# 3 Metabolic Activation of Organic Functional Groups Utilized in Medicinal Chemistry

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## 3.1 INTRODUCTION

Safety-related attrition continues to be a major concern in the pharmaceutical industry (Kramer et al., 2007). Of a total of 548 drugs approved in the period

*Mass Spectrometry in Drug Metabolism and Disposition: Basic Principles and Applications,* First Edition. Edited by Mike S. Lee and Mingshe Zhu.

from 1975 to 1999, 45 drugs (8.2%) acquired 1 or more black-box warnings and 16 (2.9%) were withdrawn from the market owing to idiosyncratic adverse drug reactions (IADRs) *that were not predicted from animal testing and/or clinical trials* (Lasser et al., 2002). IADRs (also known as type B ADRs) are unrelated to known drug pharmacology and are generally dose independent. Because the frequency of occurrence of IADRs is very low (1 in 10,000 to 1 in 100,000), these reactions are often not detected until the drug has gained broad exposure in a large patient population. *Importantly, standard regulatory animal toxicity studies have traditionally shown a poor concordance with occurrence of IADRs in humans* (Olson et al., 2000). Life-threatening IADRs noted for drugs include hepatotoxicity, severe cutaneous reactions, aplastic anemia, and blood dyscrasias. Many pharmaceutical companies have recognized this issue and have increased their efforts to implement predictive in vitro tools and identify potential safety liabilities earlier in the drug discovery process. In this way, drug candidates can be eliminated via chemical intervention or these compounds can be suspended from further development. One component of such assays is aimed at understanding a drug candidate's propensity to undergo reactive metabolite formation.

## 3.2 BIOACTIVATION OF DRUGS

Drugs are metabolized via oxidative, reductive, and hydrolytic pathways known as phase I reactions. These reactions lead to a modest increase in aqueous solubility. Phase II reactions, also known as conjugation reactions, modify the newly introduced functionality to form *O*- and *N*-glucuronides, sulfate, and acetate esters, all with increased hydrophilicity relative to the unconjugated metabolite. In most cases metabolism results in the loss of biological activity of the parent drug, and such metabolic reactions are therefore regarded as detoxication pathways. However, depending on the structural features present in some compounds, the same metabolic events on occasion can generate electrophilic, reactive metabolites. Reactive metabolites can be formed by most, if not all, of the drug-metabolizing enzymes. Common phase I oxidative and phase II conjugation enzymes involved in reactive metabolite formation include the cytochrome P450 (CYP) family of hemoproteins and uridine glucuronosyl transferases (UGTs), respectively. In some cases a single enzymatic and/or chemical reaction is involved, and in other cases several enzymatic and/or chemical reactions are involved in the formation of reactive intermediates. The biotransformation of inert chemicals to electrophilic, reactive metabolites is commonly referred to as metabolic activation (bioactivation) and is now recognized to be the rate-limiting step in certain kinds of chemical-induced toxicities. Inadequate detoxication of reactive metabolites is thought to represent a pathogenic mechanism for tissue necrosis, carcinogenicity, teratogenicity, and/or certain immune-mediated idiosyncratic toxicities.

The consequences of covalent binding of reactive drug metabolites to proteins as it relates to IADRs remain poorly understood, even after

some 40 years of research. In the case of acetaminophen, the dose-dependent hepatotoxicity observed in humans can be replicated in animals. For most other drugs, this is not the case; ADRs observed in humans cannot be reproduced in animals, which imply that there are no preclinical models to predict IADR potential of drug candidates. In addition, the downstream in vivo consequences of reactive metabolite formation and protein covalent modification as it relates to IADRs are poorly understood. Several hypotheses, however, have been proposed to explain these phenomena. The basic hypothesis that links the formation of reactive metabolites with IADRs (especially those with a possible immune component) is the process of haptenization wherein low-molecular-weight ($< 1000$ Da) reactive metabolites are converted to immunogens via binding to high-molecular-weight proteins as is the case with penicillin-induced anaphylactic reactions (Zhao et al., 2002). Examples of drugs associated with haptenization include halothane, tienilic acid, and dihydralazine, all of which are bioactivated to reactive metabolites and display mechanism-based inactivation of CYP isozymes responsible for their metabolism. Consistent with these observations, antibodies detected in sera of patients exposed to these drugs specifically recognize CYP isozymes and are responsible for their metabolism (Bourdi et al., 1992, 1996; Lecoeur et al., 1996).

Drug-metabolizing enzymes have evolved to biotransform a plethora of structurally diverse compounds encountered by the organism. These enzymes, however, cannot distinguish between molecules that are converted to reactive metabolites and those that are not. Furthermore, the likelihood of occurrence of bioactivation with a given compound will depend on several factors such as (1) the presence of functional group(s) (referred to as structural alerts or toxicophores) and/or chemical architecture, which is susceptible to bioactivation, (2) the presence of alternate metabolic soft spots within the molecule that compete with bioactivation, and (3) the detoxication of reactive metabolite and/or its precursor by metabolizing enzymes. The presence of structural alerts in a compound can be examined by visually inspecting the chemical structure or via the use of the DEREK software. DEREK for Windows is a knowledge-based expert system that is often used to identify structural alerts in a chemical (Dearden, 2003). Predictions are based largely on common occurrences of toxicophores in xenobiotics associated with some form of toxicity for which reactive metabolite formation has been observed in standard in vitro and in vivo systems.

## 3.3 EXPERIMENTAL STRATEGIES TO DETECT REACTIVE METABOLITES

With the possible exception of acyl glucuronides and cyclic iminium ions, most reactive metabolites are short-lived and are not detectable in circulation. Their formation can be inferred from the characterization of stable conjugates formed via reaction with the endogenous antioxidant glutathione (GSH). The presence

of the soft nucleophilic thiol group in GSH ensures efficient conjugation to soft electrophilic centers on some reactive metabolites [e.g., Michael acceptors (quinonoids, $\alpha,\beta$-unsaturated carbonyl compounds), epoxides (including arene oxides), alkyl halides, etc.] and yield stable sulfydryl conjugates. Qualitative in vitro assessment of reactive metabolite formation usually involves "trapping" studies conducted with reduced nicotinamide adenine dinucleotide phosphate (NADPH)-supplemented human liver microsomes and exogenously added GSH. Considering that nonmicrosomal enzymes can also participate in bioactivation, due consideration has to be given to the use of alternate metabolism vectors (e.g., liver cytosol, liver S-9 fractions, hepatocytes, neutrophils, etc.), which support the activity of non-CYP enzymes. It is noteworthy to point out that not all reactive metabolites can be trapped with GSH. "Hard" electrophiles including deoxyribonucleic acid (DNA)-reactive metabolites (e.g., aldehydes) will preferentially react with hard nucleophiles such as amines (e.g., semicarbazide and methoxylamine), amino acids (e.g., lysine), and DNA bases (e.g., guanine and cytosine) to afford the corresponding Schiff base (Sahali-Sahly et al., 1996; Olsen et al., 2005). Likewise, electrophilic iminium species, which are generated via metabolism of acyclic and cyclic tertiary amines, can be trapped using cyanide anion, which is a hard nucleophile (Gorrod et al., 1991; Argoti et al., 2005). Liquid chromatography tandem mass spectrometry is the preferred tool to detect reactive metabolite conjugates of nucleophiles (including exogenously added trapping agents and/or protein amino acids). Overall, characterization of the reactive metabolite conjugate structures provides an indirect insight into the structure of the reactive metabolite and the bioactivation pathway(s) leading to its formation.

Assessment of the amount of in vitro metabolism-dependent covalent binding to biological tissue is possible if radiolabeled drug is available (Evans et al., 2004). The assay provides quantitative estimates of radioactivity irreversibly bound to tissue but does not provide information about the nature of covalently modified proteins. 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 GSH and eliminated from the body. Once GSH is depleted, the extent of covalent binding with cellular macromolecules may increase rapidly, resulting in toxicity.

## 3.4 FUNCTIONAL GROUP METABOLISM TO REACTIVE INTERMEDIATES

### 3.4.1 Two-Electron Oxidations on Electron-Rich Aromatic Ring Systems

By far, one on the most common pathways of reactive metabolite formation is via an enzymatic two-electron oxidation process on aromatic rings containing

electron-rich functionalities in an *ortho* and/or *para* framework. The reactive intermediates derived from such bioactivation reactions can be generally categorized as quinone derivatives, which can react with GSH and/or protein nucleophiles in a typical 1,4-Michael fashion. Since 1,4-Michael addition to quinones represents a formal two-electron reduction process (regenerating the catechol/hydroquinone–nucleophile conjugate), their oxidant and electrophilic properties are intimately related. Many such enzymatic bioactivation reactions, leading to quinonelike species derived from, for example, electron-rich functional groups such as phenols, catechols, and aminophenols, have been efficiently mimicked via electrochemical techniques (Jurva et al., 2008; Madsen et al., 2008a, 2008b, 2007; Smith et al., 2003). Theoretical quantum chemical calculations have also been used to estimate the ease with which electron-rich aromatic systems undergo enzyme-catalyzed two-electron oxidations to reactive metabolites. The impact of adjacent aromatic substituents on the relative rates of oxidation can also be taken into account to rationalize differences in oxidation profiles. This approach has seen some success as demonstrated in the retrospective Ab initio analysis of acetaminophen oxidation (Koymans et al., 1989, 1990) and, more recently, with the atypical neuroleptic drug remoxipride (Erve et al., 2004). While not explored in great detail, there may be some additional scope for ab initio calculations in early discovery toward predicting oxidative instability of electron-rich aromatic ring systems as demonstrated in our studies on the hepatotoxic and nonhepatotoxic drugs, nefazodone and buspirone, respectively (Kalgutkar et al., 2005).

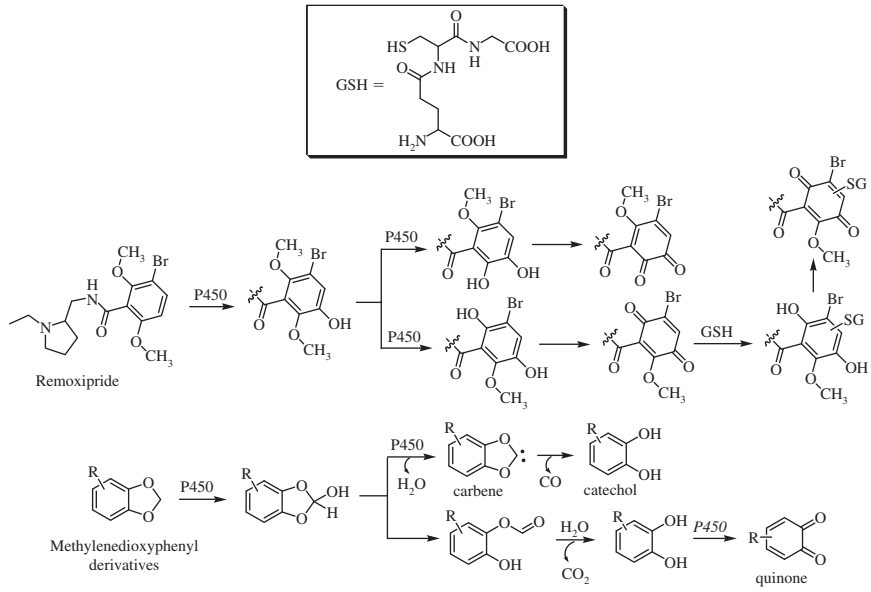
***3.4.1.1 Quinones and Quinone-Methides*** A classic way of generating *ortho*-benzoquinones is via the CYP-mediated biotransformation sequence benzene → phenol → catechol → *ortho*-benzoquinone. *para*-Hydroxylation of phenols would generate *para*-benzoquinones via the two-electron oxidation of the corresponding *para*-hydroquinone intermediates. Mono- and dialkoxyphenols where substituents are located in an *ortho*- or *para*-arrangement can undergo CYP-mediated *O*-dealkylation reactions to catechols/hydroquinones followed by oxidation to quinones. Presence of electron-withdrawing substituents confers stronger oxidant properties to the quinone, and the corresponding hydroquinone or catechol metabolites are less readily oxidized to the quinone. In contrast, electron-donating groups confer weaker oxidant properties on the quinone, and the corresponding hydroquinone or catechol metabolites are more readily oxidized. These properties are particularly important since the catechol/hydroquinone conjugates with nucleophiles such as GSH can undergo reoxidation to quinones followed by conjugation with a second molecule of GSH (Zhao et al., 2007). In the redox environment of biological systems, quinones may also cause toxicity through the formation of reactive oxygen species (Bolton et al., 2000; Monks and Jones, 2002).

There are numerous examples of endogenous molecules, xenobiotics, and drugs that form electrophilic quinones as metabolites, which in turn appear to be responsible for their toxicity as illustrated with the antipsychotic

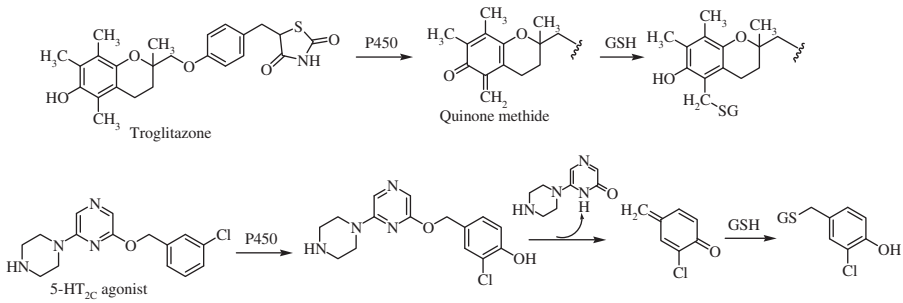
remoxipride, which has been withdrawn from commercial use due to cases of aplastic anemia associated with its use. CYP-mediated hydroxylation followed by *O*-demethylation in remoxipride generates the catechol and hydroquinone derivatives that are oxidized to quinones, amenable to trapping with GSH (Erve et al., 2004) (Scheme 1). An additional example, which will be discussed later is that of the selective estrogen receptor modulator raloxifene, wherein the electron-rich phenol architecture is oxidized to quinonoid species (Scheme 24) (Chen et al., 2002).

CYP-catalyzed demethylenation of the methylenedioxyphenyl (1,3-benzdioxole) group in natural products and/or medicinal agents also results in quinone formation via the intermediate catechol intermediate. The mechanism (see Scheme 1) involves an initial hydroxylation at the methylene carbon followed by partitioning between demethylenation yielding a catechol intermediate and formaldehyde/formate or dehydration to a carbene (Murray, 2000). Further oxidation of the catechol generates the *ortho*-benzoquinone species. The selective serotonin reuptake inhibitor paroxetine is a classic example of a drug that undergoes this pathway (Zhao et al., 2007). As such, the mechanistic details of quinone formation with paroxetine will be discussed later (see Scheme 25).

In an analogous fashion to catechols and hydroquinones, *ortho*- and *para*-alkylphenols undergo two-electron π oxidation to form quinone-methides. Quinone-methides possess a significantly reduced propensity for redox chemistry than corresponding quinones and are therefore much more reactive



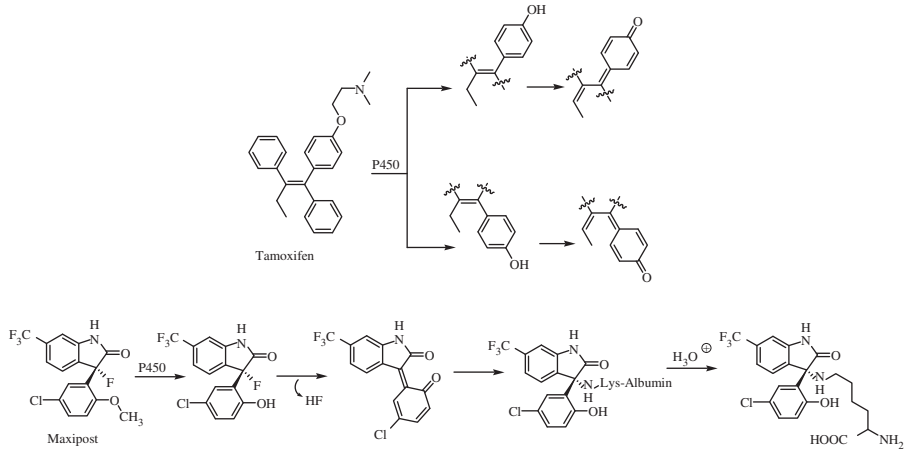

**SCHEME 1** Bioactivation pathways leading to quinone formation.




**SCHEME 2** Bioactivation pathways leading to quinone-methide formation.

electrophiles (Thompson et al., 1993). Quinone-methides react with nucleophiles in a typical Michael fashion at the exocyclic methylene carbon. As such the exocyclic methylene carbon in a quinone-methide can be considered as a hard electrophile, which can react with both hard (DNA base) and soft (GSH) nucleophiles. A basic requirement for quinone-methide formation includes the presence of an aromatic system where an alkyl and hydroxyl group are separated in either *ortho* or *para* arrangement. The alkyl group must contain an abstractable hydrogen atom for the two-electron oxidation to occur as illustrated with troglitazone (Scheme 2) (Kassahun et al., 2001). *It is noteworthy to point out that oxidation of the alkylphenol motif in troglitazone to the quinone-methide intermediate can also occur nonenzymatically* (Mingshe Zhu, personal communication). The presence of a suitable leaving group on the alkyl carbon can also render quinone-methide formation via an elimination reaction as evident in metabolism studies on the 5-hydroxytryptamine $(5\text{-HT})_{2C}$ agonist and potential antiobesity agent 2-(3-chlorobenzyloxy)-6-(piperazin-1-yl)pyrazine (Scheme 2) (Kalgutkar et al., 2007a). Elucidation of the structure of the GSH conjugate discerned in S9/NADPH and GSH-supplemented incubations of the compound is consistent with a bioactivation pathway that involves initial aromatic ring hydroxylation on the 3-chlorobenzyl motif followed by $\beta$ elimination to a quinone-methide, which is trapped by GSH.

Alkylbenzenes can also participate in this chemistry following aromatic hydroxylation by CYP enzymes. Likewise, alkoxyphenols can also form quinone-methides following oxidative dealkylation to the alkylphenol analogs as evident with tamoxifen (Scheme 3) (Fan et al., 2000; Fan and Bolton, 2001; Crewe et al., 2002). In one scenario, *O*-dealkylation occurs on the *N*, *N*-dimethylethylamine group to afford the phenol metabolite, which is oxidized to the quinone-methide. In another pathway, hydroxylation occurs on the aromatic ring geminal to the phenyl ether to the phenol metabolite, which upon further oxidation affords the quinone-methide. An interesting example of quinone-methide formation is highlighted in studies on the potassium channel opener maxipost (BMS-204352). *O*-Dealkylation of the methoxy group results in the phenol metabolite, which liberates the electrophilic *o*-quinone-methide

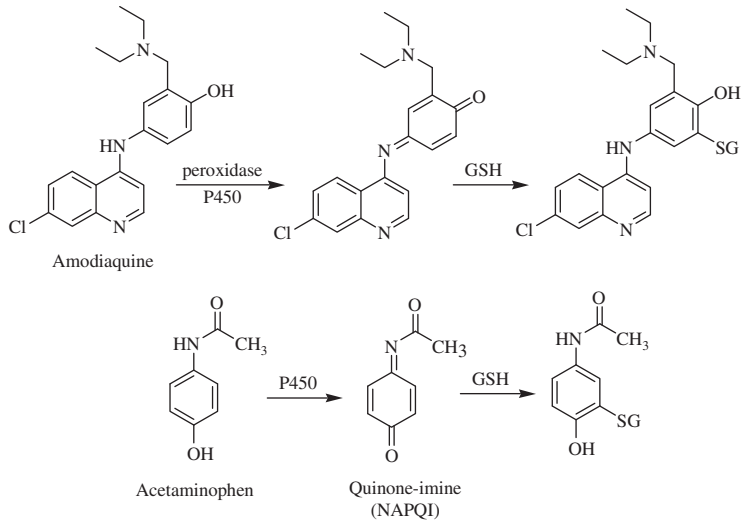



**SCHEME 3** Bioactivation of tamoxifen and maxipost to quinone-methide species.

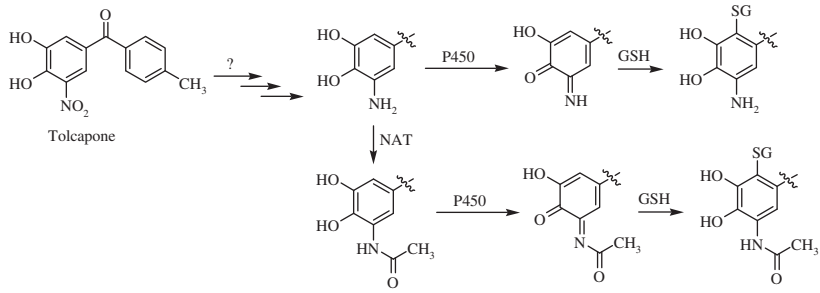
intermediate following the elimination of hydrogen fluoride (Scheme 3). The quinone-methide species is thought to be responsible for covalent binding of maxipost to albumin in vivo in animals and humans (Zhang et al., 2003, 2005). Acidic hydrolysis of plasma collected after intravenous administration of [$^{14}$C]-maxipost to rats and humans led to the characterization of a novel lysine conjugate of the des-fluoro des-*O*-methyl metabolite. The addition of lysine to the quinone-methide exocyclic carbon is consistent with the hard electrophilic character of the reactive species.

***3.4.1.2 Quinone-imines, imine-methides, and diiminoquinones*** *Ortho*- and *para*-quinone-imines are analogous to catechol and hydroquinone derivatives in that one of the carbonyl oxygen atoms is replaced by NH functionality. Quinone-imines are derived from the CYP-catalyzed two-electron oxidation of *ortho*- and *para*-aminophenols as evident in the CYP and peroxidase-catalyzed oxidation of the antimalarial agent amodiaquine (Scheme 4) (Maggs et al., 1988). Quinone-imine formation is also discerned with *ortho*- and *para*-acetamidophenols as illustrated with acetaminophen, a dose-dependent hepatotoxin in animals and humans. Two-electron oxidation of the *para*-acetamidophenol architecture in acetaminophen by CYP (Dahlin et al., 1984), peroxidases including myeloperoxidase (Corbett et al., 1992), and cyclooxygenase (Potter and Hinson, 1987) generates the electrophilic *N*-acetyl-*para*-benzoquinone imine (NAPQI) species that reacts with GSH (Scheme 4).

The metabolism of nitrobenzenes to quinone-imines arises from a six-electron reduction of the nitro group to the corresponding aniline metabolite via the intermediate nitroso and hydroxylamine analogs. Aromatic ring hydroxylation by CYP *ortho* or *para* to the aniline nitrogen then generates the aminophenol derivative. The conversion of a nitrobenzene derivative to a quinone-imine is illustrated with the catechol-*O*-methyltransferase inhibitor tolcapone, an



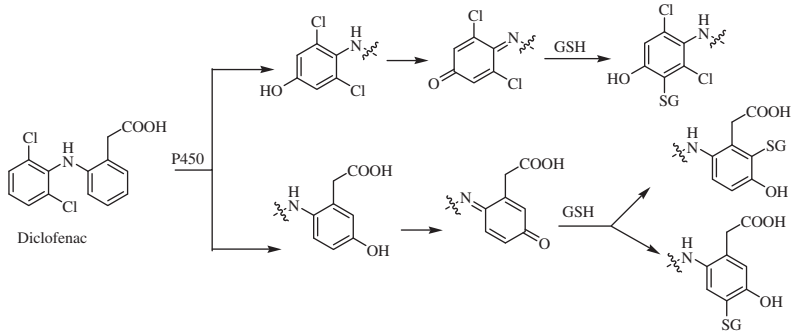

**SCHEME 4** Quinone-imine formation with amodiaquine and acetaminophen.



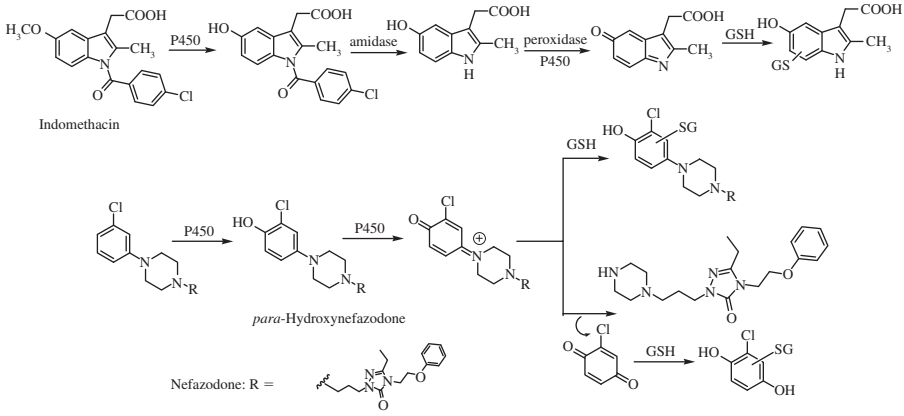

**SCHEME 5** Metabolism of *ortho*-hydroxynitrobenzene derivative tolcapone to a quinone-imine species.

*ortho*-hydroxynitrobenzene derivative. The nitro group in tolcapone is first reduced to the aniline metabolite followed by an *N*-acetyltransferase-catalyzed acetylation of the aniline nitrogen. Both the *ortho*-hydroxyaniline and acetanilide metabolites are oxidized to the corresponding quinone-imine species by CYP and peroxidase enzymes (Scheme 5) (Smith et al., 2003).

In some cases, quinone-imine formation requires an initial hydroxylation step as illustrated with the nonsteroidal anti-inflammatory drug and idiosyncratic hepatotoxin diclofenac. Thus, CYP-catalyzed aromatic hydroxylation *para* to the aniline nitrogen affords the *para*-hydroxydiclofenac isomers, which can then undergo CYP or peroxidase-mediated oxidation to the quinone-imines amenable to trapping with GSH (Scheme 6) (Tang et al., 1999; Miyamoto et al., 1997). The aniline nitrogen in quinone-imine derivatives can also be part of a heterocyclic

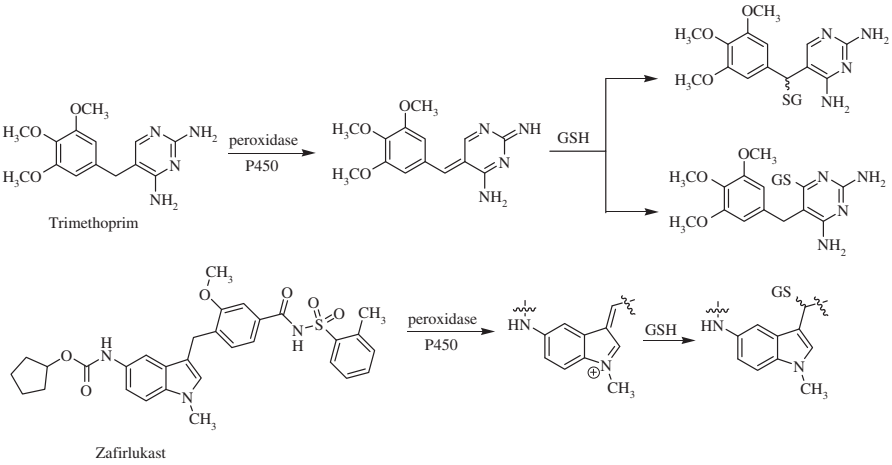



**SCHEME 6** Bioactivation of diclofenac to electrophilic quinone-imine species.




**SCHEME 7** Quinone-imine formation with indomethacin and nefazodone.

ring system as is evident with 5-hydroxyindole and *para*-hydroxyphenylpiperazine derivatives. The former situation is illustrated with the nonsteroidal anti-inflammatory drug (NSAID) and idiosyncratic hepatotoxin indomethacin. Indomethacin bioactivation is a multistep process that involves: (1) CYP-catalyzed *O*-demethylation, (2) hydrolysis of the *N*-acylindole motif, and (3) CYP- or peroxidase-mediated two-electron oxidation of the 5-hydroxyindole metabolite to the quinone-imine, which can be trapped with GSH (Scheme 7) (Ju and Uetrecht, 1998). An example of quinone-imine formation with a cyclic tertiary aniline-containing molecule is evident with the antidepressant and hepatotoxin nefazodone. The bioactivation is initiated by a CYP-catalyzed aromatic hydroxylation *para* to the piperazine nitrogen to generate *para*-hydroxynefazodone as a metabolite, two-electron oxidation, which generates the quinone-imine (Scheme 7) (Kalgutkar et al., 2005). In the case of nefazodone, the quinone-imine intermediate has been shown to undergo a hydrolytic

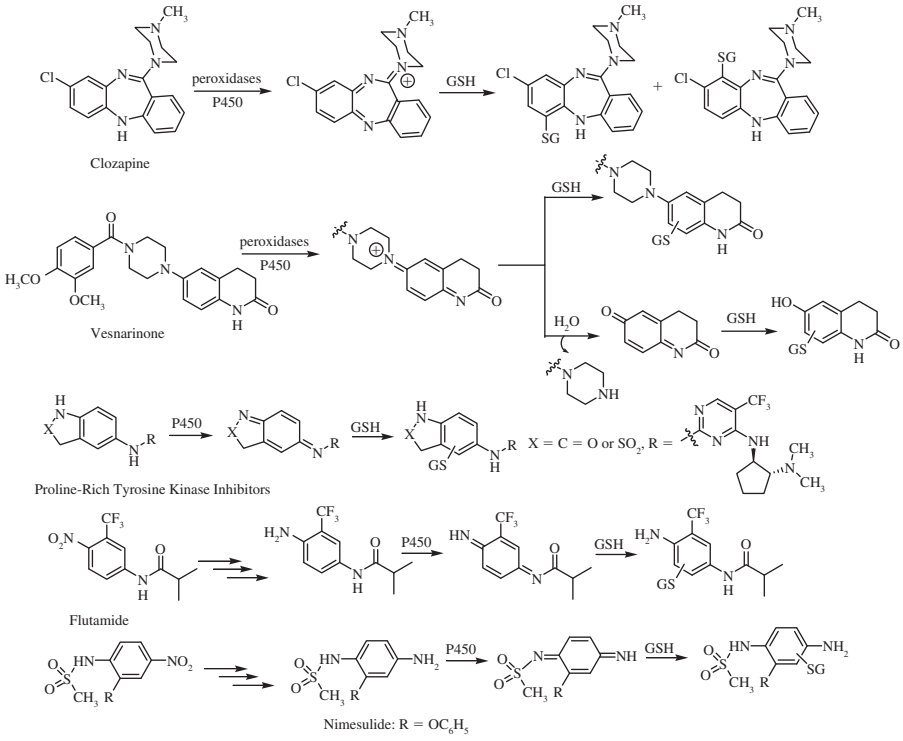



**SCHEME 8** Bioactivation pathways leading to imine-methide formation with zafirlukast and trimethoprim.

cleavage liberating the corresponding *meta*-chloro-*para*-benzoquinone derivative and the *N*-dephenylated metabolite (Scheme 7) (Kalgutkar et al., 2005).

Imine-methides are analogous to quinone-methides in that the phenolic OH group is replaced by an NH group. The underlying chemistry that accompanies two-electron $\pi$ oxidation of *ortho*- and *para*-alkylanilines to imine-methides is identical to that discerned with *ortho*- and *para*-alkylphenols, which yields quinone-methides. A classic example of a xenobiotic that undergoes imine-methide formation is evident with the pneumotoxin 3-methylindole. The pulmonary toxicity of 3-methylindole has been attributed to the action of the electrophilic imine-methide species 3-methyleneindolenine obtained by CYP-catalyzed dehydrogenation of the parent compound (Nocerini et al., 1985; Yan et al., 2007). Adducts of the imine-methide have been observed with thiol nucleophiles and DNA bases in microsomal incubations of 3-methylindole and exogenously added trapping agents (Nocerini et al., 1985; Yan et al., 2007), nucleosides, and DNA (Regal et al., 2001). The antiasthmatic drug zafirlukast is another example of a 3-methyleneindole derivative that is metabolized by CYP and peroxidases to an electrophilic imine-methide species, which can be trapped with GSH (Scheme 8) (Kassahun et al., 2005). An additional example of imine-methide formation is illustrated with the antibacterial agent trimethoprim. Unlike most imine-methides in which nucleophilic attack by GSH occurs almost exclusively on the exocyclic methylene carbon, in the case of trimethoprim, GSH preferentially adds to the pyrimidine ring (Scheme 8) (Lai et al., 1999).

Diiminoquinones are analogous to quinones in that both phenolic OH groups are replaced by NH substituents. A classic example of a drug, which forms a diiminoquinone metabolite is the antipsychotic agent clozapine wherein the *ortho-bis*-aniline framework undergoes a two-electron oxidation

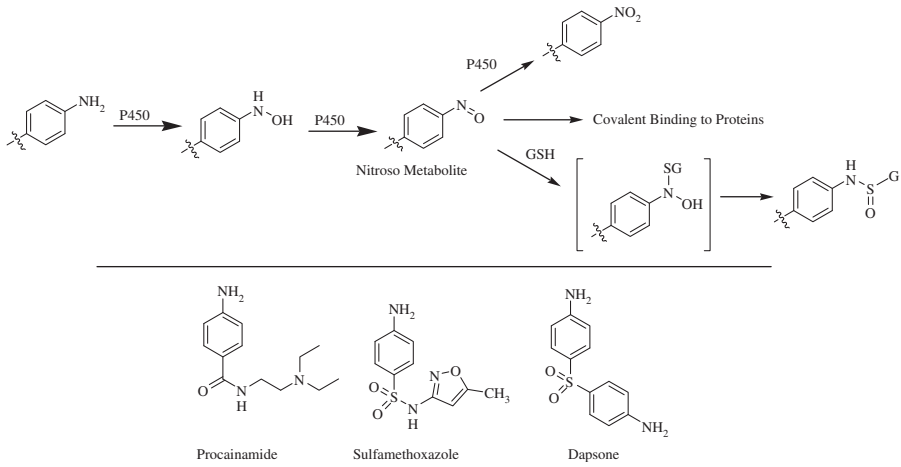



**SCHEME 9** Bioactivation pathways leading to diiminoquinones.

process by CYP and/or peroxidases to form the reactive intermediate that can be conveniently trapped with GSH (Scheme 9) (Uetrecht, 1992; Liu and Uetrecht, 1995; Gardner et al., 1998). *Ortho*- and *para*-acetamidoanilines can also participate in this chemistry as discerned with the cardiotonic agent vesnarinone (Uetrecht et al., 1994) and proline-rich tyrosine kinase (PYK 2) inhibitors (Walker et al., 2008) (Scheme 9). It is interesting to note that the diiminoquinone derivative in vesnarinone has been shown to undergo hydrolytic cleavage in a manner similar to the pathway elucidated with nefazodone (Scheme 9). Nitrobenzenes can also participate in the bioactivation pathway after reduction to the corresponding aniline metabolites as highlighted with flutamide (Wen et al., 2008) and nimesulide (Li et al., 2009) (Scheme 9).

### 3.4.2 *N*-Hydroxylation of Anilines

Besides participating in quinone-imine formation, primary anilines can also undergo an alternate CYP- or peroxidase-catalyzed bioactivation pathway that involves an initial *N*-hydroxylation on the aniline nitrogen to afford the *N*-hydroxylamine metabolite followed by further oxidation to a reactive nitroso species, which, in some cases, can be trapped with GSH (Scheme 10)



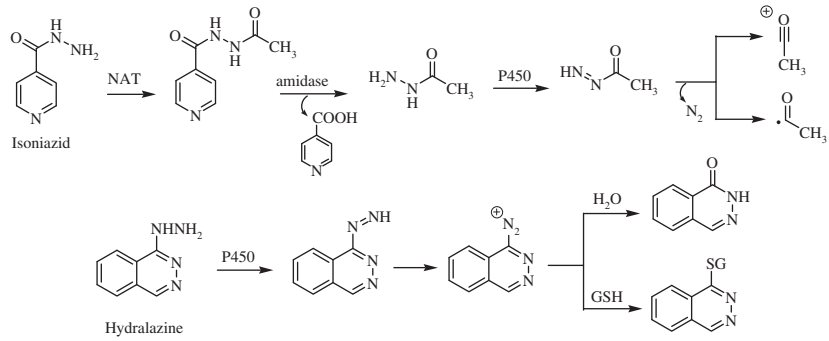

**SCHEME 10** *N*-Hydroxylation of anilines to reactive nitroso species.

(Uetrecht, 1985). Procainamide, sulfamethoxazole, and dapsone are noteworthy examples of aniline-containing drugs that exhibit this bioactivation pathway (Israili et al., 1973; Rieder et al., 1988). Masked anilines (e.g., anilides) can also generate electrophilic nitroso intermediates provided they first undergo enzymatic hydrolysis to form the primary aniline metabolite as observed with the β-adrenoceptor antagonist practolol (see Scheme 18) (Amos et al., 1978; Orton and Lowery, 1981).

### 3.4.3 Hydrazines

Drugs that contain a pendant hydrazine and/or hydrazide motif are notorious as CYP, peroxidase, and monoamine oxidase inactivators. Investigations on the mechanisms of toxicity associated with drugs that contain the hydrazine motif also implicate bioactivation of this functionality as a potential cause for tissue injury. For example, the relationship between hepatotoxicity and metabolism of the antituberculosis drug, isoniazid and its metabolites, *N*-acetylisoniazid and *N*-acetylhydrazine, in rats has been investigated and toxic doses of the radiolabeled metabolites were shown to bind covalently to liver protein in vivo. Pretreatment of the rats with the acylamidase/esterase inhibitor, *bis*-para-nitrophenyl phosphate, prevented the hydrolysis of *N*-acetyisoniazid to *N*-acetylhydrazine and isonicotinic acid and concomitantly decreased covalent binding (Timbrell et al., 1980). These results suggest that *N*-acetylhydrazine is the ultimate hepatotoxin, whose bioactivation via the intermediate *N*-acetyldiazine leads to the formation of acetylating species that reacts covalently with hepatic tissue (Scheme 11). Electron spin resonance studies have confirmed the formation of the acetyl free radical (see Scheme 11) in incubations of isoniazid in perfused rat livers.

A second example involves the antihypertensive drug hydralazine. Like other monosubstituted hydrazines, hydralazine is polymorphically acetylated




**SCHEME 11** Bioactivation of the hydrazine/hydrazide motif in drugs to reactive metabolites.

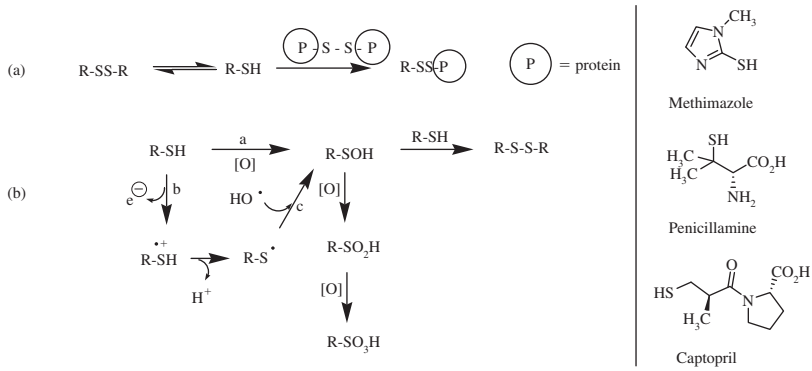
by *N*-acetyltransferase (NAT) in humans, and the manifestations of toxicity (lupus syndrome) occurs almost exclusively in slow acetylators who produce lesser amounts of acetylated metabolites and larger amounts of oxidized metabolites such as phthalazinone (Scheme 11). Evidence for the role of CYP in hydralazine bioactivation was obtained following oxidation of [$^{14}$C]-hydralazine in rat liver microsomes in the presence of NADPH, which led to reactive species (diazine and diazonium ion) that covalently bound to microsomal protein (Streeter and Timbrell, 1985). A competing detoxification pathway involves the spontaneous reaction of water or GSH with the diazonium intermediate leading to phthalazinone or the mercapturic acid conjugate, respectively.

### 3.4.4 Bioactivation of Reduced Thiols

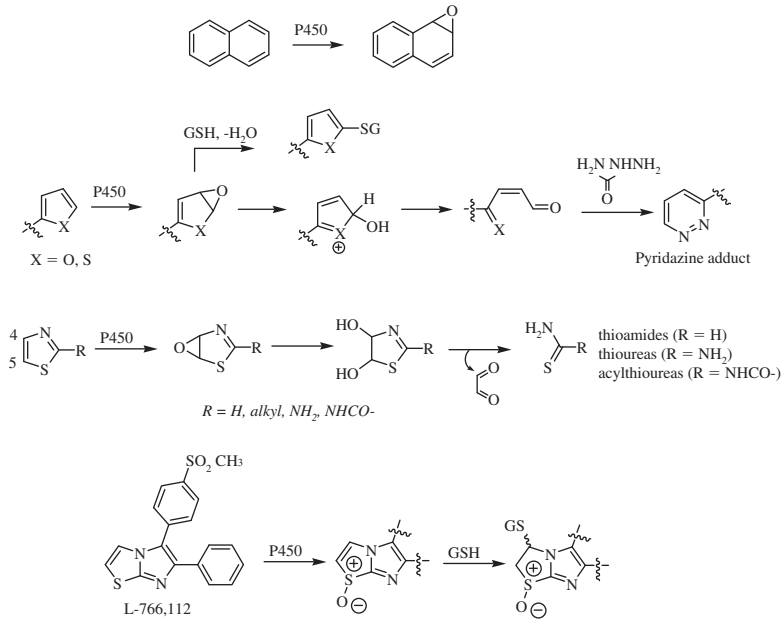
Free thiol groups react with cysteinyl-disulfide residues in proteins to form a new disulfide link with the concomitant release of a free cysteine residue within the protein (Scheme 12, pathway A). No metabolic activation is required for the covalent binding process to occur. Thiols are also readily oxidized to sulfenic acids, which react with cysteinyl residues and reduced GSH to form mixed disulfides (Scheme 12, pathway B) (Migdalof et al., 1984; Coleman et al., 1988). Methimazole, penicillamine, and captopril are examples of free thiol-containing drugs wherein evidence has been presented that traces toxicity of these compounds with oxidation of the thiol group.

### 3.4.5 Epoxidation of $sp^2$ and *sp* Centers

The isolation of a stable arene oxide metabolite of naphthalene in liver microsomal incubations provided evidence for the CYP- catalyzed epoxidation on the $sp^2$ carbons on a simple phenyl ring (Scheme 13). Carcinogenesis following exposure to polycyclic aromatic hydrocarbons (PAHs) such as benzo[*a*]pyrene is thought to arise from arene oxide formation. Arene oxides




**SCHEME 12** Oxidation of thiols to disulfides.




**SCHEME 13** CYP-catalyzed epoxidation of aromatic and heteroaromatic rings.

are usually unstable and undergo ring opening by mechanism of general acid catalysis, ultimately leading to phenols. In many instances, hydrolysis of the arene oxide by epoxide hydrolase or reaction with GSH results in the formation of dihydrodiol metabolites or dihydrohydroxy–GSH conjugates. Dehydration of the dihydroxy–GSH conjugate provides a stable rearomatized GSH adduct. Carbamazepine and alpidem represent noteworthy examples of drugs where a circumstantial link between toxicity and arene oxide formation exists

based upon characterization of stable sulfydryl conjugates both in vitro and in vivo in humans.

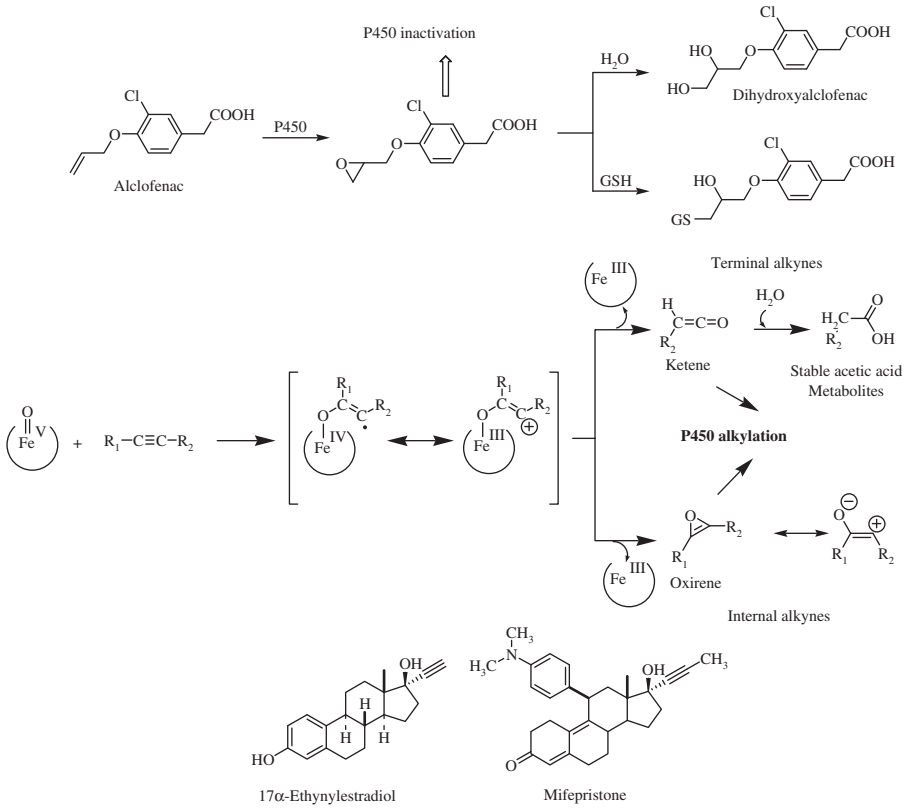
Heterocyclic rings (e.g., furans, thiophenes, and thiazoles) can also undergo epoxidation to yield electrophilic species. The mechanism involves an initial CYP-catalyzed 2,3- or 4,5-epoxidation on the furan/thiophene and thiazole rings, respectively, followed by ring opening and leads to the formation of electrophilic $\alpha,\beta$-unsaturated dicarbonyl metabolites (Scheme 13) (Zhang et al., 1996; Dansette et al., 2005; Peterson, 2006). Evidence for furan ring epoxidation has been obtained from metabolism studies on *R*-(+)-pulegone and *R*-(+)-menthofuran by human liver CYPs using $^{18}O2$ and $H2^{18}O$ and C-2 deuterium-labeled menthofuran (Khojasteh-Bakht et al., 1999). The electrophilic ,-unsaturated dicarbonyl metabolites react with soft nucleophiles via a 1,4-Michael addition across the $\alpha,\beta$-unsaturated dicarbonyl moiety or with hard nucleophiles via 1,2-addition to the aldehyde (Scheme 13) (Zhang et al., 1996; Khojasteh-Bakht et al., 1999; Erve et al., 2007).

Evidence for thiophene epoxidation has been derived from stable isotope studies on the diuretic tienilic acid and the NSAID suprofen (Scheme 13) (Koenigs et al., 1999; Dalvie et al., 2002; O'Donnell et al., 2003; Dansette et al., 2005). Incorporation of $^{18}O$ in the 5-hydroxythiophene metabolite of these compounds in microsomal incubations conducted with $^{18}O_2$ gas suggests that the source of oxygen in the 5-hydroxythiophene metabolite is exclusively derived from molecular oxygen and not from water. This is an important finding considering that previous studies have suggested thiophene bioactivation occurs exclusively via *S*-oxygenation to a Michael acceptor (Valadon et al., 1996). Additional evidence for epoxidation includes the trapping of the pyridazine derivative in microsomal incubations of suprofen (O'Donnell et al., 2003). The formation of the pyridazine conjugate of suprofen can be rationalized via the epoxidation and ring opening sequence to the $\gamma$-thioketo-$\alpha,\beta$-unsaturated aldehyde intermediate in a manner analogous to that discerned with furans.

As observed with furans and thiophenes, the C4-C5 epoxide in thiazole analogs can be hydrolyzed by epoxide hydrolase to the corresponding dihydrodiol intermediate. Ring scission of the dihydrodiol then occurs, resulting in the liberation of glyoxal and the corresponding thioamides, thiourea, and/or acylthiourea as metabolites depending on the C-2 substituent (Mizutani et al., 1994, 1996; Dalvie et al., 2002). Once formed, thiourea and acylthiourea metabolites can undergo *S*-oxidation to electrophilic sulfenic and/or sulfinic acid intermediates that can covalently modify or oxidize critical proteins leading to toxicity (Scheme 13) (Obach et al., 2008a). For many thiourea derivatives that covalently bind to microsomes, addition of GSH abolishes covalent binding via reduction of the sulfenic acid metabolites and the concomitant formation of oxidized GSH (i.e., GSSG). Thus, GSH can be involved in the detoxication of thioureas as a cellular reductant for their reactive intermediates. It is noteworthy to point out that thiazole ring oxidation can also result in the formation of an electrophilic *S*-oxide, which can covalently bind to proteins or exogenously added thiol nucleophiles as

demonstrated with L-766,112, a potent and selective cyclooxygenase-2 inhibitor (Scheme 13) (Trimble et al., 1997). Oxidative thiazole ring opening to reactive intermediates has also been proposed as the rate-limiting step in the mechanism-based inactivation of CYP3A4 by the human immunodeficiency virus (HIV) protease inhibitor ritonavir. This proposal is consistent with the observations that the HIV protease inhibitors indinavir and saquinavir, which do not possess the thiazole ring, are devoid of CYP3A4 inactivation properties (Kalgutkar et al., 2007b).

Olefins can also undergo CYP-catalyzed epoxidation; the NSAID alclofenac, which has been withdrawn from the clinic due to many cases of skin rash and hepatotoxicity, serves as a noteworthy example of this phenomenon. The epoxide metabolite of alclofenac (Scheme 14) has been detected in human urine as a stable metabolite (Slack and Ford-Hutchinson, 1980). The synthetic standard of the epoxide metabolite is a mutagen in the *Salmonella* Ames test and forms conjugates with sulfydryl nucleophiles (Slack et al., 1981; Mercier et al., 1983). The observation that the epoxide metabolite exhibits a NADPH-independent but

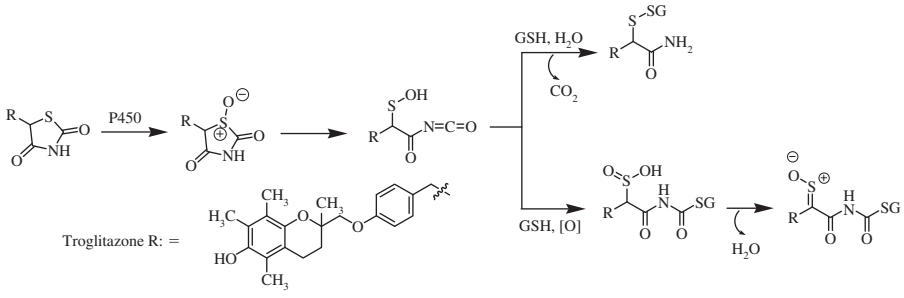



**SCHEME 14** CYP-catalyzed epoxidation of olefins and alkynes.

time-dependent loss in CYP activity suggests that this intermediate covalently modifies CYP directly and does not require further metabolic processing to reactive species consistent with the characteristics of an affinity-labeling agent (Brown and Ford-Hutchinson, 1982). The sp-hybridized carbon atoms on both terminal and internal alkynes are susceptible to epoxidation by CYP enzymes, which leads to the formation of reactive metabolites capable of alkylating CYP isozymes (Scheme 14) (Kalgutkar et al., 2007b). With terminal alkynes, the bioactivation mechanism involves oxygenation on the terminal carbon followed by a 1,2-hydrogen shift of the terminal hydrogen to the vicinal carbon to form a reactive ketene intermediate. The ketene can acylate nucleophilic residues within the CYP active site resulting in enzyme inactivation or undergo hydrolysis to the acetic acid metabolite detectable as the stable downstream product of terminal alkyne metabolism. With internal alkynes, oxidation on the internal carbon is followed by rearrangement to the oxirene, which then leads to enzyme alkylation. Synthetic estrogens 17$\alpha$-ethynylestradiol (a terminal alkyne) and mifepristone (an internal alkyne) are examples of drugs that are epoxidized on the alkyne by CYP3A4 to afford reactive metabolites, which covalently adduct to the CYP isozyme (see Scheme 14).

### 3.4.6 Thiazolidinedione Ring Bioactivation

Enzymatic conversion of the thiazolidinedione ring system to reactive metabolites was first documented with the antidiabetic agent and idiosyncratic hepatotoxin troglitazone (Kassahun et al., 2001). The mechanism of thiazolidinone ring opening (Scheme 15) involves CYP-mediated *S*-oxygenation to yield a sulfoxide intermediate, which spontaneously ring opens to the highly electrophilic $\alpha$-ketoisocyanate derivative. Isocyanate hydrolysis followed by decarboxylation to the amide, accompanied by attack of GSH on the reactive sulfenic acid, would afford one GSH conjugate. Alternatively, conjugation of the isocyanate with GSH, followed by oxidation to the corresponding sulfinic acid derivative, provides a route for a second GSH conjugate. Dehydration of the sulfinic acid intermediate is thought to lead to the formation of a third

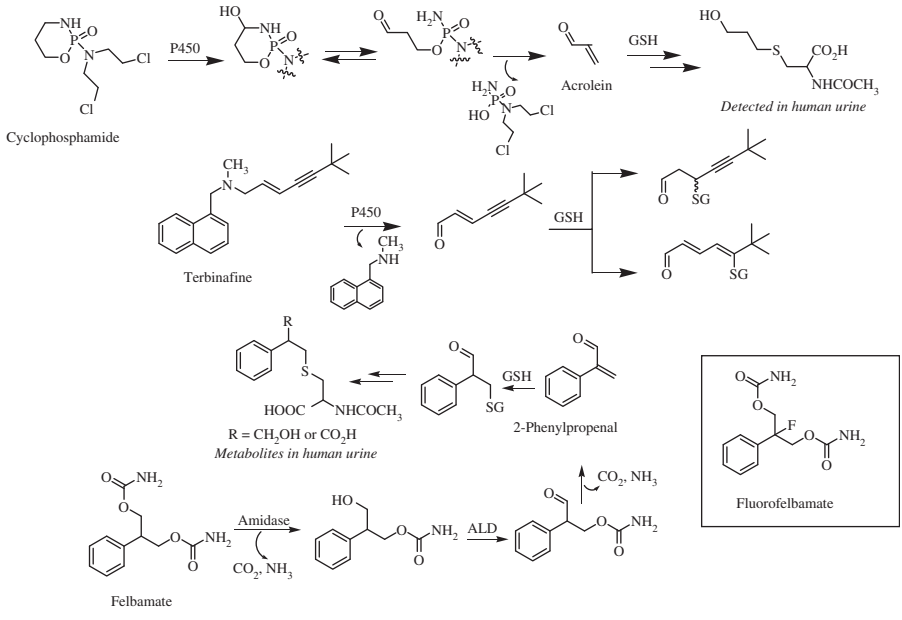



**SCHEME 15** CYP-catalyzed thiazolidinedione ring scission.

sulfine-containing GSH conjugate. In separate studies, comparison of the hepatotoxic and nephrotoxic potential of imidazolinedione, oxazolidinedione, and thiazolidinedione analogs of the nephrotoxic fungicide, *N*-(3,5-dichlorophenyl)succinimide in rats after in vivo administration indicated that only *N*-(3,5-dichlorophenyl)thiazolidinedione caused hepatic damage (Kennedy et al., 2003). It is conceivable that bioactivation of the thiazolidinedione ring to electrophilic intermediates is responsible for the observed hepatotoxicity in rats.

### 3.4.7 α,β-Unsaturated Carbonyl Compounds

α,β-Unsaturated carbonyl compounds are usually avoided in medicinal chemistry because of their intrinsic electrophilic nature. However, there are several instances where in the process of metabolism latent drugs are converted into α,β-unsaturated carbonyl intermediate(s). In most cases these reactive intermediates are believed to be responsible for the toxicological consequences associated with the parent drug. Noteworthy examples include cyclophosphamide, terbinafine, and felbamate. With cyclophosphamide, β-elimination reaction following an initial CYP-mediated hydroxylation affords the corresponding α,β-unsaturated acrolein metabolite (Scheme 16) (Ramu et al., 1995). The characterization of a hydroxypropyl mercapturic acid conjugate (see Scheme 16) in human urine is consistent with the notion that this bioactivation

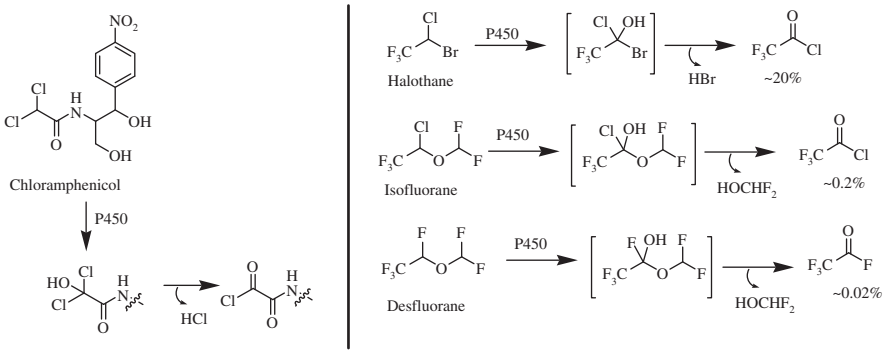



**SCHEME 16** Generation of reactive, α,β-unsaturated carbonyl metabolites in the course of drug metabolism.

pathway operates in vivo. In the case of the antifungal agent and idiosyncratic hepatotoxin terbinafine, a simple CYP-catalyzed *N*-dealkylation reaction results in the formation of the α,β,γ,δ-unsaturated aldehyde metabolite, which can be trapped in a 1,4- and 1,6-Michael fashion with GSH to yield the corresponding sulfydryl adducts (Scheme 16) (Iverson and Uetrecht, 2001). Circumstantial evidence linking felbamate bioactivation to its toxicity (aplastic anemia and hepatotoxicity) has been presented by means of the in vivo characterization of mercapturic acid (downstream metabolites of GSH) conjugates of a highly reactive α,β-unsaturated carbonyl derivative 2-phenylpropenal following felbamate administration to rats and human (Scheme 16) (Dieckhaus et al., 2002). The mechanism is believed to involve esterase-mediated hydrolysis of one of the carbamate groups to afford the primary alcohol metabolite; oxidation of which by alcohol dehydrogenase to the intermediate aldehyde derivative followed by spontaneous β-elimination of the remaining carbamoyl group generates 2-phenylpropenal. Consistent with this finding, fluorofelbamate (see Scheme 16) does not succumb to bioactivation because the fluorine atom prevents the β-elimination process, which leads to the reactive 2-phenylpropenal (Parker et al., 2005).

### 3.4.8 Haloalkanes

Alkyl halides are rarely utilized as pharmacophores in drug design because of the susceptibility of halogens toward enzymatic and nonenzymatic nucleophilic displacement reactions as illustrated with chloramphenicol and inhaled anesthetics (e.g., halothane, isofluorane, and desfluorane). In the case of chloramphenicol, CYP inactivation results from the formation of the reactive acylating agent oxamyl chloride during oxidative dechlorination by CYP (Scheme 17). The characterization of oxalic and oxamic acid metabolites of chloramphenicol analogs upon alkaline hydrolysis of inactivated protein samples and the finding that the difluoromethyl derivative of chloramphenicol does not inactivate CYP




**SCHEME 17** Bioactivation of haloalkanes to reactive metabolites.

lends further credence to the proposed pathway of inactivation (Miller and Halpert, 1986). Bioactivation of haloalkane substituents in inhaled anesthetics to reactive acylating agents is usually due to the availability of an extractable hydrogen atom on the halogenated alkyl carbon. The relative incidence of idiosyncratic hepatotoxicity due to these agents appears to directly correlate with the extent of their conversion to acyl halides by CYP, which in turn may be governed by the leaving group ability of the respective substituents within these drugs (Njoko et al., 1997). As seen in Scheme 17, 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 due to the relatively poor leaving group ability of the difluoromethoxy group compared to the bromide.

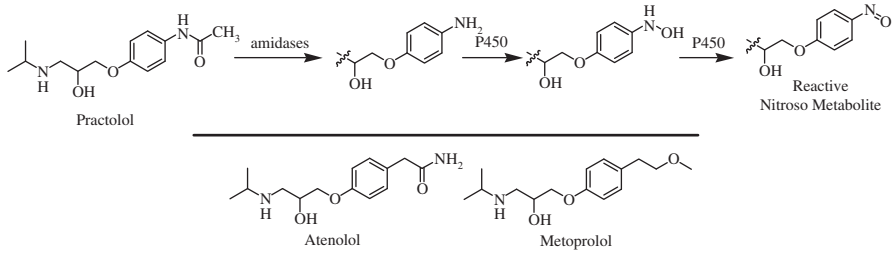
### 3.4.9 Carboxylic Acids

UGT-catalyzed glucuronidation of the carboxylic acid group in drugs results in the formation of acyl glucuronides, which are intrinsically electrophilic in nature. Protein modification can occur via a simple transacylation reaction with a protein nucleophile(s) or by acyl migration within the β-*O*-glucuronide unit to a reactive aldehyde intermediate. Detailed mechanistic discussion on this issue is provided with the NSAIDs ibufenac and ibuprofen in the following section.

## 3.5 STRUCTURAL ALERTS AND DRUG DESIGN

Anecdotal evidence obtained from visual analysis of structures of several closely related toxic and nontoxic drugs suggests that drugs, which lack toxicophores, have a superior safety record, especially with regards to IADRs (Kalgutkar and Soglia, 2005). The evidence becomes even more compelling when metabolism data supports the hypothesis as illustrated with the cardioselective β-adrenoceptor antagonists practolol, atenolol, and metoprolol. The mechanism of severe skin rashes induced by practolol is uncertain; however, a role for antinuclear antibodies, elicited by protein adducts of a reactive nitroso metabolite obtained from practolol biotransformation has been suspected (Scheme 18) (Amos et al., 1978; Orton and Lowery, 1981) in keeping with the observation that *cutaneous IADRs are not observed with atenolol and metoprolol, which lack the anilide toxicophore*. Consistent with this hypothesis are the findings that atenolol and metoprolol are metabolized by completely different pathways and are also subject to extensive urinary excretion as parent drugs (Borchard, 1990).

A second example is provided with the dibenzodiazepine derivatives and antipsychotic agents clozapine and quetiapine (Seroquel). While clozapine use

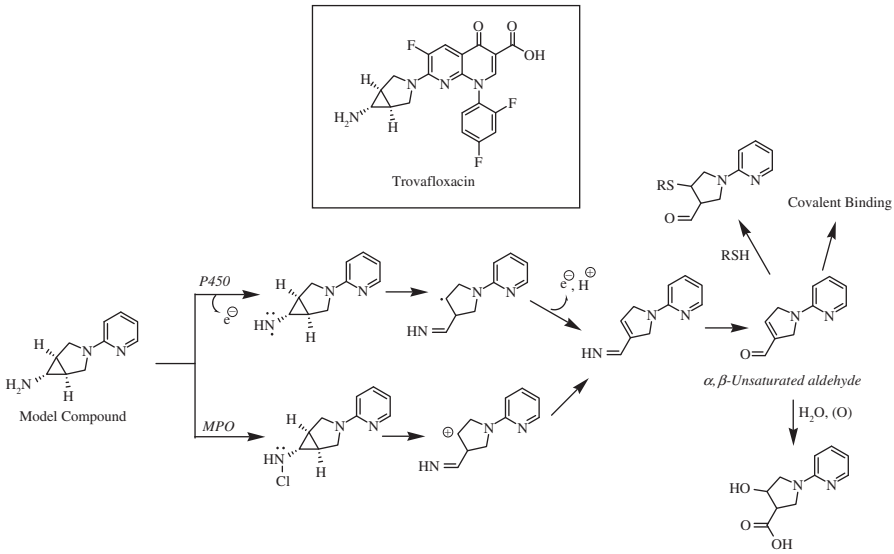



**SCHEME 18** Structure–toxicity relationships with β-adrenoceptor antagonists practolol, atenolol, and metoprolol.

is limited by a high incidence of agranulocytosis and hepatotoxicty, quetiapine does not cause these toxic events. As demonstrated earlier (see Scheme 9), evidence has been presented that links clozapine toxicity to its propensity to form a reactive imine (Liu and Uetrecht, 1995; Uetrecht, 1992). Proteins covalently modified with clozapine were also observed in neutrophils of patients being treated with clozapine, which reaffirms the relevance of the in vitro studies (Gardner et al., 1998). In the case of quetiapine the bridging nitrogen in the benzodiazepine ring is replaced with a sulfur atom; consequently this drug is not bioactivated to the reactive iminium species as shown with close-in structural clozapine analogs (Uetrecht et al., 1997). Despite administration at doses comparable to clozapine, cases of agranulocytosis with quetiapine are extremely rare.

A third example becomes evident upon comparison of trovafloxacin with related fluoroquinolone antibiotics. The rare but serious idiosyncratic hepatotoxicity (~14 cases of acute liver failure; 4 patients required liver transplants, and an additional 5 died of liver-related injuries) led to the withdrawal of trovafloxacin from the market in many countries and a black-box warning with intensive monitoring requirements in the United States. Microarray analysis (Liguori et al., 2005) revealed substantial gene expression changes following treatment of human hepatocytes with trovafloxacin as compared to other marketed fluoroquinolones. The expression profile induced by trovafloxacin was markedly distinct from other fluoroquinolones in that genes involved in oxidative stress were regulated consistently by trovafloxacin. In HepG2 cells, trovafloxacin also induced oxidative stress and depleted intracellular glutathione levels to a greater extent than other fluoroquinolones (Liguori et al., 2008). A potential role of inflammatory mediators in trovafloxacin hepatotoxicity has also been established (Waring et al., 2006).

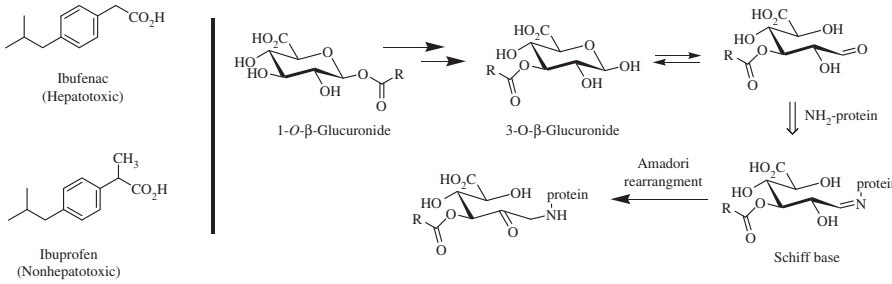
With the exception of trovafloxacin, none of the other drugs in the fluoroquinolone class of antibiotics have been associated with idiosyncratic hepatotoxicity. From a structure–toxicity standpoint, it is interesting to note the presence of the cyclopropylamine structural alert at the C7 position of the fluoroquinolone scaffold in trovafloxacin. Studies with a model compound have revealed cyclopropylamine ring bioactivation by CYP and myeloperoxidase to a reactive α,β-unsaturated aldehyde trapped as a sulfydryl conjugate




**SCHEME 19** Insights into trovafloxacin bioactivation via the use of a model compound containing the cyclopropylamine functionality.

(Scheme 19) (Sun et al., 2008). The characterization of a hydroxycarboxylic acid metabolite of trovafloxacin in preclinical species (Dalvie et al., 1996) lends further support for the metabolism of the cyclopropylamine ring in trovafloxacin to a reactive intermediate. The formation of the hydroxycarboxylic acid can occur from the addition of water to the α,β-unsaturated aldehyde via Michael addition followed by oxidation as depicted for the model compound (Scheme 19). However, the proposal for reactive metabolite formation with trovafloxacin remains a speculation since the bioactivation studies did not involve the parent fluoroquinolone and no α,β-unsaturated aldehyde or the corresponding glutathione conjugate has been detected in trovafloxacin incubations in human liver microsomes (Sun et al., 2008). Furthermore, the primary pathways of trovafloxacin clearance in humans include phase II metabolism (*N*-acetylation, acyl glucuronidation, and *N*-sulfation) (Scheme 19) with very minor contributions from phase I oxidative pathways (Dalvie et al., 1997).

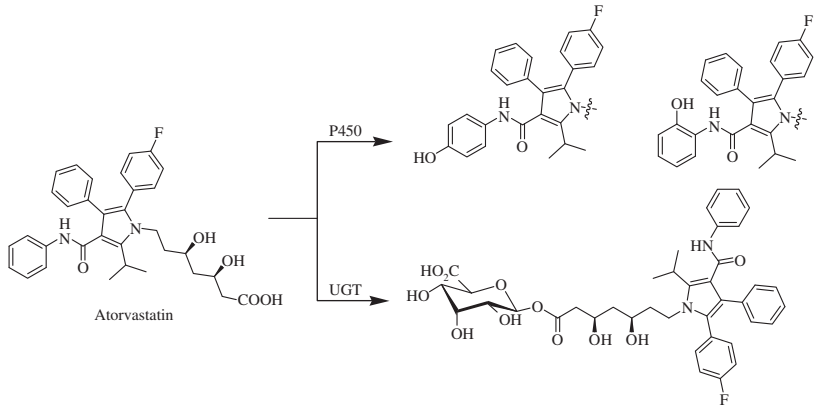
A final and perhaps an even more intriguing example of the influence that a subtle structural change can have on toxicity is highlighted with ibuprofen and ibufenac. While ibuprofen is considered to be one of the safest over-the-counter NSAID on the market, its close-in analog ibufenac was withdrawn due to severe hepatotoxicity. The daily doses of both NSAIDs were comparable and the only structural difference between the two drugs is the presence of the additional α-methyl substituent adjacent to the carboxylic acid moiety in ibuprofen (Scheme 20). Glucuronidation of the carboxylic acid moiety in most NSAIDs to the potentially electrophilic acyl glucuronide constitutes the



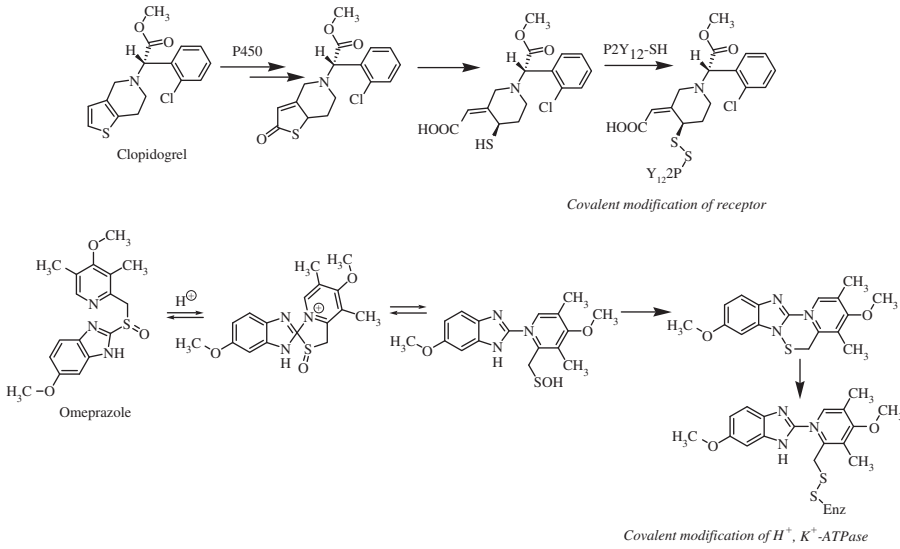

**SCHEME 20** Reaction of acylglucuronides with proteins.

principal elimination mechanism in vivo in humans (Johnson et al., 2007; Ding et al., 1993; Benet et al., 1993). As mentioned earlier, the proposed pathway of acyl glucuronide adduction with proteins involves condensation between the aldehyde group of a rearranged acyl glucuronide and a lysine residue or an amine group of the *N*-terminus, leading to the formation of a glycated protein. The formation of the 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 (see Scheme 20) (Walker et al., 2007; Wang et al., 2004; Ding et al., 1993). A structural relationship between acyl glucuronide degradation to the Schiff base and covalent binding has been established (Walker et al., 2007; Wang et al., 2004). Acyl glucuronides of ibufenac and other acetic-acid-based NSAIDs such as 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 exhibit intermediate level of acyl glucuronide rearrangement and covalent binding. Overall, these observations imply that inherent electronic and steric properties must modulate the rate of acyl glucuronide rearrangement. Thus, in the case of ibuprofen, it is likely that the presence of the α-methyl substituent slows the rearrangement of the acyl glucuronide to the electrophilic carbonyl intermediate.

In retrospect, the examples discussed above imply that by avoiding toxicophores in drug design, one would lessen the odds that a drug candidate will lead to toxicity via a bioactivation mechanism. From a medicinal chemistry standpoint, this strategy seems to be an attractive option and a path forward toward the discovery of safer drugs, especially given the lack of methodology to predict IADRs. As noted earlier, an exhaustive listing of structural alerts is far too comprehensive and also includes a simple phenyl ring. Likewise, a strategy that avoids structural alerts altogether can lead to a missed opportunity to develop potentially important medicines. Atorvastatin (Lipitor) provides an example of such a scenario as the structure not only contains the acetanilide structural alert but metabolism by CYP3A4 results in the formation of acetaminophen-like metabolites (Scheme 21) (Jacobsen et al., 2000). Furthermore, glucuronidation of the carboxylic acid moiety results in the formation of




**SCHEME 21** Chemical structures of atorvastatin and its metabolites derived from oxidative and conjugation pathways.




**SCHEME 22** Examples of commercial blockbuster drugs that require reactive metabolite formation for their pharmacologic action.

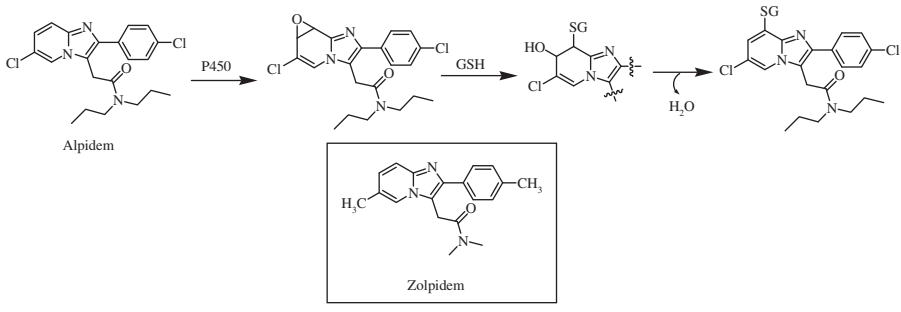
the potentially electrophilic acyl glucuronide (Prueksaritanont et al., 2002) in a manner similar to that discerned with NSAIDs (see Scheme 21).

Finally, it is pivotal to point out that several blockbuster drugs contain toxicophores, which form reactive metabolites and covalently adduct to proteins, which in some cases is essential for pharmacological activity. For instance, the blockbuster cardiovascular drug and $P2Y_{12}$ antagonist clopidogrel (Plavix) by itself is inactive and requires P450-catalyzed bioactivation of its thiophene ring to form a reactive thiol metabolite, which forms a covalent

disulfide bond with a cysteinyl residue on the $P2Y_{12}$ receptor in platelets (Scheme 22), a phenomenon that gives rise to its beneficial cardiovascular effects (Herbert and Savi, 2003; Savi et al., 2000, 2006). Likewise, the benzimidazole class of proton-pump inhibitors used to treat gastric disorders, exemplified by omeprazole by itself have no in vitro ability to inhibit the enzyme $H^+$, $K^+$-ATPase but are converted to a reactive sulfenamide intermediate in the acidic environment of the stomach. Covalent disulfide bond formation of this reactive species with an active site cytseine residue results in enzyme inactivation (Scheme 22) (Olbe et al., 2003). Irreversible enzyme inhibition on account of covalent binding (Fellenius et al., 1981) is one pharmacokinetic benefit that contributes to making omeprazole clinically superior to $H_2$-receptor antagonists initially used to treat gastric acid disorders.

## 3.6 REACTIVE METABOLITE TRAPPING AND COVALENT BINDING STUDIES AS PREDICTORS OF IDIOSYNCRATIC DRUG TOXICITY

The examples discussed above pose a significant challenge toward the reliability of structural alerts, reactive metabolite trapping, and covalent binding measurements as indicators of idiosyncratic drug toxicity. With regard to reactive metabolite formation potential with compounds possessing a structural alert, it is very important to consider mitigating factors such as reactive metabolite detoxication and alternate pathways of metabolism. This issue is highlighted with the benzodiazepine receptor ligands alpidem and zolpidem. While alpidem is hepatotoxic and has been withdrawn from the market, the commercial blockbuster zolpidem (Ambien) is devoid of the toxicity. A key structural difference in the two drugs is the replacement of the two chlorine atoms on the imidazopyridine nucleus in alpidem with two methyl groups in zolpidem. In alpidem, the imidazopyridine ring is bioactivated by CYP and leads to the formation of a reactive arene oxide that reacts with GSH to yield sulfydryl conjugates (Scheme 23), which have been detected in human excreta (Durand

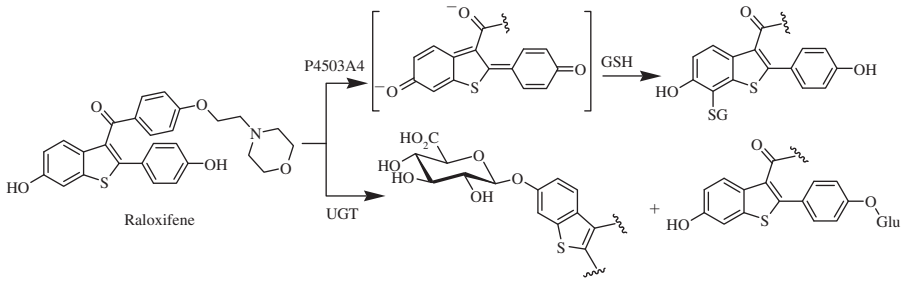



**SCHEME 23** Differential metabolism of the anxiolytic agents alpidem (hepatotoxin) and zolpidem (nonhepatotoxin).

et al., 1992). While bioactivation via epoxidation is also likely in zolpidem, the molecule does not undergo this metabolic fate; instead the two methyl groups function as metabolic soft spots and are oxidized to the corresponding alcohol and carboxylic acid metabolites (Scheme 23).

With regard to the importance of detoxication pathways, reactive metabolite formation may be discernible in standard in vitro systems; however, the principal clearance mechanism of the drug in vivo may involve a distinctly different and perhaps more facile metabolic fate that does not yield reactive intermediates as illustrated with the selective estrogen receptor modulator raloxifene. Raloxifene is known to undergo in vitro CYP3A4-catalyzed bioactivation on its phenolic groups to yield reactive quinonoid species (Scheme 24) (Chen et al., 2002); however, in vivo, glucuronidation of the same phenolic groups in the gut and liver constitute the principal elimination mechanism of raloxifene in humans (Scheme 24) (Kemp et al., 2002). Thus, the likelihood of raloxifene bioactivation in vivo is in question when compared with the phase II glucuronidation process, a phenomenon that may provide an explanation for the extremely rare occurrence of IADRs.

Although covalent binding data can provide a quantitative estimate of covalently bound radiolabeled drug to proteins and therefore an indirect measure of reactive metabolite formation, *there are no studies to date that have shown a correlation between amount of reactive metabolite formed and/or extent of covalent binding and the probability that a drug will be associated with toxicity* (Evans et al., 2004; Masubuchi et al., 2007). An example of this phenomenon is evident with the acetaminophen regioisomer, 3′-hydroxyacetanilide, which undergoes bioactivation to yield reactive metabolites that form covalently bound adducts to GSH and proteins (Rashed et al., 1989). However, despite dose normalization to provide comparable levels of covalent binding in vivo in mice, 3′-hydroxyacetanilide does not exhibit the hepatotoxicity observed with acetaminophen. To assess predictability of idiosyncratic drug toxicity, covalent binding measurements in human hepatic tissue has been examined for 18 drugs (9 hepatotoxins and 9 nonhepatotoxins) (Obach et al., 2008b; Bauman et al., 2009). This study also considers key factors such as

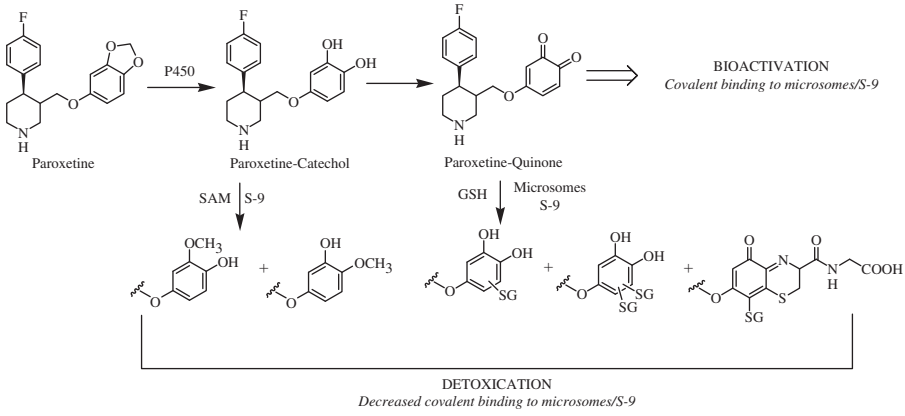



**SCHEME 24** Bioactivation and competing detoxication pathways of the selective estrogen receptor modulator raloxifene.

reactive metabolite detoxication, relative importance of bioactivation that leads to covalent binding versus overall metabolism, and daily dose for each drug. While most of the hepatotoxic drugs (e.g., acetaminophen, nefazodone, tienilic acid, etc.) demonstrate covalent binding to some degree, it is interesting to note that several nonhepatotoxic and commercially successful drugs (e.g., buspirone, diphenhydramine, paroxetine, and simvastatin) also exhibit covalent binding. A quantitative comparison of covalent binding in vitro intrinsic clearance does not separate the two groups of compounds, and, in fact, paroxetine and diphenhydramine, both nonhepatotoxins, exhibit the greatest amount of covalent binding in microsomes. Including factors such as the fraction of total metabolism comprised by covalent binding and the total daily dose of each drug improves the discrimination between hepatotoxic and nonhepatotoxic drugs in liver microsomes, S-9, and hepatocytes; *however, the approach still would falsely identify some agents as potentially hepatotoxic*.

In the case of paroxetine, mechanistic studies further confirmed the importance of parallel metabolic and detoxication pathways in attenuating covalent binding to proteins (Zhao et al., 2007). As shown in Scheme 25, the catechol metabolite obtained via ring scission of the 1,3-benzdioxole group in paroxetine can partition between *O*-methylation by catechol-*O*-methyl transferase or undergo oxidation to the reactive quinone intermediate, which is efficiently detoxicated by GSH; both pathways lead to a significant reduction in covalent binding. In humans, the *O*-methylated catechol derivatives constitute the principal metabolic fate of the drug. When coupled with the fact that the daily dose of paroxetine is low (20 mg), some insight into the excellent safety record of this drug is obtained despite the bioactivation liability.

Finally, *a drug candidate that is devoid of reactive metabolite formation and/or covalent binding to proteins in "standard" systems is not a guarantee of its safety*. Despite the in vivo observations on felbamate bioactivation, evidence for the in

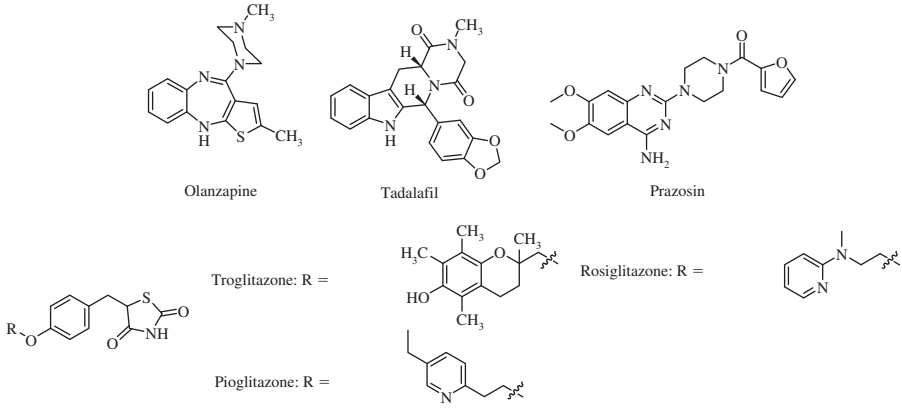



**SCHEME 25** Parallel detoxication pathways that compete with the P450-catalyzed bioactivation pathway of the anti-depressant paroxetine as explanation for its wide safety margin.

vitro metabolism of felbamate to the reactive metabolite 2-phenylpropenal in human hepatic tissue is lacking. At therapeutically relevant concentrations of radiolabeled felbamate in in vitro incubations with human liver microsomes, S-9, and hepatocytes, no GSH adducts and/or covalent binding of felbamate has been discerned (Leone et al., 2007; Obach et al., 2008b; Bauman et al., 2009). While the reason(s) for this discrepancy remain unclear at the present time, *in a drug discovery paradigm relying solely on reactive metabolite trapping and liver microsomal covalent binding as means of predicting IADR potential of drug candidates*, *felbamate would have passed the hurdle with flying colors*.

## 3.7 DOSE AS AN IMPORTANT MITIGATING FACTOR FOR IADRs

A single most important factor in migrating IADR risks appears to be the daily dose of the drug. There are no examples of drugs that are dosed at $< 20$ mg/day that cause IADRs (whether or not these agents are prone to bioactivation). There are instances of two structurally related drugs that possess identical structural alerts susceptible to bioactivation; however, the drug administered at the lower dose is safer than the one given at a higher dose. It is likely that the improved safety of low-dose drugs arises from a marked reduction in the total body burden to reactive metabolite exposure, and therefore, unlikely to exceed the threshold needed for toxicity. For example, the dibenzodiazepine derivative olanzapine (Zyprexa) (Scheme 26) forms a reactive iminium metabolite very similar to the metabolite observed with clozapine, yet olanzapine is not associated with a significant incidence of agranulocytosis (Gardner et al., 1998). One difference between the two drugs is the daily dose; clozapine is given at a dose of $>300$ mg/day, while the maximum recommended daily dose of olanzapine is 10 mg/day. A second illustration of this concept is evident




**SCHEME 26** Examples of low daily dose drugs devoid of IADRs despite bioactivation liability.

upon comparison of the thiazolidinedione derivatives troglitazone, rosiglitazone, and pioglitazone. The idiosyncratic hepatotoxicity with troglitazone, which led to its withdrawal from the marketplace, has not been discerned with rosiglitazone and pioglitazone. As noted earlier, both the chromane and the thiazolidenedione ring systems in troglitazone succumb to CYP3A4-catalyzed bioactivation to reactive metabolites (Kassahun et al., 2001; He et al., 2004). While rosiglitazone and pioglitazone do not contain the chromane ring system found in troglitazone, they do contain the thiazolidinedione scaffold. Consistent with the findings with troglitazone, both rosiglitazone and pioglitazone have been shown to undergo thiazolidinedione ring scission mediated by P450 enzyme(s) in human microsomes resulting in reactive metabolites that are trapped by GSH (Baughman et al., 2005; Alvarez-Sanchez et al., 2006). While bioactivation of the thiazolidenedione ring is a common theme in these drugs, a key difference lies in their daily doses—troglitazone (200–400 mg/day) and rosiglitazone and pioglitazone (<10 mg/day).

Additional examples of this phenomenon are illustrated with tadalafil (Cialis) and the antihypertensive prazosin (Minipress) (see Scheme 26). The methylenedioxyphenyl group in tadalafil undergoes P4503A4-catalyzed bioactivation to an electrophilic catechol, a process that also leads to the suicide inactivation of P4503A4 activity in vitro (Ring et al., 2005). However, to date there are no reports of IADRs or P4503A4 drug–drug interactions associated with tadalafil use at the recommended dose of 10–20 mg/day. Likewise, there are no reports of IADRs with prazosin at the recommended daily dose of 1 mg/day, despite the bioactivation of the pendant furan ring to electrophilic intermediates, trapped with GSH and semicarbazide (Erve et al., 2007).

## 3.8 CONCLUDING REMARKS

The issue of reactive metabolites continues to receive widespread interest in the pharmaceutical industry. Should evidence for reactive metabolite formation cause abandonment of an otherwise attractive drug candidate or initiate the oftentimes challenging and time-consuming task of eliminating/minimizing their formation. The current evidence suggests that detection of reactive metabolites for a chemical series does not warrant an instant demise of the compounds per se, but this occurrence does trigger some additional due diligence, including the evaluation of competing detoxication pathways of the reactive metabolite and its precursor by phase I/phase II enzymes. Likewise, estimates of human dose can assist in decision making when considering advancement of drug candidates that form reactive metabolites. It is noteworthy to point out that the adduction of GSH with reactive metabolites is not necessarily a bad attribute; instead, it confirms the ability of the endogenous sulfydryl antioxidant to efficiently scavenge the electrophilic reactive intermediate. It is only in cases where the concentration of the

reactive metabolite formed is so high that the endogenous antioxidant pool is depleted and leads to toxicity as has been demonstrated with acetaminophen. It is important to emphasize that bioactivation is only one aspect of the overall risk–benefit assessment for advancing a drug candidate into development. Consequently, data from reactive metabolite trapping and covalent binding studies need to be placed in proper and broader context with previously discussed factors such as the daily dose and alternate routes of metabolism/detoxication. Likewise, appropriate consideration needs to be given for drug candidates for potential treatment options for unmet and urgent medical need.

The ability to predict the potential of a drug candidate to cause IADRs is dependent on a better understanding of the pathophysiological mechanisms of such reactions. IADRs are too complex to duplicate in a test tube, and their idiosyncratic nature precludes prospective clinical studies. Genetic factors also appear to have a crucial role in the induction of IADRs. A fruitful approach may therefore lie in focused and well-controlled phenotype/genotype studies of the rare patients who have survived this type of injury. For instance, results of a 500,000 single nucleotide polymorphism analysis in population exposed to the HIV agent abacavir-associated hypersensitivity reaction suggest that the known human leucocyte antigen B (HLA-B) gene region could be identified with as few as 15 cases and 200 population controls in a sequential analysis and as such has been instituted in practice to avoid the side effects (Hughes et al., 2009). An additional area of research includes studies on the identities of the protein targets of reactive metabolites discerned with toxic versus nontoxic drugs and on the combined application of covalent binding measurements with transcriptomic, metabonomic, and proteomic technologies in an effort to discern (and thereby predict) the characteristics of a toxic response. Until a better understanding of the risk of toxicity arising from the formation of reactive metabolites is developed, the advancement of potent (low-dose) drug candidates with only a limited propensity to form reactive intermediates would appear to be the most favored strategy in an ideal world.

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