioactivation, but the one administered at the lower dose is much safer than the one given at a higher dose (Figure 2.11) (e.g., the alpidem/zolpidem and the ticlopidine/clopidogrel comparisons, previously mentioned). Olanzapine forms the same reactive iminium species that is presumably thought to be responsible for clozapine-induced agranulocytosis (Gardner et al. 1998), yet occurrence of agranulocytosis with olanzapine is extremely rare. A major difference between the two drugs is the dose; clozapine is administered at doses exceeding several 100 mg QD, whereas the recommended daily dose of olanzapine is 10 mg QD.

Additional examples of potential low therapeutic dose as a mitigating factor for ADRs are depicted with tadalafil, a PDE5 inhibitor for the treatment of erectile dysfunction, the antidepressant paroxetine, the hypnotic quazepam, the antihypertensive prazosin, and the estrogen ethinylestradiol. The benzodioxalane group in tadalafil is bioactivated by P450 to the corresponding reactive catechol metabolite, a process that also results in the mechanism-based inactivation of P4503A4 activity in vitro (Ring et al. 2005). However, there are no reports of ADRs (especially

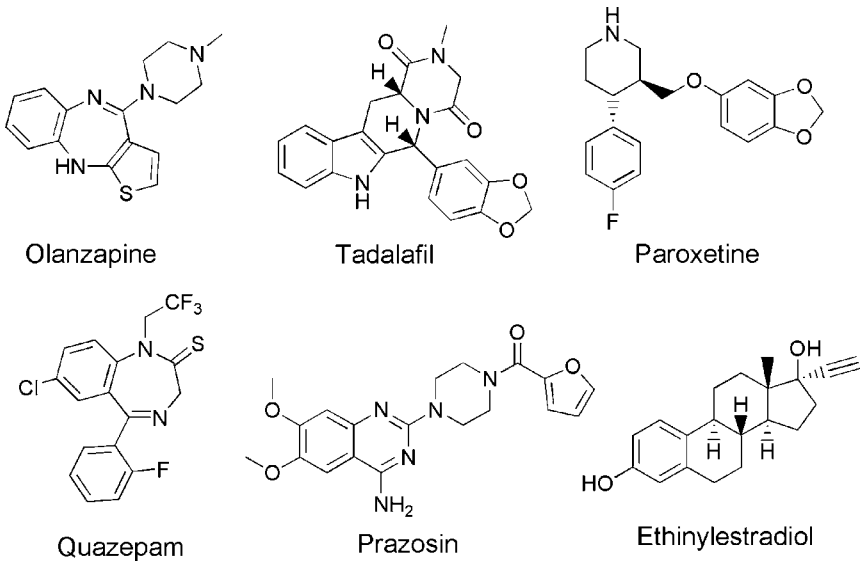


**Figure 2.11** Examples of low-dose drugs devoid of IADRs despite forming reactive intermediates.

hepatotoxicity) associated with tadalafil use at the recommended dose of 10–20 mg QD and, furthermore, tadalafil also does not produce significant changes in the clearance of drugs metabolized by P4503A4 (Ring et al. 2005). In a similar fashion, paroxetine undergoes a P4502D6-catalyzed scission of its benzodioxalane group to the reactive catechol metabolite, which is known to partition between further oxidation to reactive *O*-quinone [Dr. Chandra Prakash, Pfizer Global Research & Development, unpublished observations] or undergo O-methylation catalyzed by catechol-*O*-methyltransferase (Haddock et al. 1989). Cleavage of the benzodioxalane group in paroxetine also results in the mechanism-based inactivation of P4502D6 (Bertelsen et al. 2003). Despite these liabilities, ADRs including hepatotoxicity associated with paroxetine use are extremely rare (Carvajal et al. 2002), a feature that may be linked to a moderately low dose of 20–50 mg $day^{-1}$ and/or to the immediate detoxification of the catechol metabolite by O-methylation.

S-oxidation of the thioamide substituent in quazepam followed by hydrolysis of the potentially electrophilic S-oxide intermediate to the corresponding amide constitutes the rate-limiting step in the metabolism of the drug in humans (Kato et al. 2003), yet there are no reports of potentially life-threatening toxicity linked to the use of this agent, which is administered at a daily dose of 15 mg. Likewise, there are no adverse findings with the use of the antihypertensive agent prazosin at the recommended daily dose of 1 mg, despite the presence of the furan and the dimethoxybenzene rings in its structure (Jaillon 1980). Finally, ethinylestradiol, the major estrogenic component of many oral contraceptives, undergoes bioactivation at two different regions within its architecture (oxidation of the phenol and the acetylenic substituents to the corresponding catechol and ketene intermediates) that results in suicide inactivation of P4503A4 and a high degree of covalent binding to hepatic tissue (Lin, Kent and Hollenberg 2002), but does not elicit a toxicological response in the clinic. The extremely low oral dose of 0.035 mg must represent a significant mitigating factor.

## 2.6. Exploring Biochemical Mechanisms of Toxicity Other Than (or in Addition to) Bioactivation

There are cases where mechanisms other than (or in addition to) bioactivation can result in a toxicological outcome as was discussed earlier with the alpidem/zolpidem case. A second example is the bioactivation and formation of glutathione adducts for the nontricyclic antidepressant nefazodone. Severe hepatotoxicity resulting in death or the need for a liver transplant has been reported for nefazodone (Choi 2003) and the incidence (29 cases of hepatic injury per 100,000 patient years) far exceeds that associated with other antidepressants on the market (generally less than 4 cases of hepatic injury per 100 000 patient years). Recently, nefazodone was voluntarily withdrawn from the market. Following incubation of nefazodone with human liver microsomes and recombinant P4503A4 fortified with GSH, a GSH adduct associated with the 3-chlorophenyl piperazine moiety (Figure 2.12) was detected (Kalgutkar et al. 2005b). The authors suggested that formation of this GSH adduct was mediated via

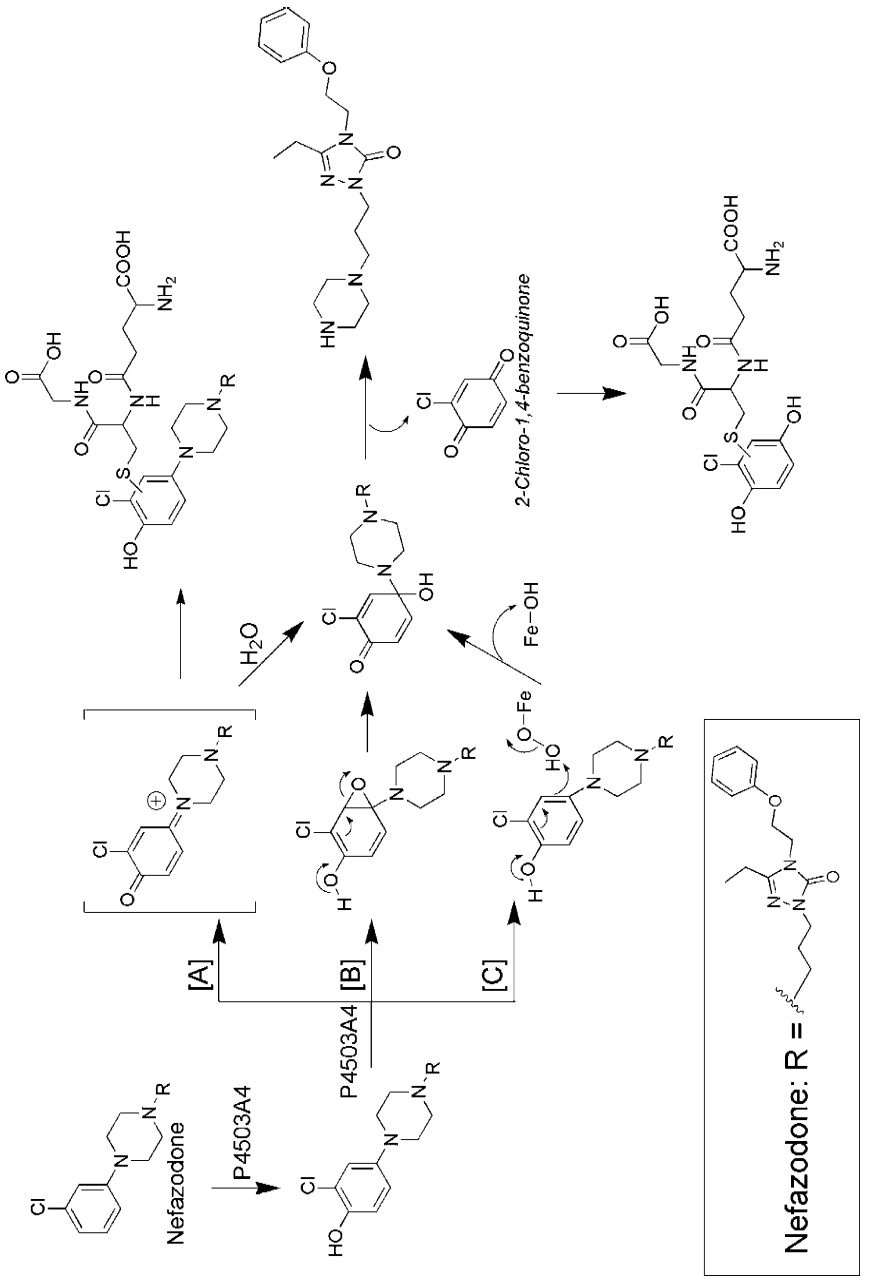


**Figure 2.12** Bioactivation pathways of the nontricyclic antidepressant agent nefazodone.

para-hydroxylation of the chlorophenyl ring followed by two-electron oxidation to the reactive quinone-imine (Figure 2.12, pathway A). A novel N-dearylated metabolite was detected as well and it was speculated that its formation should be accompanied by release of 2-chloro-1,4-benzoquinone. Indirect evidence for release of 2-chloro-1,4-benzoquinone was obtained via trapping of this intermediate with GSH (Figure 2.12, pathways B and C) and detection of the adduct by LC-MS/MS. Although these data point to a potential liability for nefazodone, it must be emphasized that an unambiguous link between the formation of reactive metabolites and the observed hepatotoxicity has not been established. Moreover, recent evidence suggests that the parent compound itself is responsible for the clinical hepatotoxicity (Kostrubsky et al. 2006). In vitro and in vivo studies were performed, which indicate that nefazodone inhibits the human bile salt export pump (BSEP) in the liver. Interestingly, the structurally related drug and anxiolytic agent buspirone, which is not associated with overt hepatoxicity, does not form GSH adduct(s) (Kalgutkar et al. 2005b) and is not a BSEP inhibitor (Kostrubsky et al. 2006). In vivo rat studies showed an increase in serum bile acids following administration of nefazodone, but not for buspirone. In vitro hepatocyte studies in the presence of 1-aminobenzotriazole (ABT), a nonspecific mechanism-based inhibitor of P450 enzymes, increased the cytotoxicity. ABT inhibited the hydroxylation of nefazodone, which is the first step in formation of GSH adducts. These two observations together suggest that the metabolic bioactivation is not the mechanism responsible for nefazodone toxicity. This example highlights the difficulty associated with finding the root mechanistic cause for toxicity.

## 2.7. Concluding Remarks

There can be little debate on the value of predicting potential adverse events associated with drug candidates as early as possible in the overall discovery/development process, since safety-related issues continue to significantly contribute to overall attrition statistics in the pharmaceutical industry. In an increasing number of cases, a broader understanding of the molecular basis for idiosyncratic pathomechanisms has aided to replace a vague perception of a class effect with a sharper picture of an individual molecular peculiarity. A low-risk strategy in drug discovery could potentially preclude the use of structural alerts susceptible to bioactivation altogether, but this is likely to limit a detailed exploration of SAR around a chemical series of interest. Thus, a proactive approach between medicinal chemists, pharmacologists, and metabolism scientists in establishing detailed pharmacological SAR alongside screening for bioactivation potential is more appropriate and balanced especially in the lead optimization stage. If the chemical matter is susceptible to bioactivation, then efforts to minimize metabolic activation by iterative chemical interventions should be considered. If an alternate structural series that does not form reactive metabolites is available, then this series could be progressed as a suitable replacement for further optimization of pharmacology and/or absorption, distribution, metabolism, and excretion (ADME) properties. The potential for toxicity of a new drug candidate depends on a variety of factors

including its overall disposition (extent of metabolism via bioactivation pathway relative to latent metabolic or nonmetabolic fate), daily dose (20 mg or less), therapeutic regimen (acute versus chronic), and the intended target population; factors that need to be taken into consideration when making a final decision to develop a drug candidate that is bioprocessed to reactive intermediates. Likewise, appropriate consideration needs to be given for drug candidates for potential treatment options for unmet and urgent medical needs. Despite decades of research in the arena of bioactivation and toxicity, accurate prediction of the toxicity potential of a drug candidate susceptible to bioactivation remains elusive. Future advances in the areas of immunology, genetics, and proteomics will hopefully provide a coherent relationship between covalent modification of biomacromolecules by reactive metabolites and a toxicity outcome. Toxicogenomics also represents a powerful new tool that may show gene and protein changes earlier and at treatment levels below the limits of detection of traditional measures of toxicity. Finally, considering that the meaning of the term "idiosyncrasy" literally translates to a characteristic belonging to, and distinguishing, an individual, the "one-size-fits-all" paradigm in drug discovery/development may need to be examined in greater detail.

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