A Comprehensive Listing of Bioactivation Pathways of Organic Functional Groups

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Abstract: The occurrence of idiosyncratic adverse drug reactions during late clinical trials or after a drug has been released can lead to a severe restriction in its use and even in its withdrawal. Metabolic activation of relatively inert functional groups to reactive electrophilic intermediates is considered to be an obligatory event in the etiology of many drug-induced adverse reactions. Therefore, a thorough examination of the biochemical reactivity of functional groups/structural motifs in all new drug candidates is essential from a safety standpoint. A major theme attempted in this review is the comprehensive cataloging of all of the known bioactivation pathways of functional groups or structural motifs commonly utilized in drug design efforts. Potential strategies in the detection of reactive intermediates in biochemical systems are also discussed. The intention of this review is not to "black list" functional groups or to immediately discard compounds based on their potential to form reactive metabolites, but rather to serve as a resource describing the structural diversity of these functionalities as well as experimental approaches that could be taken to evaluate whether a "structural alert" in a new drug candidate undergoes bioactivation to reactive metabolites.

Key Words: Bioactivation, reactive metabolite, P450, inactivation, liver, idiosyncratic, toxicity, glutathione, drugs, amines.

INTRODUCTION

Adverse Drug Reactions (ADRs)

ADRs are one the most common causes for pharmaceutical product recalls and labeling changes [1]. An estimated 100,000 deaths per year are attributed to ADRs making it the sixth leading cause of death in the United States. Certain ADRs are not recognized as potential medical problems prior to approval due to the insufficient number of patients in clinical trials as the incidence rate can be extremely low (1 in 3,000 to 1 in 10,000) [2]. Failure to account for variability among patients in terms of gender, genetic background, co-administered drugs, co-existence of other diseases, and concurrent effects on drug metabolism and/or excretion represent putative factors that can lead to ADRs [3].

ADRs are further categorized into predictable and unpredictable (idiosyncratic) reactions [4]. About 80% of all ADRs are predictable, and are generally identified in preclinical toxicological evaluations. If toxicity is observed in animals at systemic drug exposures near those anticipated to be clinically efficacious, the drug is generally abandoned from further development. Of greater concern than predictable toxins are drugs that are associated with idiosyncratic toxicity. These ADRs cannot be explained by 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 [5]. An additional complication is that the adverse events typically emerge after weeks or months of therapy, and are only observed in a small fraction of the total patients receiving the implicated drug. Because of this, idiosyncratic adverse drug reactions (IADRs) are not usually identified as such until after approval and widespread use.

Role of Reactive Metabolites in ADRs

Some drugs are known to elicit ADRs prior to metabolism. However, most drugs that elicit an ADR are first metabolized to proximate and ultimate toxic species, a process often referred to as metabolic activation or bioactivation. Since the liver is the major site of metabolism, it is often the target for toxicity [6]. In most cases hepatic metabolism results in the loss of biological activity, and such metabolic reactions are therefore regarded as detoxification pathways. However, it has become increasingly clear that the biotransformation of some xenobiotics results in the formation of reactive intermediates and/or metabolites [7, 8]. In fact, the formation of reactive metabolites represents a frequent underlying mechanism that gives rise to hepatotoxicity. Most bioactivation reactions involve either oxidation or reduction and can often be attributed to the action of cytochrome P450 enzymes [9], flavin monooxygenases (FMOs) [10], monoamine oxidases (MAOs) [10] and the heme-containing peroxidases (e.g. cyclooxygenases (COX) and myeloperoxidase (MPO)) [11]. In addition, bioactivation can arise *via* conjugative pathways (e.g. sulfation and glucuronidation) [12-15]. Such bioactivation events can result in

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the formation of electrophilic and/or free radical metabolites which are thought to cause toxicity by covalently altering essential cellular macromolecules, potentially resulting in the inactivation of drug metabolizing enzymes, the disruption of intracellular calcium homeostasis, or the general loss of cellular membrane integrity. Although not all toxicological manifestations are attributable to reactive metabolites, and not all covalent binding events lead to a deleterious biological consequence, a vast body of evidence suggests that inadequate detoxification of reactive metabolites is a pathogenic mechanism for tissue necrosis, carcinogenicity, teratogenicity and immune-mediated toxicity. Table **1** lists several drugs that exhibit some form of toxicity and are known to produce reactive metabolites.

Bioactivation - Underlying Science

Living tissues contain proteins and nucleic acids, essential for life-sustaining processes. For proteins, appropriate tertiary and quaternary structure needs to be maintained in order for them to function properly. For nucleic acids, the nucleotide sequence must be maintained intact in order for genes to be appropriately transcribed to functional proteins. It is these two fundamental elements of the biochemical processes of life that can be disrupted by xenobiotics. Xenobiotics including drugs, environmental pollutants and non-nutritive organic substances in foodstuffs can disrupt the proper functioning of proteins and nucleic acids by either binding covalently or non-covalently to them. Non-covalent binding is, by its nature, a transient and reversible process such that non-covalent interactions are not as prone to irreversible disruption of the target protein or nucleic acid. However, covalent modification of proteins and nucleic acids represents an irreversible process whereby the victim macromolecule is permanently damaged by the xenobiotic. In some cases, these events manifest into cell death, and in other cases the damaged macromolecule can trigger a deleterious autoimmune response [32].

Xenobiotic Metabolism and Bioactivation

Metabolism of xenobiotics including drugs is typically categorized as phase 1 and phase 2 reaction types [33-35]. In phase 1 reactions, xenobiotics are metabolized *via* oxidative, reductive, and/or hydrolytic pathways, with P450-mediated oxidation reactions predominating. P450-catalyzed oxidation reactions include hydroxylation, heteroatom-dealkylation and -oxygenation, epoxidation and dehydrogenation reactions along with many less frequently observed oxidative reactions [36]. In the vast majority of cases, phase 1 reactions lead to metabolites that are less toxic and more polar than the parent xenobiotic. However, in a small number of cases, usually associated with specific functionalities within xenobiotics, phase 1 metabolism gives rise to a reactive metabolite. Phase 2 metabolism is the term generally used to describe conjugation reactions. In these reactions, substituents introduced during phase 1 metabolism or those already present in the xenobiotic are derivatized to water-soluble metabolites. Examples include glucuronidation, sulfation, acetylation, methylation, aminoacylation or glutathione conjugation. In many cases (e.g. glucuronidation and sulfation) of phase 2 metabolism, a substituent is introduced that makes the metabolite more polar and thus easier to excrete. In almost all cases phase 2 metabolism results in an overall detoxification of the xenobiotic, but in some cases phase 2 metabolism is also known to generate reactive metabolites.

Thus, the process of bioactivation is intrinsically linked to xenobiotic metabolism. In most cases, the process of metabolism is beneficial to the organism in that toxins are converted to less toxic and more readily excreted metabolites. However, in some cases this process "goes wrong" in that a chemically inert molecule is converted to a chemically reactive metabolite. Drug metabolizing enzymes have evolved to process a plethora of structurally diverse xenobiotics 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 factors: (1) does the molecule possess a functionality that is susceptible to bioactivation and (2) does binding of the molecule in the catalytic site of the drug metabolizing enzyme present the suspected substituent for bioactivation. While the former can be often answered by visual examination of the structure, the latter must be tested experimentally. Furthermore, even if a compound is converted to a reactive metabolite, there is no assurance that toxicity *via* reaction with biomacromolecules is bound to occur.

Table 1. Examples of Drugs that are Known to Generate Reactive Metabolites and Also Exhibit Toxicity

Drug (ADR) [References]	Drug (ADR) [References]
Acetaminophen (hepatotoxicity) [16]	Vesnarinone (agranulocytosis) 17]
Clozapine (agranulocytosis) [18]	Halothane (hepatoxicity) [19]
Tacrine (hepatotoxicity) [20]	Ticlopidine (agranulocytosis) [21]
Indomethacin (hepatotoxicity) [22]	Phenacetin (hepatotoxicity) [23]
Diclofenac (hepatotoxicity) [24]	Felbamate (aplastic anemia) [25]
Carbamazepine (hypersensitivity) [26]	Tienilic Acid (hypersensitivity) [27]
Valproic acid (hepatotoxicity) [28]	Zimelidine (Guillain-Barre syndrome) [29]
Phenytoin (hepatotoxicity) [30]	Remoxipride (aplastic anaemia) [31]

Placing Reactive Metabolites in the Proper Context

There are myriad examples of drugs that are hepatotoxic or cause idiosyncratic toxicity for which bioactivation mechanisms have been described [1, 8, 37]. These examples provide a strong circumstantial link between bioactivation and toxicity. These examples also provide guidance in that functionalities (structural alerts) that are susceptible to bioactivation could be potentially avoided in future drug design efforts. However, when considering drug bioactivation and reactive metabolite formation in a new chemical entity, several factors must be accounted for before making a go/no-go decision. First, not all drugs possessing functionalities susceptible to bioactivation are bioactivated and second, not all drugs that are bioactivated lead to toxicity. Thus, by avoiding structural alerts in drug design, one lessens the odds that the new drug will lead to toxicity *via* a bioactivation mechanism. However, presence of structural alerts within a new drug candidate is not a necessary criterion for toxicity. In many cases, experiments can be done and data gathered to aid in the prediction of whether a drug candidate possessing a structural alert will eventually cause toxicity in the clinic.

There are several other variables that will impact whether a drug candidate that possesses a structural alert will eventually lead to ADRs. These include: (a) dose: drug-induced hepatotoxicity and drug-induced autoimmune disease are more frequently associated with compounds administered at high total daily doses (Fig. **1**). Thus, for two compounds possessing a structural alert, it is frequently the case that a low dose compound will not cause toxicity whereas a higher dose compound will [8, 38]. Examples include olanzapine versus clozapine and pioglitazone versus troglitazone [38]. There are no examples of drugs that are dosed below 10 mg/day that cause IADRs, (b) usage: drugs that are used chronically are more prone to idiosyncratic ADRs than those used in an acute setting. In many cases, toxicity is evident only after several weeks of administration, thus agents that are administered for two weeks or less are rarely associated with bioactivation-related toxicities, (c) detoxification of reactive metabolites: as stated above, in addition to phase 1 metabolism that can give rise to reactive metabolites, there are several phase 2 reactions that can result in inactivation of these intermediary reactive metabolites. Thus, if a drug has a substituent that can be bioactivated, consideration needs to be made for the relative formation versus deactivation of the reactive metabolite. *In vivo*, the reactive metabolite may be formed but immediately metabolized to an inactive metabolite (e.g. glutathione conjugation of electrophilic intermediates), and finally, (d) multiple metabolic pathways: a drug possessing a structural alert may be more readily metabolized or excreted by a process that does not involve bioactivation of that structural alert. Thus, multiple factors must be taken into account when considering the possibility that a drug known to undergo bioactivation to reactive metabolites will eventually cause toxicity. The science has not fully developed such that a reliable prediction of bioactivation/toxicity can be successfully accomplished.

Experimental Strategies

In vivo (preclinical species) and *in vitro* approaches to examine the bioactivation potential of promising drug candidates using animal or human reagents may offer some value in toxicity predictions. Some current approaches for the identification of reactive metabolites include: (1) trapping electrophilic reactive intermediates *in situ*: This approach has proven fairly successful in identifying drug candidates that produce reactive metabolites *via* incubating a compound of interest with liver microsomes in the presence of exogenously added nucleophiles such as *N*-acetylcysteine or glutathione (GSH) and its derivatives [39]. This methodology has been frequently utilized to demonstrate bioactivation of structurally diverse functionalities/motifs as shown in Table **2**. However, as indicated in Table **2**, not all reactive metabolites will react with a nucleophilic sulfydryl group; instead some (e.g. aldehydes and ketones) may preferentially react with other nucleophilic amine-based peptides such as lysine and histidine [40, 41], whereas others (e.g. iminiums) may react with cyanide [42, 43]. In these cases, proteins such as bovine pancreatic ribonuclease A and keyhole limpet hemocyanin that contain a representative array of amino acids have been used to demonstrate biochemical reactivity [44, 45]; (2) covalent binding of reactive intermediates to biological macromolecules: Covalent binding of reactive intermediates to proteins is generally recognized as an initial and crucial event in the etiology of many chemical-induced toxicities [7]. It is becoming increasingly clear that amino acid alkylation in proteins by electrophiles is determined by the nature of the electrophile (i.e. hard or soft), available nucleophiles and steric constraints imposed by the tertiary structure of the protein. In general, covalent binding studies require the incorporation of a radiolabel in a stable (chemically and metabolically) position of the molecule. The extent of protein modification is based on the amount of radiolabel covalently bound to the protein following *in vitro* incubation with liver microsomes or hepatocytes or after *in vivo* administration in a preclinical species; (3) enzyme inactivation studies: In some cases, P450-mediated oxidative bioactivation of drugs to reactive metabolites may also lead

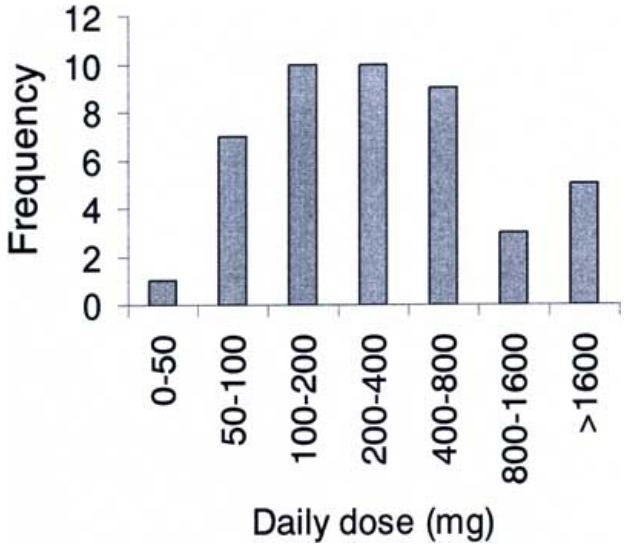


Fig. (1). Typical daily doses of 45 drugs whose use is associated with idiosyncratic adverse drug reactions.

to irreversible inactivation of the P450 enzyme by the reactive species prior to its release from the active site [46]. Because bioactivation is required for enzyme inactivation, these drugs are classified as mechanism-based inactivators or suicide substrates. 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; (4) immunochemical approaches for identifying and characterizing protein targets of reactive metabolites: This approach entails the synthesis of an immunogen (i.e. surrogate protein-hapten adduct), antibodies against which are then raised *via* immunization of animals (e.g. rodents, rabbits) with the modified protein. Following several challenges with the immunogen, the antiserum is screened for the antibody titer using an enzyme-linked immunosorbent assay (ELISA). The antibody then can be used to identify haptenized protein *via* Western blot analysis [47-49]. While this method can be a very powerful technique for the identification and characterization of cellular constituents that have undergone covalent modification by a reactive metabolite, it is generally a low-throughput and a time consuming method for the detection of reactive intermediates; and finally, (5) metabolite identification: An understanding of metabolic pathways and the biochemical mechanisms by which metabolites are synthesized can give insight to the potential of a compound to yield a reactive species [39]. Both *in vitro* and *in vivo* approaches can and should be performed to obtain a more complete understanding of the biotransformation pathways that a particular compound may undergo. Final products such as carboxylic acids derived from alkynes, products of aromatic hydoxylation, mercapturic acids in urine, acyl glucuronides, quinones or quinone-like intermediates (quinone imines or quinone methides), etc, provide evidence that reactive species were generated (Table **3**).

Challenges We Face

A significant portion of attrition during drug development is often due to hepatotoxicity. In an attempt to identify the potential for serious liver injury, promising new candidates are generally extensively evaluated for liver toxicity in animal models prior to administration to humans. However,

Table 2. Nucleophiles Commonly Used to Trap Reactive Metabolites in Biological Matrices

Functional Group	Reactive Metabolite(s)	Trapping Agent	Biological Matrix
Aromatic amines	Quinone-imine or nitroso metabolite	Thiol (glutathione or *N*-acetylcysteine)	Liver microsomes (LM)
Hydrazine, hydrazides	Diazene or diazonium ion	Thiol (glutathione or *N*-acetylcysteine)	LM, hepatocytes (H)
Nitroarenes	Nitroso	Thiol (glutathione or *N*-acetylcysteine)	LM (anaerobic conditions)
Fused azaheterocycles	Nitrenium ion	Thiol (glutathione or *N*-acetylcysteine)	LM, neutrophils, MPO
Benzylamines	Nitroso, oxime	Thiol (glutathione or *N*-acetylcysteine)	LM
Foramides	Isocyanate	Thiol (glutathione or *N*-acetylcysteine)	LM
Sulfonylureas	Isocyanate	Thiol (glutathione or *N*-acetylcysteine)	LM, H
Thioureas	*S*-Oxides, isocyanate	Thiol (glutathione or *N*-acetylcysteine)	LM, H
Hydroquinones	*p*-benzoquinone	Thiol (glutathione or *N*-acetylcysteine)	LM
o- or *p*-Alkylphenols	*o* or *p*-Quinone methide	Thiol (glutathione or *N*-acetylcysteine)	LM
Methylenedioxyphenyl	*o*-quinone	Thiol (glutathione or *N*-acetylcysteine)	LM
3-Methyleneindoles	Imine-methide	Thiol (glutathione or *N*-acetylcysteine)	LM
Furans	α, β-Unsaturated dicarbonyl	Thiol (glutathione or *N*-acetylcysteine), amines	LM
Thiophenes	α, β-Unsaturated dicarbonyl	Thiol (glutathione or *N*-acetylcysteine), amines	LM
Thiazoles	Thioamide, glyoxal	Amines (to trap glyoxal)	LM
Thiazolidinediones	Isocyanate, *S*-oxide	Thiol (glutathione or *N*-acetylcysteine)	LM
Arenes, bromoarenes	Arene oxide	Thiol (glutathione or *N*-acetylcysteine)	LM
Alkynes	Ketenes, oxirene	Thiol (glutathione or *N*-acetylcysteine)	LM
α, β-Unsaturatedcarbonyl	Intrinsic electrophilicity	Thiol (glutathione or *N*-acetylcysteine)	pH 7.4 buffer
Aliphatic amines	Iminium ion	Cyanide	LM, H
Alkylhalides	Acylhalides	Amines (e.g. d_5-aniline)	LM

Table 3. Stable Metabolites Derived From the Bioactivation of Functional Groups

Functional Group	Stable End Product of Bioactivation
Aniline, Masked anilines	*N*- or *o*- or *p*-Hydroxyaniline, nitroarene
Nitroarenes	Anaerobic reduction of the nitro group
Aliphatic amines (cyclic, tertiary amines)	Stable iminium ion or aminoaldeyhde or aminoacid
Cyclopropylamines	Stable α, β-unsaturatedcarbonyl metabolite
Cyclic secondary amines	*N*-Hydroxy or nitroxide metabolite
Benzylamines	*N*-Hydroxy or oxime metabolite
Formamides	Stable isocynates or hydrolysis products thereof
Sulfonylureas	Stable isocyanate or hydrolysis products thereof
Thiourea	*S*-Oxide, sulfinic acid
Thiols	Disulfides, sulfonic acid
Methylenedioxyphenyl	Catechol, guaiacol
5-Alkoxyindole	5-Hydroxyindole
Furans	γ-Ketocarboxylic acid metabolite
Thiophenes	5-Hydroxythiophene, ketocarboxylic acid
Thiazoles	Thioamide, α, β-dicarbonyl metabolite
Arenes	Stable dihydrodiol
Alkyne	Acetic acid
Carboxylic acid	Acyl glucuronide

not all human hepatotoxins can be identified in preclinical studies due to differences in species dependent metabolism. Furthermore, the number of animals used in toxicological studies is far too low to pick up low frequency toxicities. Since IADRs in humans cannot be predicted from preclinical toxicological assessments, the sure means of minimizing reactive metabolite-mediated safety issues are to eliminate the possibility of reactive metabolite formation or reduce the formation of reactive metabolites to undetectable levels using the approaches stated above.

BIOACTIVATION PATHWAYS OF FUNCTIONAL GROUPS / STRUCTURAL MOTIFS USED IN DRUG DESIGN

Aromatic Amines (Anilines)

Occurrence and Frequency

The carcinogenic, mutagenic, and cytotoxic properties of anilines are fairly well understood as a result of extensive studies on aromatic and heteroaromatic amine-containing drugs and environmental toxins (For a recent review see [8]). Noteworthy examples of aniline-containing drugs include the type IA antiarrhythmic agent procainamide, the sulfonamide antimicrobial sulfamethoxazole, used in combination with trimethoprim for the treatment of *Pneumocystis carinii* pneumonia in HIV-infected patients, and the sulfonamide dapsone, which remains the mainstay in leprosy treatment (Fig. **2**). The utility of procainamide has been hampered by the relatively high incidence of procainamide-induced lupus erythematosus whereas the sulfamethoxazole/trimethoprim combination is associated with a much higher incidence of hypersensitivity reactions in AIDS patients, ranging from 30 to 80% in various studies. Dapsone therapy is often associated with dose-dependent toxicity towards erythrocytes (methaemoglobinemia) in all individuals, and in rare instances with dose-dependent white cell toxicity such as agranulocytosis, fever, and rashes that result in discontinuation of therapy.

Mechanism

The obligatory step in the bioactivation of all anilines involves *N*-hydroxylation on the primary amine nitrogen leading to the formation of the *N*-hydroxylamine intermediate **1** as illustrated with procainamide (Fig. **2**). These reactive *N*-hydroxylamine metabolites can undergo either phase II conjugation to generate the more reactive *N*-*O*-sulfate **3** and/or *N*-*O*-acetyl conjugates **4**, or two-electron oxidation to the more reactive nitrosoamine intermediates (compound **2** such as in the case of procainamide). The excellent leaving group tendency of the sulfonyloxy group and the acetoxy groups in the *N*-*O*-sulfate and/or *N*-*O*-acetyl conjugates is thought to lead to a highly reactive nitrenium that may be the ultimate reactive intermediate involved in DNA adduct formation. In the case of procainamide, *N*-

acetylation of its primary amino group to afford *N*-acetylprocainamide (**5**), a reaction catalyzed by the polymorphic *N*-acetyltransferase (NAT2), constitutes its major metabolic fate (see Fig. **2**). Consistent with the metabolic profile, clinical studies have indicated that patients of the rapid acetylator phenotype require a longer period of time to develop lupus erythematosus than slow acetylators [50]. As a consequence, *N*-hydroxyprocainamide (**1**) and its two electron oxidation product nitrosoprocainamide (**4**) that have been observed as metabolites of procainamide in humans (CYP2D6 catalyzed) are thought to be responsible for the ADRs since [^{14}C]-procainamide but not [^{14}C]-*N*-acetylprocainamide covalently binds to mouse liver microsomes (NADPH-dependent) *in vitro* as well as *in vivo* and this binding is prevented by the addition of reducing agents [51]. The isolation of a stable GSH-based sulfinamide conjugate **7** indicates that GSH efficiently scavenges the reactive nitrosoprocainamide **4** to form the initial unstable mercaptal derivative **6** that rearranges to **7** and thus, prevents covalent binding to hepatic tissue (see Fig. **2**) [52]. Recent clinical studies have resulted in the detailed evaluation of the role of CYP2D6 in the *in vivo* metabolism of procainamide in humans [53]. A key finding in this report included the ability to measure nitroprocainamide (**8**), the stable six-electron oxidation product of procainamide, in extensive CYP2D6 metabolizers, but none in poor metabolizers. Concomitant administration of the CYP2D6 inhibitor, quinidine, prevented the detection of nitroprocainamide in all individuals further confirming the isozyme's role in procainamide bioactivation in humans. Similar metabolism findings have also been observed with sulfamethoxazole.

Structure-Bioactivation Relationships

An obvious requirement for bioactivation is the presence of an oxidizable aniline nitrogen. For instance, structure-toxicity relationship studies on dapsone analogs indicate the parent compound as the most potent inducer of methaemoglobinemia in human erthrocytes in the presence of rat liver microsomes and that replacement of the sulfone group with S, O, NH, CH_2, or C=O functionalities decreases the toxic effects. A significant correlation between hemotoxicity and the Hammett constant σ_p suggests that the electron-withdrawing properties of the 4-substituent in dapsone influences the rate of oxidation of the primary amine nitrogen [54].

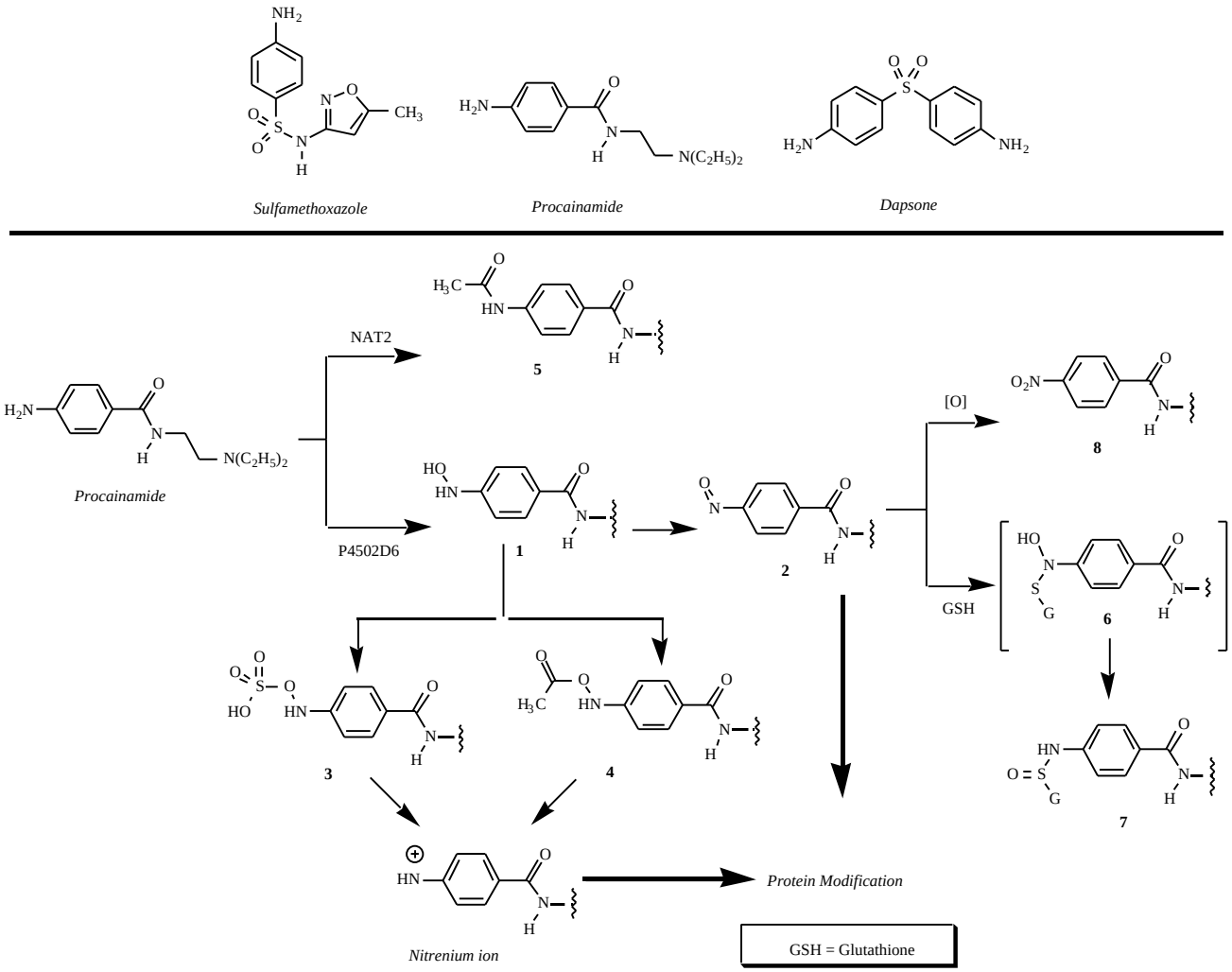


Fig. (2). Proposed Mechanism for the Bioactivation of Procainamide.

Masked Anilines (Anilides) and *p*-Aminophenols

Occurrence, Frequency and Mechanism

Acetaminophen (*p*-acetamidophenol) is the most widely studied hepatotoxin in humans and animals (for a comprehensive review on the pharmacological and metabolism of this drug see [55]). Hepatic failure from acetaminophen overdose has been reported to cause ~ 120 deaths/year in the UK alone [4]. The role of metabolism in acetaminophen toxicity has been well characterized (Fig. **3**). At therapeutic levels, acetaminophen undergoes phase II conjugation reactions and is primarily excreted in the urine as the corresponding *O*-glucuronide (55% of the administered dose) and *O*-sulfate (30% of the dose). Although considered to be safe for humans at the recommended doses, ingestion of a toxic dose of acetaminophen (greater than the recommended 4 grams/day; although some patients develop liver failure after ingesting a few tablets) leads to a P450-dependent hepatotoxicity *via* a CYP3A4-mediated two-electron oxidation to the reactive *N*-acetyl-*p*-benzoquinone imine (NAPQI) species. NAPQI can be readily trapped as the corresponding GSH or *N*-acetylcysteinyl conjugates *in vitro* liver microsomal systems supplemented with the sulfydryl nucleophiles. Besides CYP3A4, baculovirus-expressed and purified human CYP1A2, 2A6 as well as 2E1 also catalyze the oxidation of acetaminophen to NAPQI, although at toxic doses, CYP2E1 has been found to be the more efficient catalyst for acetaminophen bioactivation. Thus, CYP3A4 is probably the most important P450 isozyme involved in acetaminophen metabolism at therapeutic concentrations, whereas the ethanol inducible CYP2E1 becomes significantly involved at high plasma levels of the drug and after serious intoxication, respectively. Several groups also have established the involvement of peroxidases such as MPO and COX in acetaminophen bioactivation to NAPQI. At normal doses of acetaminophen in most species including humans, only a small amount of NAPQI is formed that upon conjugation with GSH affords the corresponding GSH adduct (4% of the dose), subsequently characterized as the corresponding mercapturate conjugates. Under these conditions, no adverse effects are discernible following acetaminophen administration. After an overdose, however, or when specific P450 isozymes are increased, hepatic GSH is depleted more extensively and can no longer compensate for a massive production of NAPQI. Thus, mitochondrial GSH depletion is normally correlated with acetaminophen hepatotoxicity. Acetaminophen, most likely *via* NAPQI, also can form adducts with proteins, oxidize cysteine residues on proteins, covalently bind to liver and renal DNA and eventually disrupt cellular homeostasis.

Also of interest in this context is the antimalarial agent, amodiaquine (AQ), which is effective against chloroquine-resistant and -sensitive isolates of *P. falciparum*. This observation resulted in an increase in its use for the treatment for malaria; however, life-threatening agranulocytosis and hepatotoxicity in ~ 1 in 2000 patients during prophylactic administration led to its withdrawal [56]. Despite this drawback, AQ is still used for the treatment of acute malaria if the risk of infection outweighs the potential for drug toxicity. That bioactivation plays a significant role in the observed ADRs is strongly suggested from the observations that in neutral solutions, the 4-aminophenol moiety in [^{14}C]-AQ undergoes autooxidation or peroxidase-catalyzed two-electron oxidation to the corresponding quinone-imine intermediate **10** that arylates microsomal protein or conjugates to *N*-acetylcysteine resulting in the formation of C'5-protein (**11**) or sulfydryl (**12**) adducts (Fig. **4**) [57]. The observation that antioxidants prevent the covalent binding of AQ to proteins implicates the formation of the semiquinone imine intermediate **9** as the initial one-electron oxidation

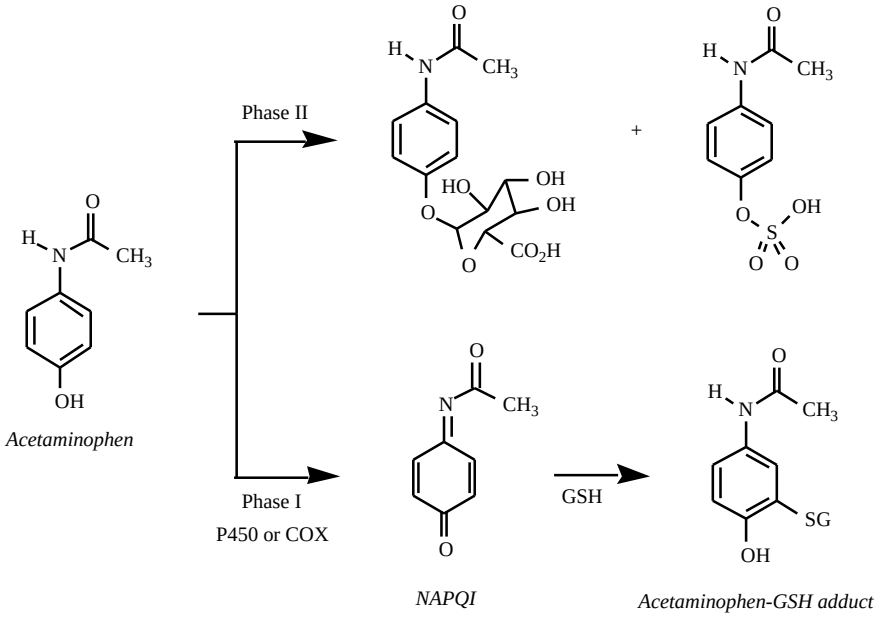


Fig. (3). Metabolism/Bioactivation of Acetaminophen.

product of AQ. The synthetic availability of the reactive AQ-quinone-imine metabolite has also proved useful in establishing the immunogenic properties of AQ in rats and humans by subsequent detection of AQ-specific antibodies in these species.

Information on the drug metabolizing enzymes responsible for AQ metabolism is scarce and studies on [^{14}C]-AQ bioactivation in phorbol ester-activated neutrophils resulted in irreversible binding of radiolabel to protein, depletion of intracellular GSH, and the identification of the corresponding C'5 glutathionyl adduct of AQ. These studies further substantiate the role of peroxidases, such as MPO that are present in PMN, in the bioactivation of AQ in humans.

The nonsteroidal anti-inflammatory drug diclofenac (Fig. **5**) represents an aniline derivative that undergoes CYP3A4 and CYP2C9 catalyzed aromatic hydroxylations (*para* to the aniline nitrogen) resulting in the formation of the corresponding *p*-aminophenol metabolites **13** and **14**, respectively. These metabolites are known to undergo further P450 or MPO catalyzed two-electron oxidation to the corresponding electrophilic quinone-imine species **15** and **16**, respectively, [58] and this event is thought to contribute to the hepatoxicity associated with diclofenac use. That diclofenac bioactivation involves the formation of the reactive quinone-imines **15** and **16** is consistent with the detection of the corresponding GSH adducts **17** (**17a** and **17b**) and **18**, respectively, *in vitro* liver microsomal incubations in the presence of GSH and also in male rats administered with diclofenac. The involvement of P450 in diclofenac bioactivation is consistent with the observation that covalent binding of radiolabeled diclofenac to rat liver microsomes is dependent upon the presence of NADPH [59]. For additional information on the bioactivation of masked anilines and *p*-aminophenols, the reader is advised to refer to our recent review on this subject [8].

Structure/Bioactivation Relationships

An obvious strategy that avoids generation of "reactive" quinone-imine intermediates from 4-aminophenolic compounds involves the replacement of the corresponding phenolic function with a "non-oxidizable" group. For instance, the amodiaquine derivative (see Fig. **4**) in which the C'4 phenolic OH group is replaced with a fluoro substituent is incapable of forming the reactive quinone-imine intermediate [60]. In the cases of anilino derivatives such as diclofenac, it seems logical to block the para position on the aromatic ring thus preventing the formation of the corresponding 4-aminophenol metabolite. A significant challenge is to retain the biological activity against the given enzyme or receptor upon incorporating these changes.

Hydrazines

Occurrence and Frequency

Hydrazines are commonly used as intermediates in the synthesis of a variety of pharmaceuticals including hydralazine (antihypertension), isoniazid (antituberculosis), phenelzine (antidepressant) and procarbazine (anticancer). Apart from their many therapeutic benefits, hydrazines are known to elicit toxicity. For instance, isoniazid contains the following warning on its label. “Severe and sometimes fatal hepatitis associated with isoniazid therapy has been reported and may occur or may develop even after many months of

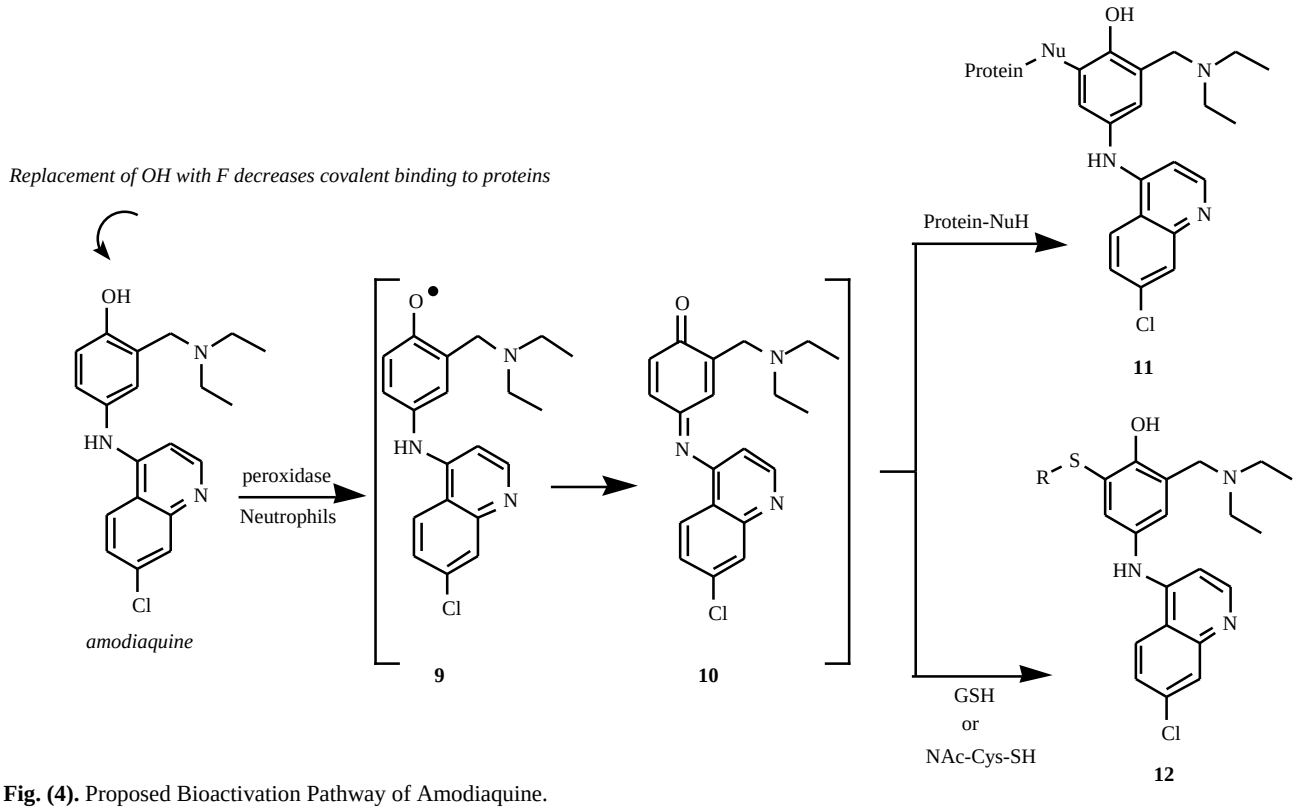


Fig. (4). Proposed Bioactivation Pathway of Amodiaquine.

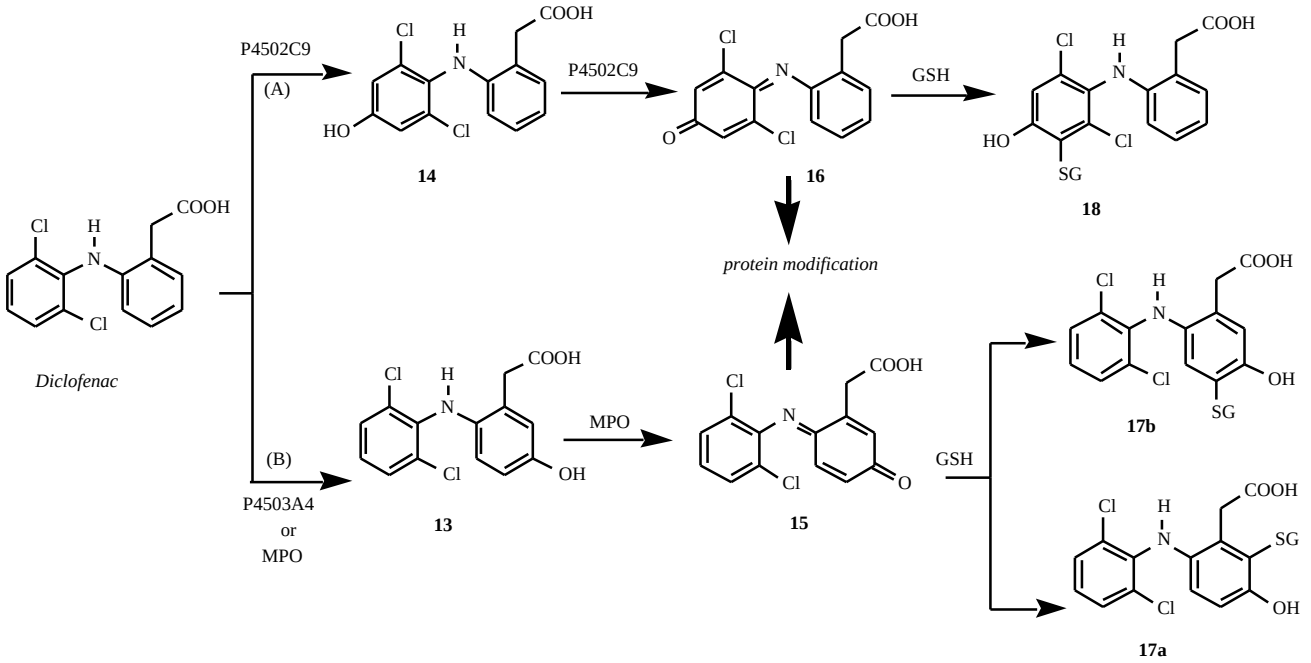


Fig. (5). Proposed Bioactivation Pathway of the Anti-Inflammatory Agent Diclofenac.

treatment". The risk of developing hepatitis with isoniazid is age related. Approximate case rates by age are: less than 1 per 1,000 for persons under 20 years of age, 3 per 1,000 for persons in the 20-34 year age group, 12 per 1,000 for persons in the 35 -49 year age group, 23 per 1,000 for persons in the 50-64 year age group, and 8 per 1, 000 for persons over 65 years of age. Furthermore, the risk of hepatitis increases with daily consumption of alcohol. Precise data to provide a fatality rate for isoniazid-related hepatitis is not available; however, in a U. S. Public Health Service Surveillance Study involving 13, 838 persons taking isoniazid, there were 8 deaths amongst 174 cases of hepatitis. The major adverse effect of the widely used antihypertensive drug hydralazine is an autoimmunological syndrome that resembles systemic lupus erythematosus (SLE) or rheumatoid arthritis. The development of this syndrome is dose-dependent, displaying a dramatic increase with daily doses greater than 200 mg. Like hydralazine, the structurally related antihypertensive drug, dihydralazine also is known to induce immunoallergic hepatitis. The CYP1A2 autoantibodies found in the serum of patients suffering from dihydralazine-mediated hepatitis suggest a key role for P450 in the overall toxicological process.

Mechanism

Investigations on the mechanism of toxicity mediated by several hydrazine-based and hydrazide drugs implicate bioactivation as a potential cause of tissue injury. For instance, administration of ^{15}N-hydrazine (**19**) sulfate to mice results in ~ 20% of the dose being excreted as nitrogen gas suggesting a rapid conversion of the parent compound into metabolites other than the major mono- and diacetylated hydrazine derivatives **20** and **21**, respectively (Fig. **6**). The evolution of nitrogen gas is consistent with the formation of the reactive diimide intermediate **22**, a strong reducing agent that concomitantly reduces styrene (**23**) to ethylbenzene (**24**) in mouse liver microsomal incubations (see Fig. **6**). Likewise, metabolism studies on phenylhydrazine (**25**) also have provided evidence for the formation of phenyldiazine (**26**), phenyldiazonium (**27**), and the putative phenyl radical (**28**) as reactive intermediates. The bioactivation of arylhydrazines and aryldiazenes with simple iron porphyrins and hemeproteins has been studied and involves the formation of sigma-bonded aryl-iron complexes, which in the case of P450, results in the shifting of the aryl group to the porphyrin nitrogens and the corresponding formation of *N*-arylprotoporphyrin IX regioisomers that subsequently lead to enzyme inactivation. Compared with alkyl- or aryl-substituted hydrazines, hydrazine, itself, is a much less potent inducer of red blood cell hemolysis, despite the fact that it is readily oxidized to the reactive diimide. These observations implicate that the alkyl- or the aryl substituent in these compounds is responsible for the hemolytic and hepatotoxic events presumably *via* the alkyl or aryl free radicals that oxidize essential cysteinyl residues in proteins or covalently react with biomacromolecules. Some evidence for this proposal has been provided by the trapping of free radicals formed upon bioactivation of hydrazine derivatives by horseradish peroxidase (HRP), an event that also demonstrated enhanced cytotoxicity of the parent compounds in mouse fibroblasts.

Hepatotoxicity associated with the antituberculosis drug, isoniazid (**29**) (Fig. **7**) is also linked to the bioactivation of the hydrazine group. The relationship between hepatotoxicity and metabolism of isoniazid and its metabolites, *N*-acetylisoniazid (**30**) and *N*-acetylhydrazine (**31**) in rats has been investigated and toxic doses of [^{14}C]-**30** and [^{14}C]-**31** 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 (**30**) to **31** and isonicotinic acid (**32**) and

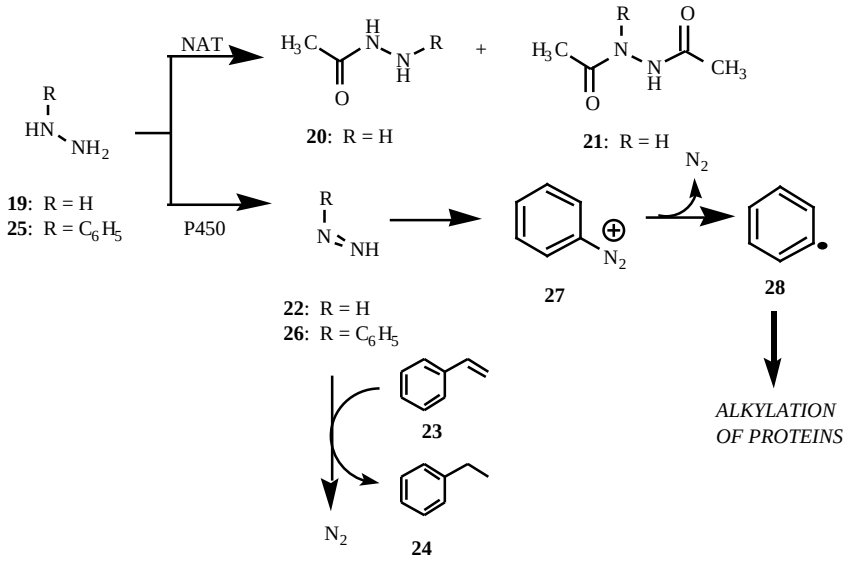


Fig. (6). Proposed Mechanism of Bioactivation of Hydrazine and Phenylhydrazine.

concomitantly decreased the covalent binding of *N*-acetylisoniazid (Fig. **7**). These results suggest that *N*-acetylhydrazine (**31**) is the ultimate hepatotoxin, whose bioactivation *via* the intermediate *N*-acetyldiazine **33** leads to the formation of reactive acetylating species (electrophilic **34** or free radical **35**) that reacts covalently with hepatic tissue (see Fig. **7**). Electron spin resonance studies have confirmed the formation of the acetyl free radical **35** in incubations of isoniazid in perfused rat livers.

Like other monosubstituted hydrazines, hydralazine is polymorphically acetylated in man, and the development of SLE occurs almost exclusively in slow acetylators who produce lesser amounts of acetylated metabolites such as **36** (detoxification pathway) and larger amounts of oxidized metabolites such as phthalazinone (**39**) (bioactivation pathway) (Fig. **8**). Evidence for the role of P450 in the bioactivation pathway was obtained following oxidation of [^{14}C]-hydralazine in rat liver microsomes in the presence of NADPH which led to reactive intermediates (diazine **37** and diazonium ion **38**) that covalently bound to microsomal protein as indicated by incorporation of radioactivity. A competing detoxification pathway involves the spontaneous reaction of water or GSH with the diazonium intermediate **38** leading to phthalazinone (**39**) or the mercapturic acid conjugate **40**, respectively.

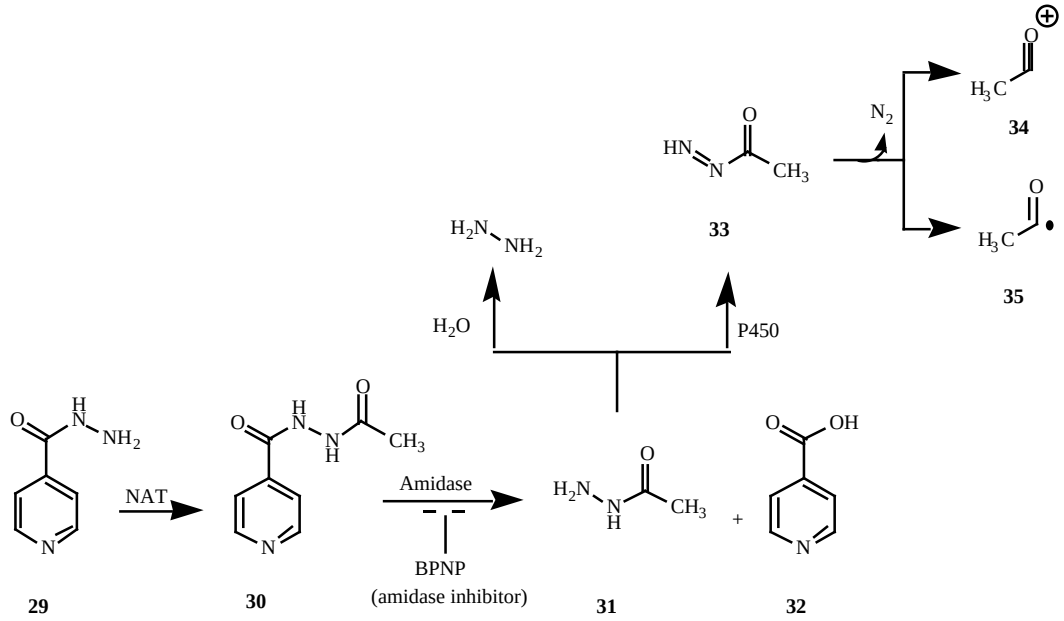


Fig. (7). Proposed Mechanism of Isoniazid Bioactivation.

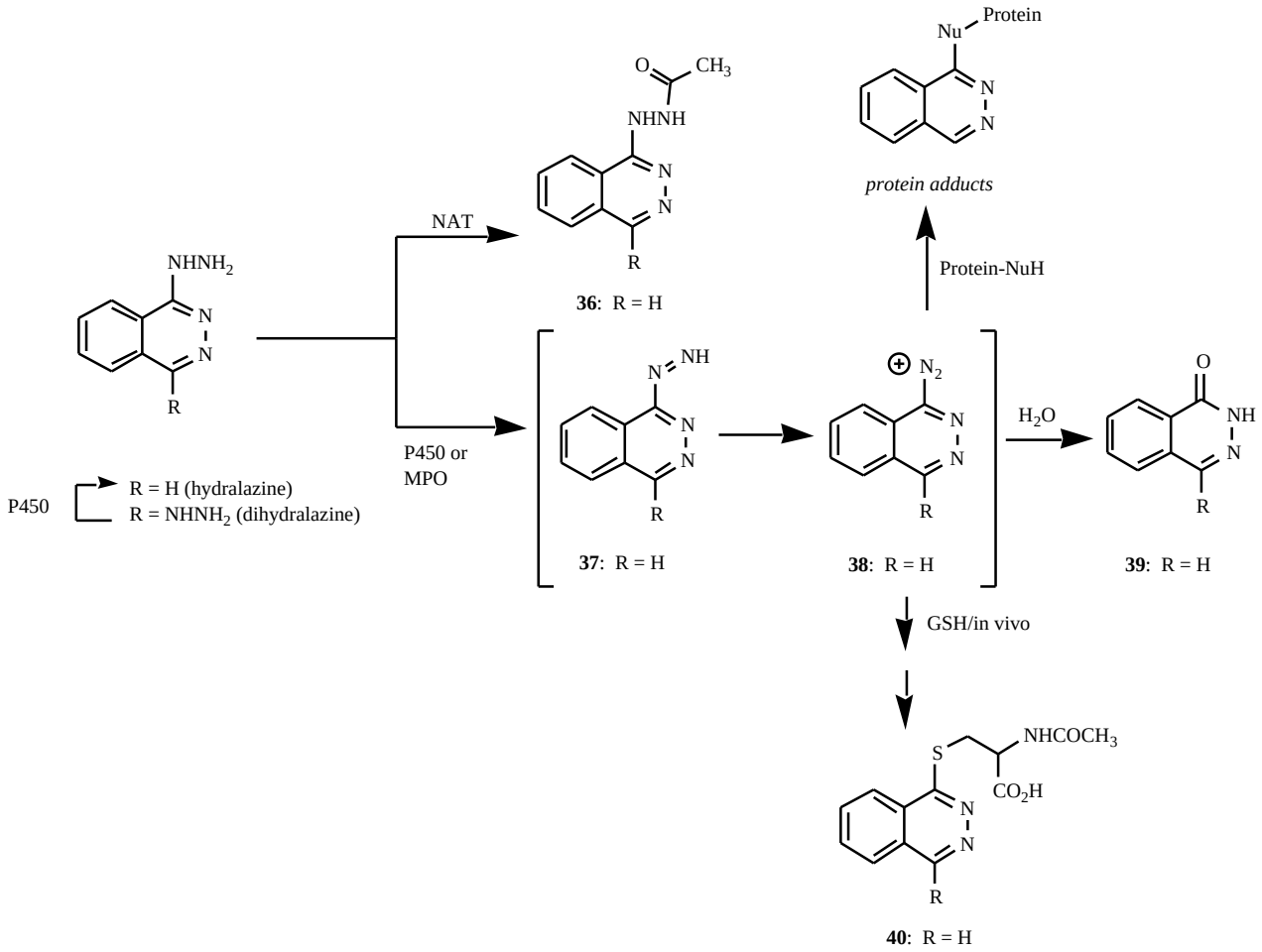


Fig. (8). Proposed Mechanism of Bioactivation of Hydralazine.

Structure/Bioactivation Relationships

Although specific structure/bioactivation relationship studies on hydrazines and hydrazides have not been conducted, it is assumed that upon metabolism all aryl/alkyl hydrazines and hydrazides will lead to reactive diazene/ diazonium intermediates.

Nitroarenes

Occurrence and Frequency

The toxicological properties of nitroarenes have been the subject of many studies for the past fifty years [61]. As early as the 1950s, 4-nitroquinoline-1-oxide (**41**) (Fig. **9**) was reported to be a potent carcinogen with reduction of the nitro group as the rate-limiting step in its bioactivation. Nitroaromatic compounds are ubiquitous in nature, and several marketed drugs also contain nitroaromatic or nitroheteroaromatic groups within the core structure. Examples include nitrofuran containing antibacterial agents nitrofurazone (**42**) and nitrofurantoin (**43**) (see Fig. **9**). Nitrofurantoin is associated with pulmonary fibrosis and a few other types of IADRs [62]. Nitroimidazoles (e.g. metronidazole, (**44**)) comprise another class of drugs that has been used in humans and animals as antibacterial agents. The pharmacological activity of this class of compounds is dependent on the reduction of the nitro group to reactive intermediates. Chloramphenicol (**45**) is an antibiotic that is associated with a relatively high incidence of aplastic anaemia, which has been linked to the reduction of the nitro group to the corresponding nitroso metabolite [63]. Tolcapone (**46**) was the first of a new class of catechol-*O*-methyltransferase inhibitors approved for the treatment of Parkinson's disease. However, it is associated with a relatively high incidence of liver toxicity that eventually led to its withdrawal in several countries [64]. A recent investigation has linked hepatotoxicity to the reduction of the nitro group in **46** to the corresponding amine metabolite **47**, which undergoes a two-electron oxidation affording the quinone-imine **48** [65]. Interestingly, the structurally related compound entacapone (**49**) does not appear to possess the liability associated with **46**. The basis for this discrepancy is unclear, although recent evidence [65] suggests that the reduction of the nitro group is not a significant metabolic fate of **49** in humans. Furthermore, **49** appears to be a better substrate for human glucuronosyl transferases than **46** [66], and so exposure of **49** and its reactive metabolite(s) in humans is likely to be less.

Mechanism

A common metabolic fate of most nitroaromatic compounds involves their six-electron reduction to the corres-

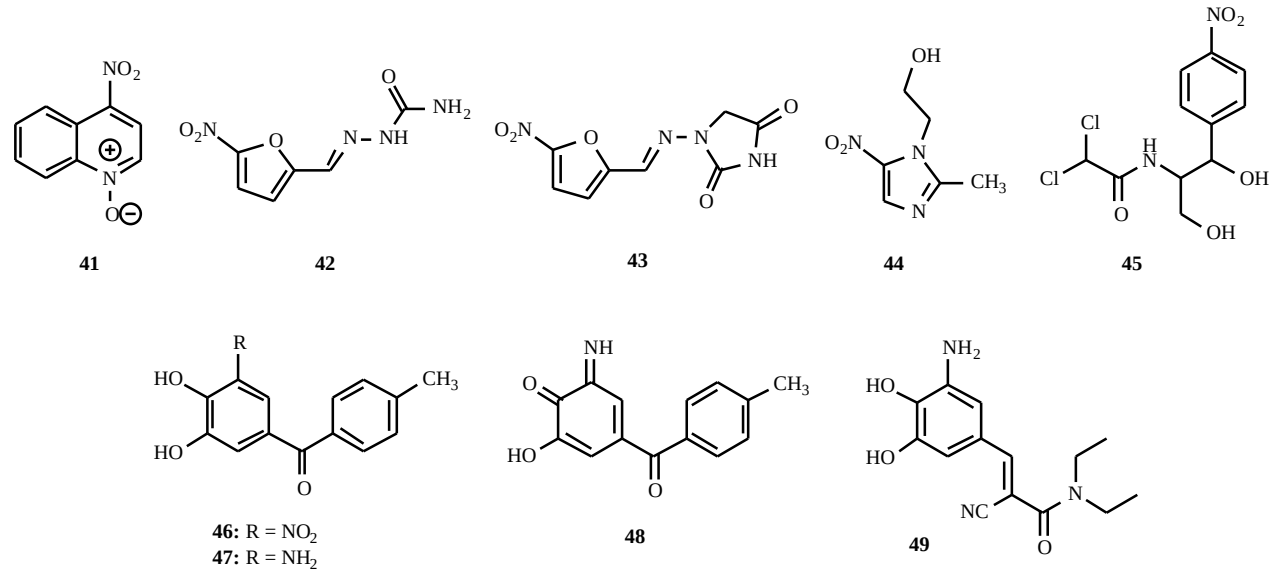


Fig. (9). Structures of Representative Nitroarene Analogs.

ponding aniline metabolite **53** (Fig. **10**), which is catalyzed by P450 enzymes, xanthine oxidase, aldehyde oxidase and quinone reductase [67]. It has been proposed that the nitro anion radical **50** is the first intermediate in the reduction of the nitro group. The reoxidation of this radical by molecular oxygen to regenerate the parent nitro compound and a superoxide anion radical may explain the inhibition of this metabolic pathway by molecular oxygen. Reduction of the nitro to the nitroso intermediate **51** is the rate-limiting step, which is followed by the formation of a *N*-hydroxylamine species **52**. Several transient radical intermediates are formed along the way and these species have been implicated in causing DNA damage in the form of DNA strand breaks. Additional DNA damage results from covalent adduct formation arising from several activated metabolites including the *N*-hydroxylamine and the *O*-esterified derivative **54** that results from enzymatic sulfation of the hydroxylamine. A detoxification pathway involves *N*-acetylation of the aniline nitrogen to the corresponding amide **55**.

Although, reduction of the nitro group in metronidazole (**44**) to the corresponding heterocyclic amine, 5-aminoimidazole **57** (Fig. **11**), is essential for its pharmacological activity, the process also generates reactive metabolites that bind covalently to cellular proteins and DNA [68, 69]. Thus, the interaction of **57** with DNA is considered to be essential for efficacy as well as the carcinogenic properties associated with **44**. Mechanistic studies on the anaerobic covalent binding of the related 5-nitroimidazole drug, ronidazole (**58**), in several biological systems have demonstrated the retention of the imidazole ring in the protein bound product but loss of the C4 proton on the imidazole ring and the carbamoyl group in ronidazole [70-73]. An identical labeling pattern was observed after reductive activation by dithionite suggesting that covalent binding is mediated by the same species in chemical and biological systems. Studies of the stoichiometry of dithionite utilization during the covalent binding of reductively activated ronidazole to immobilized sulfydryl groups demonstrated that four electrons are required for maximal covalent binding, implicating the 5-(hydroxylamino)imidazole (**59**) as the reactive intermediate.

The anaerobic reductive activation of metronidazole and ronidazole in human hepatic tissue (NADPH-dependent) and dithionite has been investigated (Fig. **11**) [67]. Thus, 4-electron reduction of the 5-nitro group in these compounds generates the corresponding 5-(hydroxylamino)imidazole derivatives **56** and **59**, respectively, that undergo nucleophilic attack by DNA bases or active site amino acid nucleophiles at the C4 position with concomitant loss of water. These data also aid in explaining the loss of the C4 imidazolyl hydrogen and the carbamoyl group in **58**. For instance, nucleophilic attack on the C4 position in the 5-(hydroxylamino)imidazole metabolite **59** results in the formation of the protein bound imidazole derivative **61** *via* the rearrangement (loss of the C4 proton) of the unstable imine intermediate **60**. Alternately, rearrangement of **60** leading to the elimination of the carbamoyl group could generate the highly reactive imine-methide **62** that could undergo nucleophilic attack on the exocyclic olefin leading to the cross-linked adduct **63**.

Structure/Bioactivation Relationships

Since reduction of the nitro group plays a critical role in the bioactivation of nitroarenes, the energy barrier for the reduction of nitro group can be altered either electronically or by altering the orientation of the nitro group in respect to the plane of the aromatic ring [74]. For example, those compounds in which the nitro group is perpendicular to the aromatic system exhibit a drastic reduction in their mutagenicity and tumorigenicity potential compared to analogs in which the nitro group is coplanar with the aromatic ring.

Fused Azaheterocycles

Occurrence and Frequency

Clozapine (**64**) (Fig. **12**) is a very effective antipsychotic agent, but its use is limited by a high incidence of agranulo-

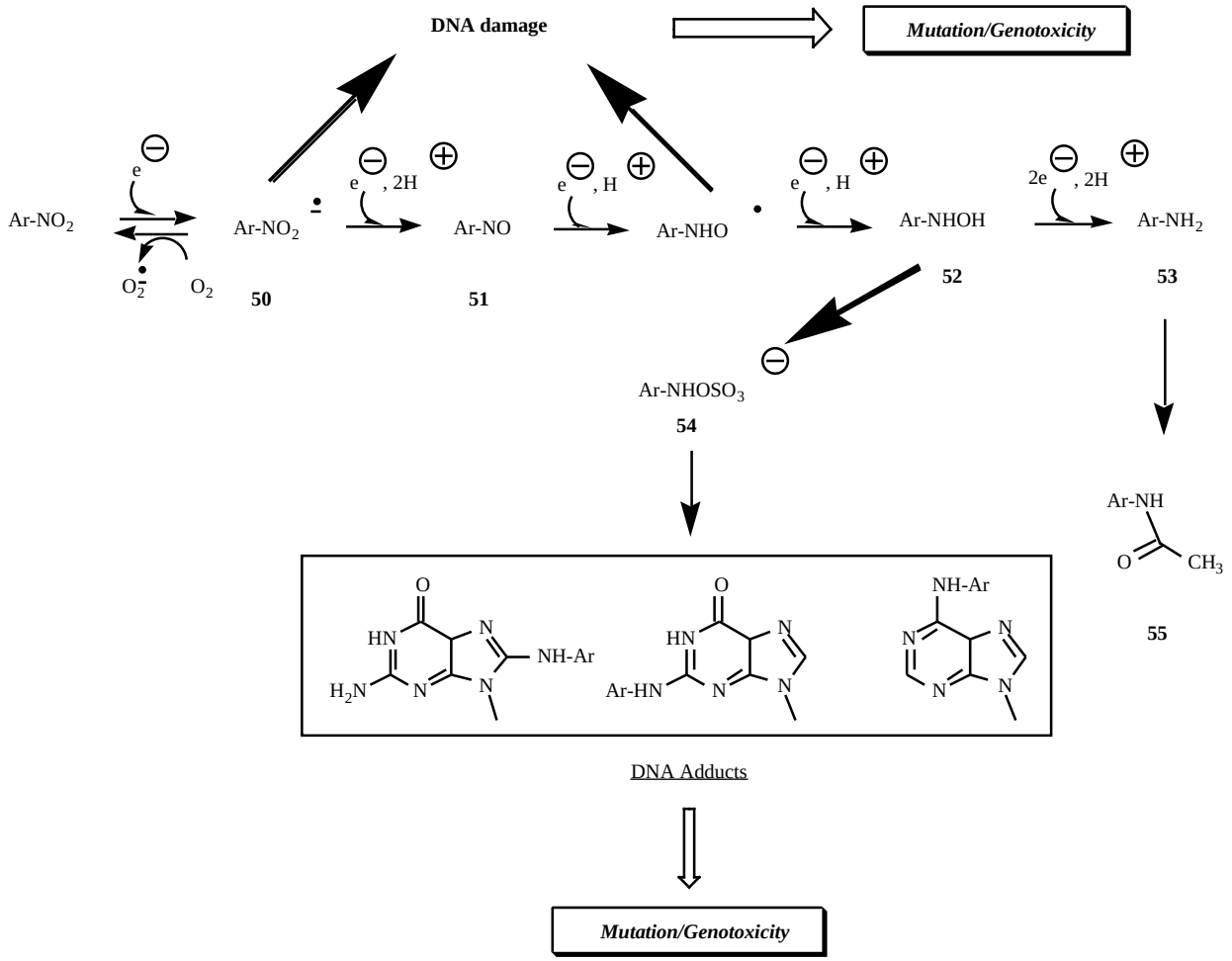


Fig. (10). Reductive Bioactivation of Nitroarenes.

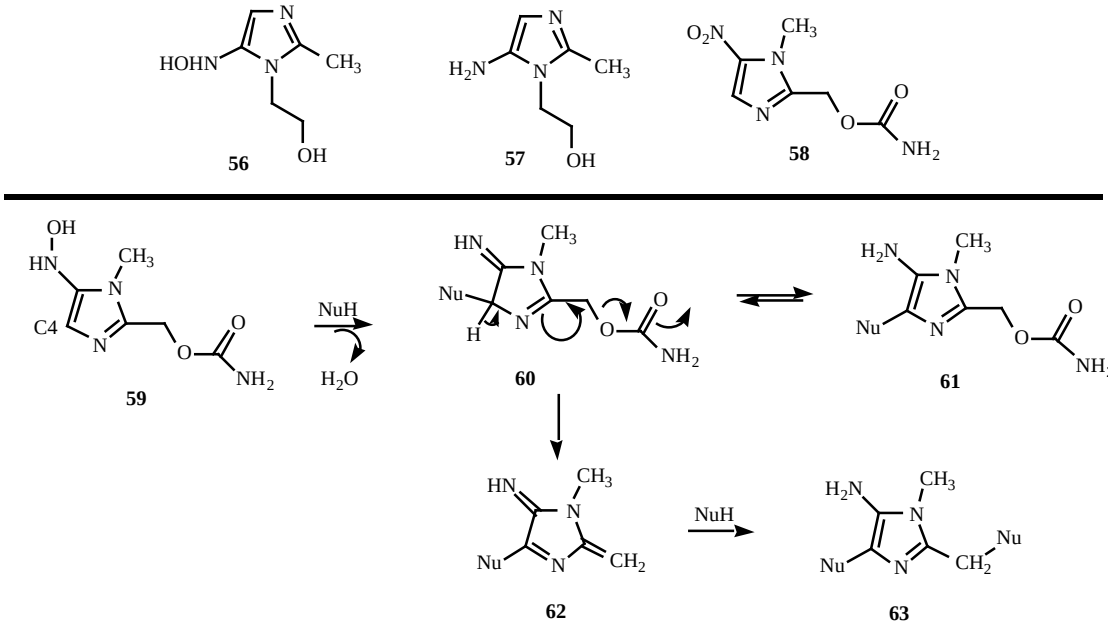


Fig. (11). Proposed Mechanism of Bioactivation of 5-Nitroimidazoles (metronidazole and ronidazole) to Reactive Species.

cytosis [75], and it is also associated with hepatotoxicity [76] and myocarditis [77]. Examination of bone marrow from patients with clozapine-induced agranulocytosis reveals the absence of myeloid precursors implicating a neutrophil precursor in bone marrow may be the target of toxicity. The dibenzodiazepine ring in clozapine is readily bioactivated by liver tissue [78] and activated neutrophils [79] to a reactive intermediate, and subsequent covalent binding to liver tissue and neutrophils with radiolabeled **64** has also been documented. It is believed that the reactive intermediate is the corresponding nitrenium ion **66**, which may play a causative role in clozapine-induced agranulocytosis [80].

Mianserin (**67**) (see Fig. **12**) a clozapine analog, was developed in the early 1970s and has advantages over the related tricyclic antidepressants in terms of fewer cardiotoxic side effects [81]. However, mianserin therapy also is associated with a number of adverse reactions including skin rashes, hepatotoxicity, and blood dyscrasias [82-84]. The role of metabolism in mianserin toxicity also has been evaluated. P450 is known to bioactivate **67** to the corresponding reactive iminium ion **68** (Fig. **12**) [85-87]. Studies with radiolabeled **67** demonstrated the NADPH-dependent, irreversible incorporation of radiolabel in microsomal protein and also demonstrated their cytotoxicity towards human mononuclear leukocytes included in the microsomal incubation [88].

Mechanism

Bioactivation of **64** is presumably catalyzed by MPO that is present at high concentrations in neutrophils and is also present in neutrophil precursors in the bone marrow. Upon activation of neutrophils the cells undergo a respiratory burst and NADPH oxidase is activated. NADPH oxidase converts oxygen to superoxide which both spontaneously and enzymatically is converted to additional oxidant species including hydrogen peroxide. MPO uses hydrogen peroxide and chloride anion to produce HOCl, the principal neutrophil oxidant [89]. It has been shown that both HOCl and MPO can oxidize **64** to **66** *via* the *N*-chloro intermediate **65** (Fig. **12**) [89]. The nitrenium ion **66** can react with cellular constituents in neutrophils or with GSH affording conjugates **69** and **70**.

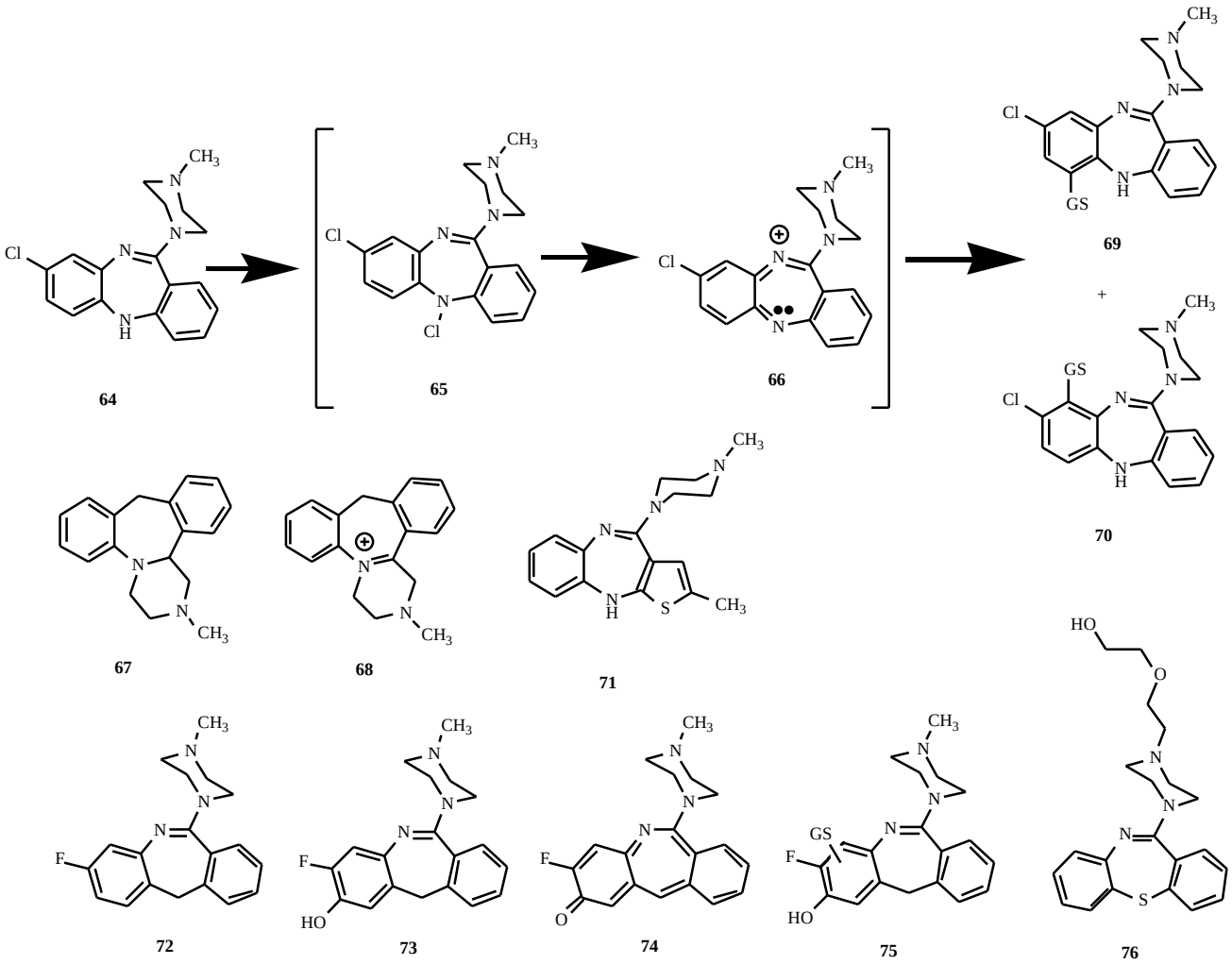


Fig. (12). Bioactivation of Clozapine and Related Fused Azaheterocycles.

Structure/Bioactivation Relationships

Several analogs of **64** and **67** have entered clinical trials or are marketed as drugs. In the light of the findings on clozapine and mianserin bioactivation, the enzymatic reactivity of these derivatives has also received much attention. The available data suggest that any modification that replaces the bridgehead nitrogen and/or replaces the bridgehead methine hydrogen will produce compounds with a lower propensity for bioactivation and toxicity. Olanzapine (**71**) is a close structural analog of **64** that retains the bridgehead nitrogen. Unlike **64** the use of **71** is not associated with a high incidence of agranulocytosis. This is despite the finding that **71** also forms a reactive nitrenium ion in *in vitro* metabolism studies [90]. This nitrenium ion can be trapped with GSH and protein adducts of **71** are generated in *in vitro* systems. The lack of agranulocytosis upon dosing of **71** may be due to the lower dose of **71** compared to **64** (10 mg/day versus 300-450 mg/day) that presumably would result in a lower systemic exposure to the reactive nitrenium ion. In addition **71**, unlike **64**, is metabolized in humans to an *N*-glucuronide metabolite that is likely to circumvent the bioactivation pathway. This difference may also be a factor in explaining the lack of toxicity observed upon olanzapine administration.

Fluperlapine (**72**) is an analog of **64** in which the bridgehead nitrogen is replaced with a methylene group. This precludes the formation of a nitrenium ion with fluperlapine. However, the development of fluperlapine was discontinued due to a low incidence of agranulocytosis. The major metabolic fate of **72** in human hepatic tissue is its conversion to 7-hydroxyfluperlapine (**73**), catalyzed by P450 [91]. Uetrecht and coworkers have revealed that **73** is readily oxidized by MPO, hypochlorous acid, or activated neutrophils to a reactive quinone-imine species **74** that leads to the formation of GSH adducts such as **75** and binds to human neutrophils *in vivo* [92]. They propose that bioactivation of **73** to **74** represents an obligatory step in the mechanism of fluperlapine-induced agranulocytosis.

Quetiapine (**76**) is a clozapine analog that contains a sulfur atom in place of the bridgehead nitrogen. The clinical use of **76** has not been associated with a high incidence of drug-induced agranulocytosis, despite being given at high doses (> 300-450 mg/day). The finding that analogs similar in structure to **76** (presence of bridgehead sulfur) do not undergo bioactivation in HOCl or activated neutrophils [93] appears to be consistent with the observed safety profile of **76**.

The use of electrochemical oxidation methodology as a tool for evaluating the ability of clozapine-like analogs to form reactive nitrenium ions and structural modifications to decrease the oxidative instability have also appeared in the literature [94]. In addition, the bioactivation of several clozapine analogs has been investigated *in vitro* using both neutrophil and HOCl systems [93]. Analogs that were most susceptible to nitrenium ion formation contained the bridgehead nitrogen. When the nitrogen was replaced with sulfur or oxygen, no bioactivation was discernible. Studies also have been conducted in an attempt to understand the structural requirements for mianserin bioactivation [95]. Replacement of the bridgehead nitrogen atom in mianserin with a methine group significantly reduces cytotoxicity as does substituting a methyl group for a hydrogen atom at the carbon adjacent to the bridgehead nitrogen atom. These observations further confirm iminium ion formation as the putative pathway towards toxicity.

Aliphatic Amines (Cyclic and Acyclic Tertiary Amines)

Occurrence, Frequency and Mechanism

Many naturally occurring and synthetic drugs directed at central biogenic amine receptors contain basic tertiary amine centers as seen with pyrrolidine, piperidine, *N*-methylpyrrole, or morpholine rings in addition to acyclic *N*, *N*-dimethylamino groups. The most important metabolic transformation that cyclic tertiary amines undergo is the P450 or MAO catalyzed ring α-carbon oxidation that generates the corresponding iminium metabolites (exocyclic iminium **77** or endocyclic iminium **78**) (Fig. **13**) [96]. Cyclic tertiary amines undergo oxidative *N*-dealkylation *via* oxidation of the exocyclic iminium intermediate **77** to afford the corresponding secondary amine and an aldehyde. Unlike the acyclic regioisomer **77**, hydrolysis of **78** to the corresponding aminoaldehyde **79** is reversible, giving rise to possible further metabolic processing of **78**. Intermediate endocyclic iminiums such as **78** are often oxidized by the liver cytosolic enzyme aldehyde oxidase to the biologically less active lactams (compd. **80**) (Fig. **13**). If special structural features are present in the substrate molecule or if the cyclic iminium intermediate is generated in extrahepatic tissue lacking aldehyde oxidase, iminiums such as **78** undergo alternative chemical transformations that can produce toxic metabolites.

Notable examples of xenobiotics in which iminium ion formation is thought to be the rate-limiting step in adverse effects include the nigrostriatal neurotoxin 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP, **81**) (Fig. **13**) and the previously discussed tetracyclic antidepressant mianserin [8, 97, 98]. In the case of **81**, the intermediate 2, 3-dihydropyridinium species $MPDP^+$ (**82**), derived from the MAO-B mediated bioactivation in the brain, undergoes a further two electron oxidation to the corresponding pyridinium species MPP^+ (**83**) [99-101]. Pyridinium **83** is actively transported into dopaminergic nerve terminals by the dopamine uptake system where it localizes in the mitochondrial matrix and inhibit complex I of the mitochondrial electron transport chain leading to cessation of oxidative phosphorylation and ATP depletion [102-105]. Interestingly, **82** is an excellent substrate for hepatic aldehyde oxidase that catalyzes its detoxification to the lactam **84** [106]. Unlike liver homogenates, brain homogenates do not detoxify **82** to **84**, presumably because of the absence of aldehyde oxidase activity in the brain [107]. The neurotoxicological importance of this bioactivation was demonstrated in experiments in which irreversible, selective MAO-B inhibitors protected monkeys and mice against **81**-induced Parkinson's disease [108-110].

An additional compound of interest in this context is haloperidol (HP, **85**), a neuroleptic agent that causes severe side effects including parkinsonism and tardive dyskinesia that, in many cases, is irreversible [111]. This 4-piperidinol derivative resembles **81** in that it bears an aryl group at the C-4 position of the piperidine ring. The proposed pathway for the 4-electron oxidation of **85** to the MPP^+-like

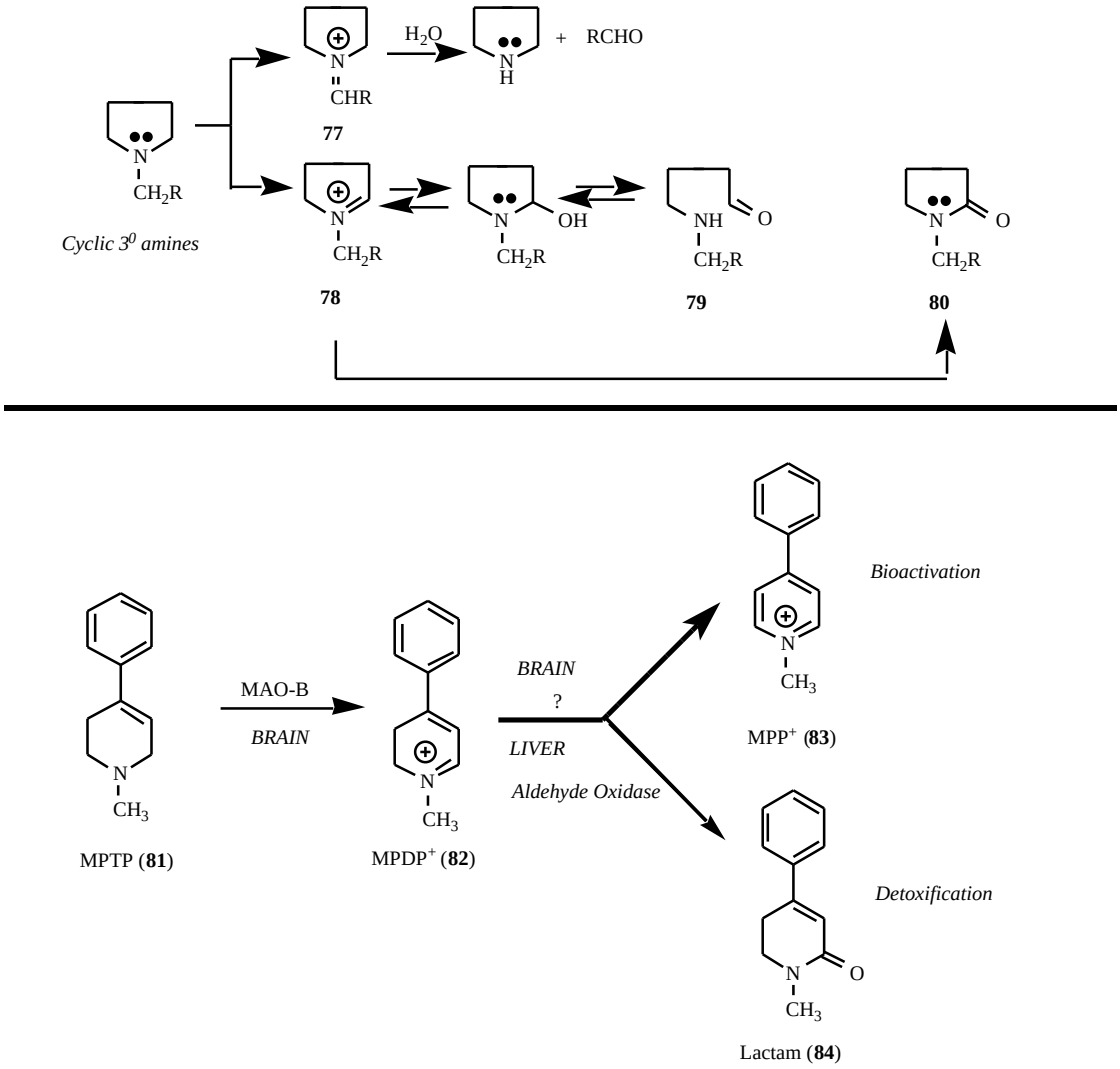


Fig. (13). α-Carbon Oxidation of Cyclic Tertiary Amines Including the Nigrostriatial Neurotoxin MPTP.

intermediate HPP^+ (**89**) involves initial P450 catalyzed conversion of **85** to the cyclic iminium species **86** which, via the free base amino enol **87**, can undergo rapid conversion to the $MPDP^+$-like 2, 3-dihydropyridinium species $HPDP^+$ (**88**) (Fig. **14**) [112-114]. Subsequent disproportionation of **88** affords the pyridinium **89** and the dehydrated HP metabolite **90** (which resembles **81** even more closely). Recent studies have shown that CYP3A4 selectively catalyzes the conversion of **85** and **90** to **89** in human liver microsomes [115]. The formation of **89** has been unambiguously characterized in the urine of rats [113] and baboons [116] treated with **85** or **90** and in the urine and the brains of humans treated with **85** [112]. Like **83**, **89** is a potent inhibitor of complex I of the mitochondrial respiratory chain [117]. It is interesting to note that unlike MPTP and haloperidol, the structurally related *N*-substituted-4-arylpiperidin-4-ol derivative and antidiarrheal agent loperamide (**91**) does not induce Parkinson's-like effects, despite a CYP3A4 catalyzed metabolism to the putative neurotoxic pyridinium species **92** (Fig. **15**) [118]. The wide safety margin of loperamide (compared with MPTP and haloperidol) despite metabolism to a potentially neurotoxic pyridinium species likely stems from a combination of factors that include a therapeutic regimen normally restricted to a few days and the fact that loperamide and perhaps LPP^+ are p-glycoprotein substrates and are denied entry into the CNS [119, 120]. The differences in safety profile of haloperidol and loperamide despite a common bioactivation event supports the notion that not all compounds undergoing bioactivation *in vitro* will necessarily elicit a toxicological response *in vivo*.

Two other tetrahydropyridines of interest in this context are the 4-(4-fluorophenoxy)-1,2,3,6-tetrahydropyridine derivative **93** and the 1,2,5,6-tetrahydro-3-pyridinecarboxylic acid derivative **94** (Fig. **15**). Toxicological studies in monkeys established the neurotoxicity of **93** [121]. Neurotoxic complications also were observed in the clinic with **94**, a candidate drug under development as an anticonvulsant agent [122]. Some of these drug-induced CNS disorders were evident in patients for several weeks after

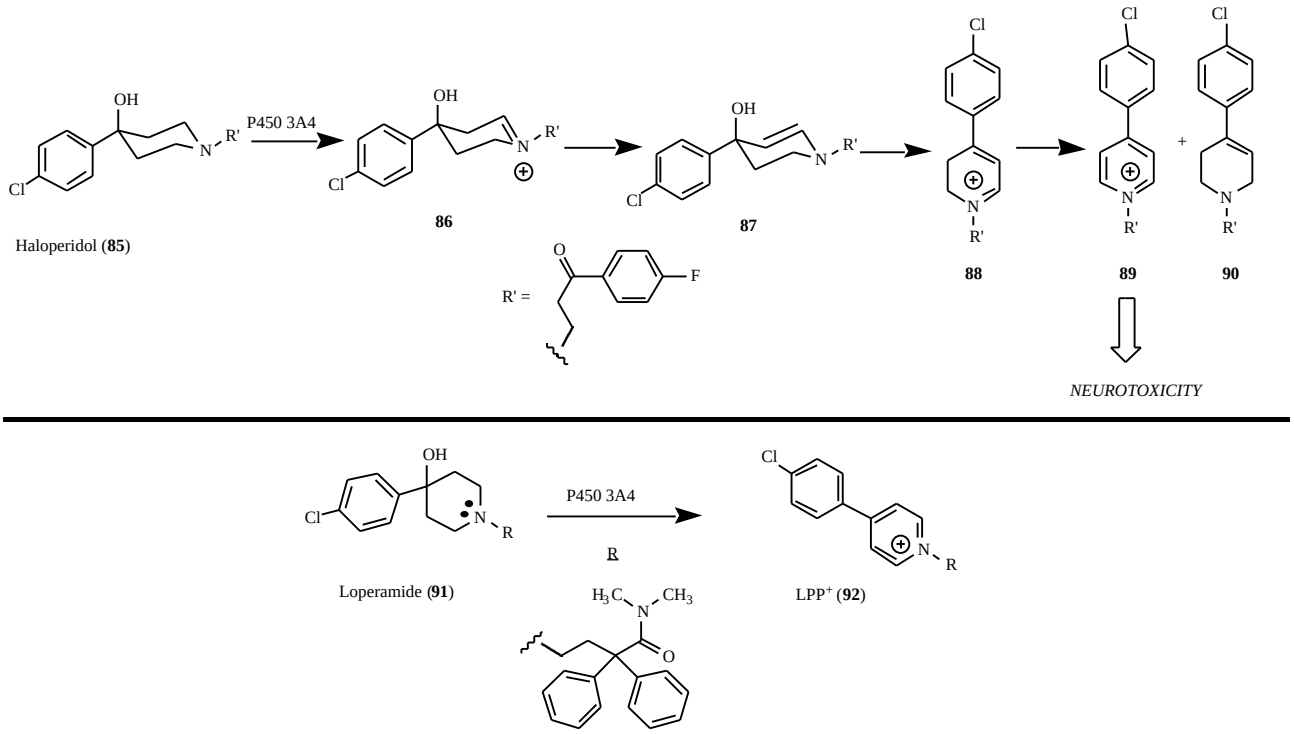


Fig. (14). Bioactivation of the Neuroleptic Agent Haloperidol and the Antidiarrheal Agent Loperamide in Human Liver Microsomes.

initial exposure. Although details concerning the mechanism of neurotoxicity of **93** and **94** remain unclear, it is noteworthy to point out that the major urinary metabolite of **93** and **94** are the corresponding pyridinium species **95** and **96**, respectively [123].

Structure/Bioactivation Relationships

Based on the structural diversity of cyclic tertiary amine substrates that undergo P450 or MAO catalyzed bioactivation to the corresponding iminium species, it is impossible to conduct structure-activity relationship (SAR) analysis. Thus, any cyclic tertiary amine containing an endocyclic oxidizable α-carbon atom should be considered as potential candidate(s) for conversion to iminium metabolites. Although replacement of abstractable α-carbon hydrogens may represent a potential strategy to prevent iminium formation, often times such approaches result in the net loss of potency against pharmacological target.

Cyclopropylamines

Occurrence and Frequency

The cyclopropylamine substructure is present in several pharmacologic agents including the antidepressant tranyl-

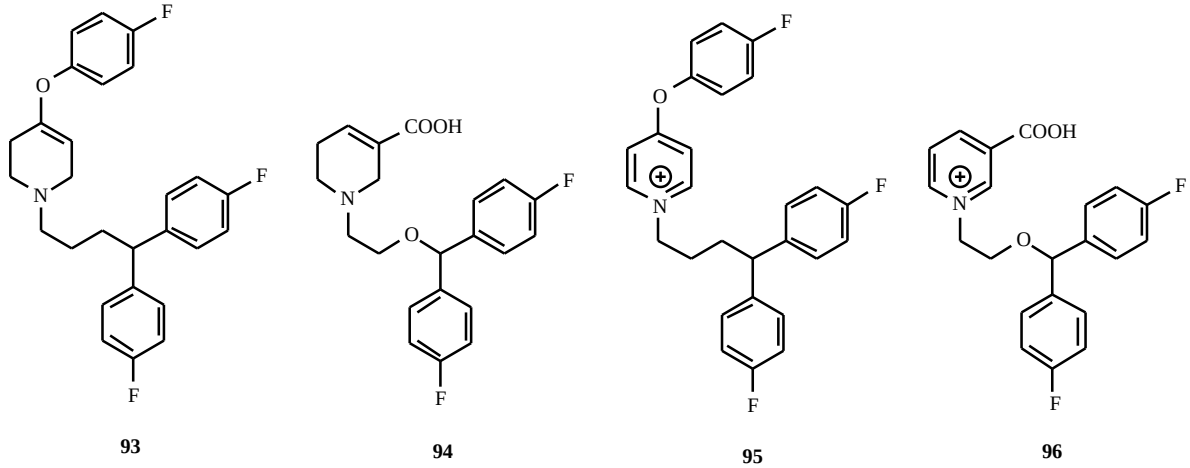
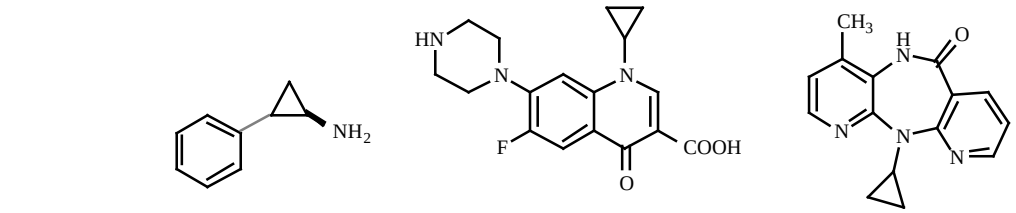


Fig. (15). Additional Examples of Neurotoxic Cyclic Tertiary Amines.

cypromine (**97**), the fluoroquinoline antibiotic ciprofloxicin (**98**) and the non-nucleoside reverse transcriptase inhibitor nevirapine (**99**) (Fig. **16**). The presence of a cyclopropylamine moiety within a potential drug candidate should be viewed with some caution since this group is a well-known mechanism-based inactivator of cytochrome P450 [124, 125]. Cyclopropylamines have also been implicated in the suicide inactivation of other human enzymes, such as the inactivation of MAO by tranylcypromine [126] and benzylcyclopropylamine [127], and alcohol dehydrogenase (ADH) by the mushroom toxin coprine [128].

Mechanism

The precise bioactivation mechanism of cyclopropylamines to reactive intermediates is still not well understood. For enzymes which are unequivocally known to oxidize amines by a single electron transfer (SET) mechanism and be irreversibly inhibited by cyclopropylamines, such as MAO-B, a cyclopropyl ring fragmented distonic cation radical intermediate **100** or resulting α, β-unsaturatedcarbonyl metabolite **101** are the reactive enzyme modifying species [126, 127]. However, in the case of cytochrome P450, for which the mechanism of cyclopropylamine oxidation has no definitive consensus, the suicide intermediate may be generated either by SET or by direct hydroxylation (HAT) at the α-carbon of the cyclopropyl group. In either case, the electrophilic species is believed to covalently modify either the P450 active site or apoprotein. However, if *N*-dealkylation of the cyclopropylamine does proceed (by either SET or HAT), the resulting 3-carbon metabolites **102** and **103** can readily modify protein side chains (Fig. **16**). The α, β-unsaturatedcarbonyl metabolite **101** is an excellent Michael acceptor, while cyclopropanone hydrate (**103**) is a potent inhibitor of peroxidases [129, 130] and ADH [128].



97 **98** **99**

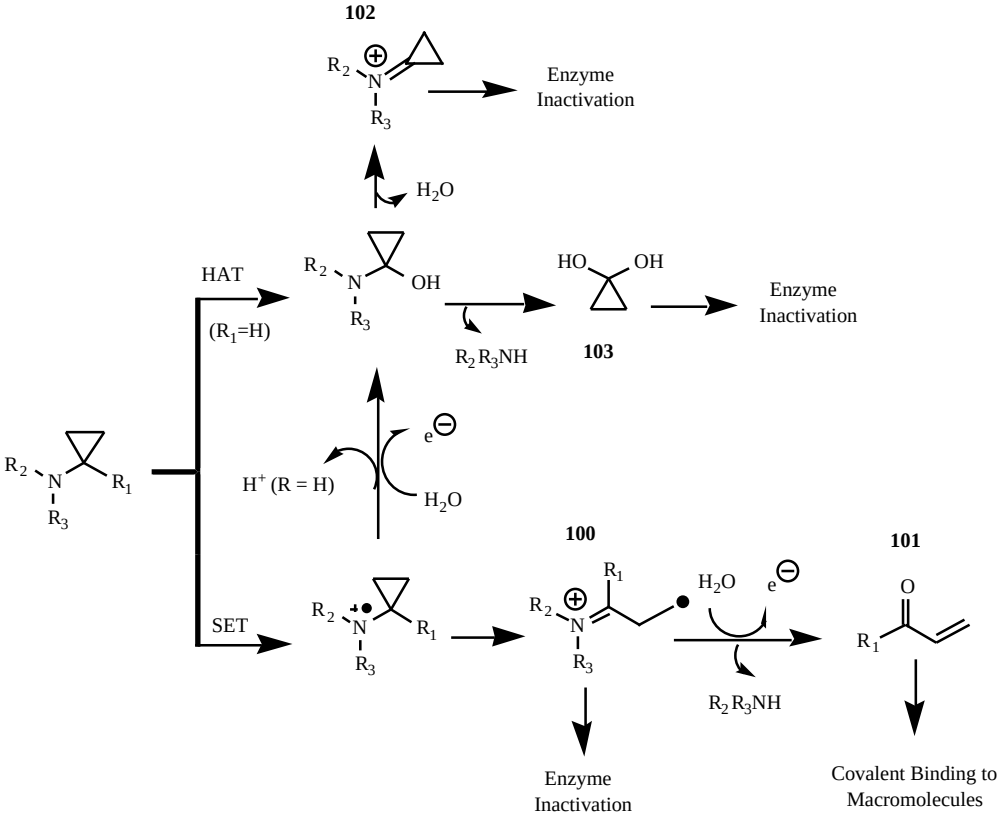


Fig. (16). Examples of Pharmacologically Active Compounds Containing the Cyclopropylamine Group and Bioactivation by P450 and MAO Enzymes.

Structure/Bioactivation Relationships

The structural features necessary for bioactivation of cyclopropylamines remain unclear. Recent studies have demonstrated that for some substrates removal of the α-carbon hydrogen on the cyclopropyl ring hinders P450 oxidation of the moiety, but not its metabolism by SET enzymes [129, 131, 132]. Furthermore, although cyclopropyl*amines* regardless of the presence of an abstractable α-carbon hydrogen have been demonstrated to inhibit P450 activity, cyclopropyl*anilines* with or without an α-carbon hydrogen do not inhibit P450 activity [131]. These findings suggest that lowering the oxidation potential (i.e. pKa) of the cylopropylamine nitrogen may indeed prevent the mechanism-based inactivation of P450 by cyclopropylamines, while leaving its metabolic lability by SET enzymes unchanged. In order to address the possibility of cyclopropylamine bioactivation, the following experiments should be contemplated: (a) cyanide trapping of the iminium and/or carbinolamine intermediates *in vitro* [129, 132, 133]. Structure elucidation of the cyano adduct should provide information regarding whether the cyclopropyl ring has fragmented or remained intact and (b) demonstrate the formation of the α, β-unsaturatedcarbonyl metabolite (or other cyclopropyl ring-fragmented carbonyl metabolite) *in vitro* incubations of parent cyclopropylamine with P450 or MAO using 2, 4-dinitrophenylhydrazine derivatizing agent [129, 132]. Alternatively, after extracting the incubation mixture to remove amine metabolites, the aqueous layer can be esterified with phenyl diazomethane under acidic conditions [134]. The detection of benzyl propionate by LC-MS/MS or GC/MS will suggest that the cyclopropyl ring has remained intact throughout enzymatic oxidation, and the reactive species (if any) is formed *via* a ring-intact carbinolamine or is cyclopropanone hydrate itself.

Benzylamines

Occurrence and Frequency

Although the presence of benzylamine substituents is not a common occurrence in pharmaceuticals, there are a few examples of drugs that contain *N*, *N*-disubstitutedbenzylamine motifs including antifungals (e.g. naftifine (**104**), terbinafine (**105**) and butenafine (**106**)) and the antiparkinson's drug deprenyl (**107**) (Fig. **17**). A few drug candidates containing the primary benzylamine moiety have also been

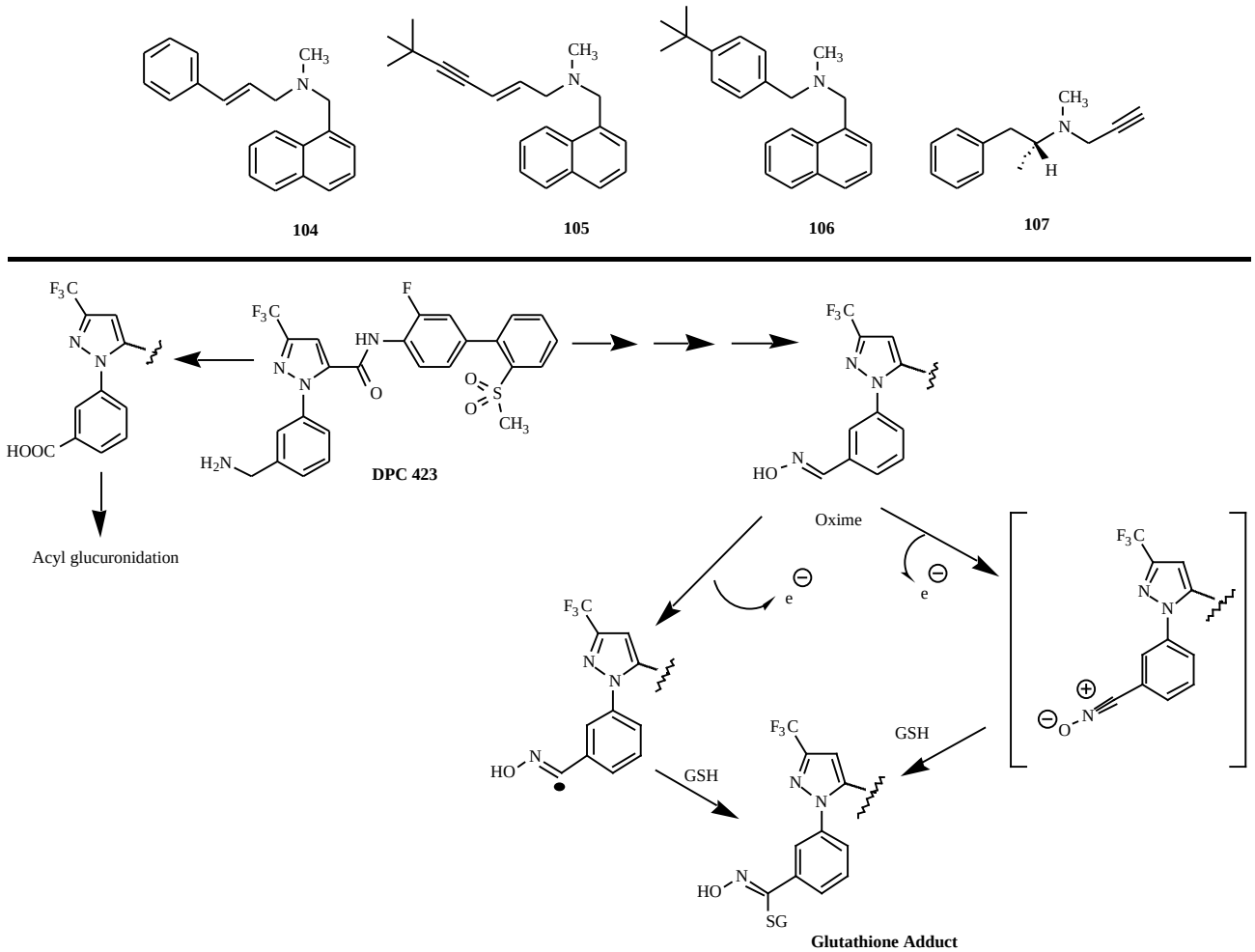


Fig. (17). Bioactivation of Benzylamine-Containing Compounds.

considered for development including antifungal agents [135], NK-1 receptor antagonists [136], factor Xa inhibitors [137] and analgesics [138]. However the development of many compounds (e.g., factor Xa inhibitors) were suspended because of toxicities observed in various preclinical species.

Mechanism

The mechanism by which primary benzylamines cause toxicity in preclinical species remains unclear. Studies conducted *in vitro* and *in vivo* with the factor Xa inhibitors DPC 423 and DPC 602, revealed the existence of a number of reactive metabolites [139, 140]. It was found that the aminomethyl portion in the benzylamine moiety was a metabolic soft spot, leading to a plethora of metabolites including aldehydes, carboxylic acids, sulfamates, hydroxylamines and GSH conjugates. The carboxylic acids were conjugated to form potentially reactive acylglucuronides. The oxidation of benzylamine nitrogen led to a hydroxylamine, which was subsequently further oxidized to a reactive nitroso intermediate that rearranged to an oxime. This oxime metabolite was shown to be a potential precursor to a reactive nitrile oxide that was trapped as a GSH adduct. The proposed bioactivation pathways are shown in Fig. (**17**).

Further studies on the metabolism of parent benzylamine indicated the formation of reactive metabolites different from those observed with DPC 423 or DPC 602 (Fig. **18**) [141]. Thus, the formation of hydroxylamine-derived metabolites was not discernible in the incubations but the production of reactive metabolites *via* arene oxide formation and by several other novel metabolic pathways was observed. A novel pathway included the formation of a new carbon-nitrogen bond that led to a potentially reactive intermediate, Ar-CH_2-NH-(CO)-X, capable of interacting with various nucleophiles including glutathione (Fig. **18**). This reactive intermediate is thought to arise *via* the formation of either a formamide or carbamic acid metabolite. The formation of formamide as metabolites of alkyl- or arylalkylmines has been described previously. The formylation reaction, usually observed only *in vivo*, leads to formamides that are capable of being further oxidized to reactive isocyanates.

Structure/Bioactivation Relationships

Although structure-bioactivation relationship studies on benzylamines have not been conducted, it is assumed that benzylamines, especially the unsubstituted ones, will be subject to the bioactivation process. Secondary metabolism of *N*-substitutedbenzylamines could potentially lead to the formation of primary amines and their subsequent bioactivation. Substitution on the benzylic carbon may also hinder some of the bioactivation sequences involving α-carbon oxidation.

Bioactivation of Cyclic Secondary Amines to Reactive Nitroxide Radicals

Occurrence and Frequency

In addition to formation of reactive iminium species, there are rare instance when cyclic tertiary amines are bioactivated to reactive nitroxide radicals. An example of a cyclic tertiary amine that demonstrates such behavior is cocaine. Cocaine is mostly metabolized *via* ester hydrolysis, but a small portion is metabolized *via N*-demethylation to norcocaine followed by *N*-hydroxylation of norcocaine and further oxidation of the *N*-hydroxy metabolite to the nitroxide radical (Fig. **19**) [142-145]. The latter species has been observed by EPR spectroscopy. Cocaine is capable of producing severe hepatocellular necrosis in laboratory animals and humans and this adverse event has been linked to the oxidative metabolism of cocaine.

Mechanism

P450 is thought to catalyze all three oxidative steps, but the role of FMOs cannot be ruled out. Incubation of radiolabeled cocaine with liver microsomes leads to incorporation of radioactivity into the protein [146]. However, the toxicity induced by cocaine appears to be due to oxidative stress by the nitroxide undergoing one electron redox cycling with the hydroxylamine. The nitroxide can spontaneously oxidize NADH and NADPH, which in turn oxidize GSH to oxidized glutathione (GSSG), resulting in GSH depletion and increased lipid peroxidation.

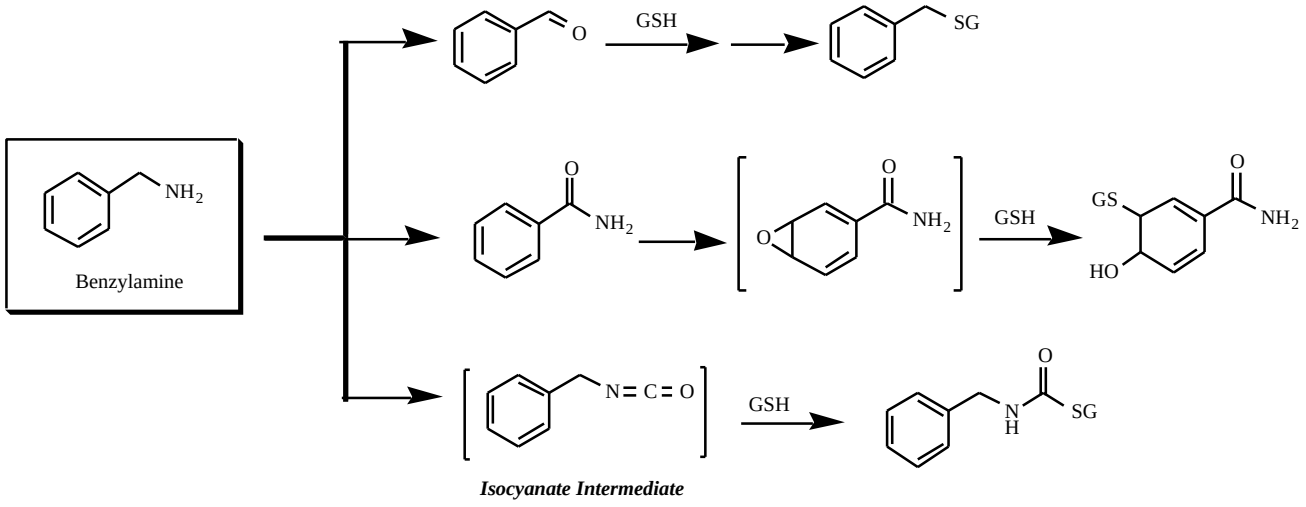


Fig. (18). Bioactivation of Benzylamine.

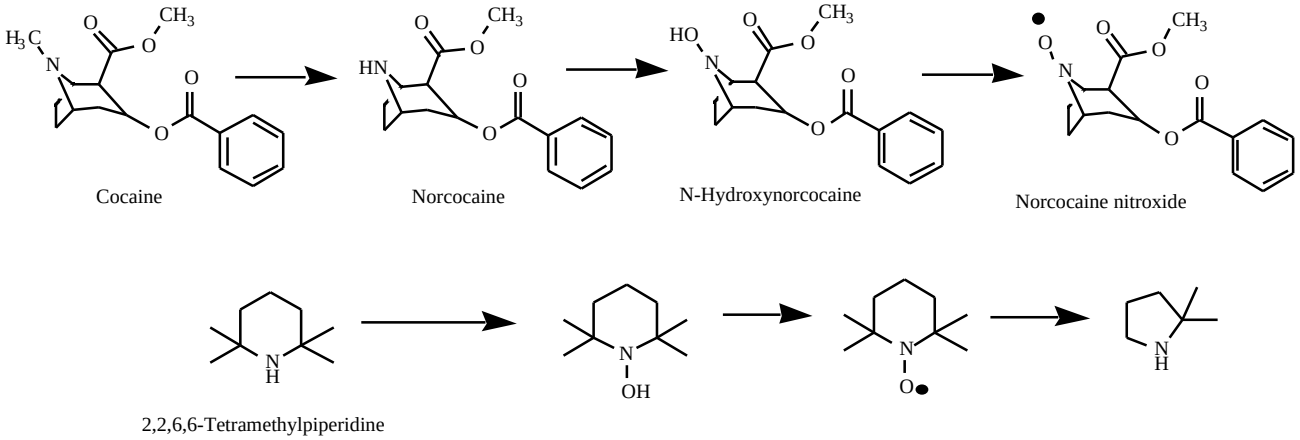


Fig. (19). Bioactivation of Cyclic Secondary Amines to Reactive Nitroxide Radical.

Structure/Bioactivation Relationships

No SAR work has been done to address the structural determinants that cause a compound to be metabolized to a nitroxide radical. In norcocaine, the secondary amine nitrogen is highly accessible (unhindered) in the fused ring structure, and this could possibly be an underlying reason as to why oxidation to the hydroxylamine and nitroxide occur. Recent work with 2, 2, 6, 6-tetramethylpiperidine as a model structure has shown an unusual ring contraction reaction proposed to occur *via* a nitroxide intermediate [147]. The secondary piperidine nitrogen, which like norcocaine has alkyl substitutions on the α-carbons, undergoes *N*-hydroxylation followed by a second oxidation to the nitroxide radical. Reaction with ferric iron is proposed as the step that initiates the ring contraction, with release of one of the α-carbon as a carbonyl with the corresponding alkyl substituents (acetone in the case of 2, 2, 6, 6-tetramethylpiperidine). Thus, it can be postulated that secondary alicyclic amines possessing substitutions on the α-carbons may be most susceptible to this type of metabolic pathway and hydroxylamine and possibly ring-contracted metabolites can be indicative of the formation of the nitroxide radical intermediate. However, the aneasthetic ketamine, in which, the secondary nitrogen also undergoes conversion to a nitroxide radical [148], leaves the possibility that acyclic aliphatic secondary amines can also undergo this bioactivation reaction. As the examples pertaining to nitroxide radical formation are rare, the strategy of routinely checking for such pathways may not be warranted. However, some experimental approaches that could be applied when considering the possibility include: (a) does the secondary cyclic amine undergo *N*-hydroxylation in microsomes? Hydroxylamines are easily detectable by mass spectroscopy, (b) *in vitro* examination of the effect of the parent amine on oxidative stress markers in hepatocytes (e.g. measurement of GSSG or lipid peroxidation) and (c) detection of nitroxide radical from ESR studies.

Formamides

Occurrence and Frequency

N-Alkylformamides are important industrial chemicals often utilized as synthons for chemical syntheses and as solvents. *N, N*-Dimethylformamide (DMF, $HCON(CH_3)_2$) is a representative compound that is frequently used as a organic solvent. DMF is also used in the manufacture of synthetic fibers, leathers, films, and surface coatings. DMF has been found to be hepatotoxic in rodents and in humans after occupational exposure to high concentrations of the DMF vapor [149-151]. The monomethyl derivative *N*-methylformamide (NMF) possesses antineoplastic activity against a variety of mouse tumors. The potential application of NMF as a chemotherapeutic agent in the treatment of cancer in humans has been evaluated in several phase-I and -II clinical trials, but no beneficial therapeutic effect has been discerned from these studies. Patients in these clinical trials however suffered from nonspecific malaise, nausea, and dose-limiting liver damage [152]. The illicit synthesis of amphetamine also involves a formamide intermediate (produced *via* the Leukart-Wallach Reaction), which has shown to be a potent hepatotoxic agent in rodents [153]. Bladder carcinogenesis has been observed with *N*-[4-(5-nitro-2-furyl)-2-triazolyl]formamide [154]. Studies with (±)-*N*-(1-methyl-3,3-diphenylpropyl)formamide also showed evidence of hepatic necrosis in rats [155].

Mechanism

The mechanism by which NMF causes liver damage in mice has been intrinsically linked to its metabolism (Fig. **20**) [150]. Studies have shown that P450 enzymes, especially CYP2E1 is involved in NMF bioactivation [156]. Induction of this enzyme by ethanol could potentially increase the likelihood of hepatotoxicity. NMF is extensively metabolized in rodents and humans and oxidation to the highly reactive methyl isocyanate (CH_3NCO) is thought to constitute the rate-limiting step towards hepatotoxicity. Due to the extreme instability of low molecular weight isocyanates, the intermediacy of an isocyanate metabolite from formamides such as NMF has been inferred from the formation and presence of the appropriate GSH or *N*-acetylcysteine conjugate(s) *in vivo*. However, the formation of relatively stable isocyanate intermediate from larger molecules containing the formamide moiety has been well demonstrated. Studies have also shown that the sulfydryl conjugates could release methyl isocyanate under physiological conditions [155].

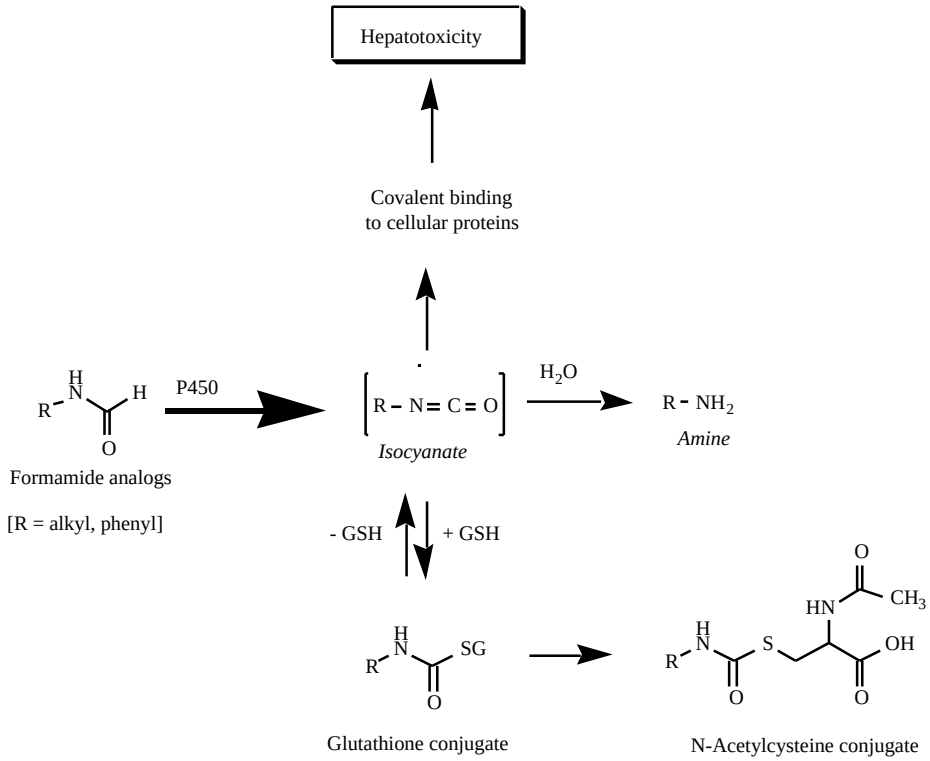


Fig. (20). Bioactivation of the Formamide Group.

Suggestions have been made that sulfydryl conjugates could act as potential transporters for the reactive methyl isocyanate to distant sites where toxicity could be produced [157].

Structure/Bioactivation Relationships

Although specific studies have not been performed, it is postulated that all formamides, which have the ability to be metabolized to the reactive isocyanate intermediate, can be potentially toxic. The hepatotoxicity of formamides is not limited to only small molecules such as NMF and DMF. Compounds such as *N*-formylamphetamine and (±)-*N*-(1-methyl-3, 3-diphenylpropyl)formamide are also hepatotoxic in rats and the existence of the reactive isocyanate intermediate has been demonstrated by identification of the corresponding sulfydryl conjugates. Alkyl substitution on the formamide nitrogen leading to the tertiary formamide leads to a reduction in the formation of sulfydryl conjugates. However, *N*-dealkylation can easily precede the metabolic activation of the secondary formamide to reactive isocyanate metabolites as is observed with DMF. The formation of formamides from some primary amines has also been demonstrated; however this has been shown to be a fairly minor metabolic pathway.

Imides (Including Hydantoin and Barbiturates)

Occurrence and Frequency

Classic examples of drugs in which teratogenic effects in pregnant women have been associated with an imide moiety include the anti-convulsant phenytoin and the immunomodulatory agent thalidomide (Fig. **21**). Major human fetal abnormalities related to thalidomide administration during pregnancy include absence of limbs, short limbs, hypoplasticity of the bones, absence of bones, external ear abnormalities, facial palsy, and congenital heart defects. Mortality at or shortly after birth has been reported at about 40%. Furthermore, in addition to the reports of increased incidence of congenital malformations, such as cleft lip/palate and heart malformations in children of women receiving phenytoin, there have more recently been reports of a fetal hydantoin syndrome [158]. This consists of prenatal growth deficiency, microcephaly and mental deficiency in children born to mothers who have received phenytoin or barbiturates. There have also been isolated reports of malignancies, including neuroblastoma, in children whose mothers received phenytoin during pregnancy.

Mechanism

The finding that imide teratogenicity (e.g. phenytoin, trimethadione, or nirvanol) in mice is reduced or enhanced upon pretreatment with COX inhibitors (e.g., aspirin) or phorbol esters (known to stimulate phospholipase A_2, thereby releasing the cyclooxygenase substrate arachidonic acid intracellularly) strongly suggests COX's involvement in imide bioactivation [159-161], a proposal which has been confirmed by ESR analysis of incubation mixtures containing COX/phenytoin or thalidomide/phenyl-α-tert-butyl nitrone, that revealed the presence of a unstable nitrogen-centered radical **108** and a stable carbon-centered radical **109** (Fig. **21**) [162]. The structures of the enzymatically-generated radicals were shown to be identical to those generated upon photochemical oxidation of imides. That the addition

of 2'-deoxyguanosine to the incubation mixtures resulted in a 5-fold increase in its oxidation to 8-oxo-2'-deoxyguanosine strongly suggests a role for cyclooxygenase in phenytoin or thalidomide bioactivation to free radical intermediates such as **108** that initiate DNA oxidation, which may constitute a common molecular mechanism of teratologic initiation described (Fig. **21**, Pathway A) [163].

Structure/Bioactivation Relationships

Based on the detection of phenolic metabolites of phenytoin and thalidomide in liver microsomal mixtures, earlier reports suggested the involvement of a reactive arene oxide **110** (Fig. **21**, pathway B), a proposal further supported by the observations that ring-opened thalidomide metabolites (see Fig. **21**) retain the teratogenic effects of the parent. However, the possibility that the teratogenic potential of these ring-opened metabolites are due to their further metabolism (*N*-dealkylation on the pthalimide) to a reactive pthalimide species remains to be studied. Overall, most of the recent evidence favors the single electron oxidation on the imide nitrogen to the free radical and so a critical requirement for teratogenicity is the presence of an unsubstituted imide nitrogen. Additional evidence for its critical requirement includes the association of teratologic effects with the imide anti-convulsants mephenytoin and its *N*-demethylated active metabolite nirvanol with the *L*-isomers that do not form arene oxides and the teratogenic activity of phenytoin analogs such as trimethadione, which has no phenyl rings and cannot form an arene oxide [159]. To address the possibility of bioactivation of imides and consequent thalidomide-

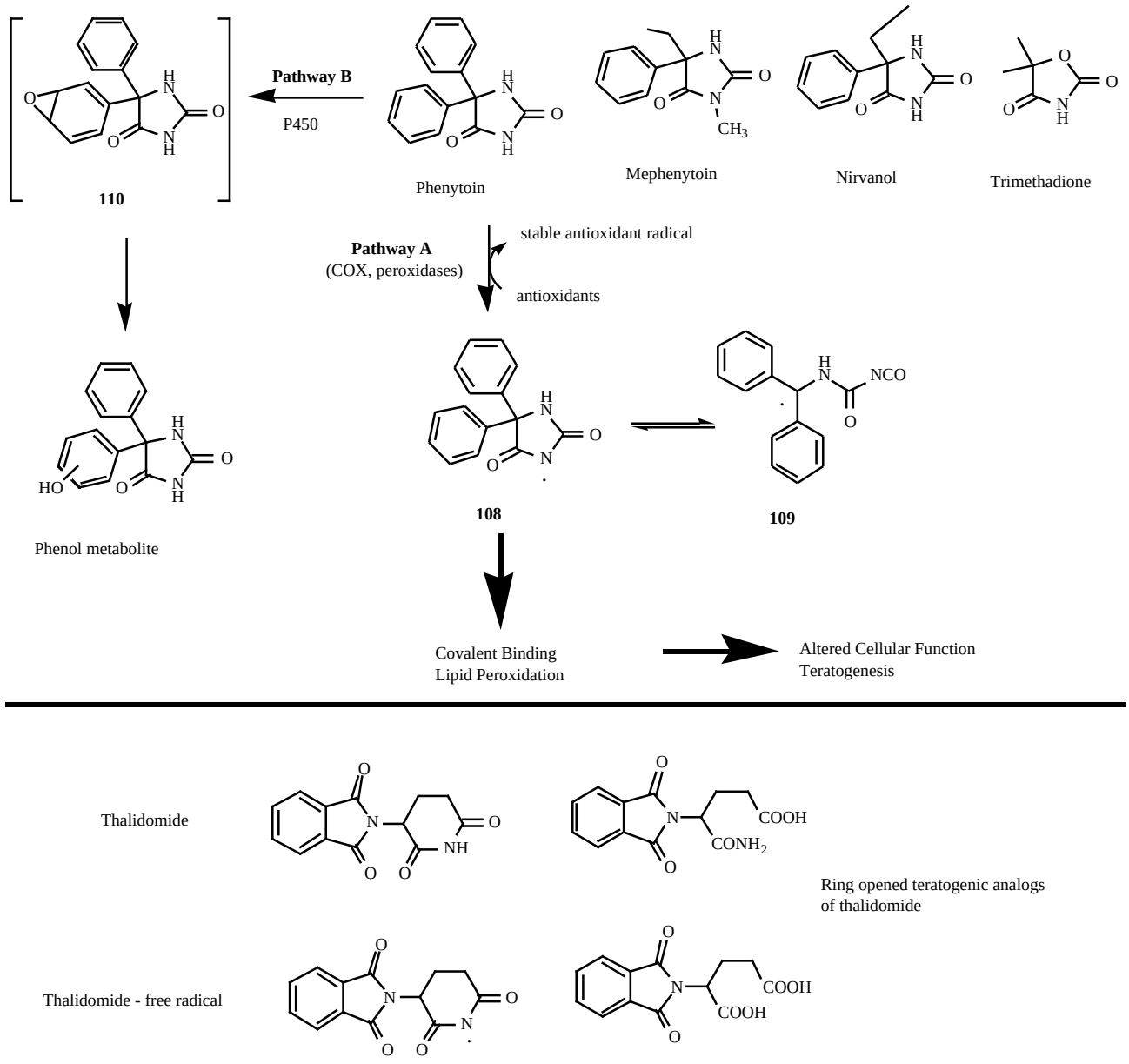


Fig. (21). Proposed Mechanisms for the Bioactivation of Imides.

like teratogenic effects, the following experiments should be contemplated: (1) Evaluation of teratogenic potential of imide-containing compounds, (b) Incubation of imides with P450 and/or peroxidases (including MPO or COX-1/-2 isozymes) in the presence of 2'-deoxyguanosine and monitoring the increase in its oxidation to 8-oxo-2'-deoxyguanosine as previously described [163], (3) *In vitro* experiments in embryonic cell cultures or stem cells documenting the teratogenic potential of the imide and prevention of these effects in the presence of COX inhibitors and/or by antioxidants as previously described [164, 165] and (4) *In vivo* experiments where pretreatment of animals (mouse and/or rabbits) with peroxidase inhibitors or peroxidase stimulants inhibits or enhances teratogenicity [166]. It is noteworthy to point out that since the bioactivation involves the generation of free radicals, thiols nucleophiles such as GSH will "quench" the free radical rather than react with it and most of the methodologies recommended are highly specialized.

Sulfonylureas

Occurrence and Frequency

Although diarylsulfonylureas such as sulofenur (LY186641) (Fig. **22**) have demonstrated utility as antitumor agents against a wide range of cancers (kidney, lung, ovaries, stomach and breast), dose-limiting toxicities in humans, which include methmoglobinemia and hemolytic anemia, have hampered wide usage. In pediatric patients with refractory malignant solid tumors, methemoglobinemia was observed at all doses during a phase I clinical trial. The dose-limiting toxicity has been linked to the bioactivation of the sulfonylurea group to the corresponding reactive *p*-chlorophenylisocyanate metabolite **111** [167]. An additional example of toxicity associated with a sulfonylurea derivative is highlighted with tolbutamide (Fig. **22**). Tolbutamide has been employed widely as a hypoglycemic agent in the treatment of non-insulin-dependent diabetes mellitus. However, the compound possesses teratogenic properties in several animal species and humans [168] and its use is contraindicated in pregnant diabetic patients. It has been proposed that the teratogenic effects in rats are linked to tolbutamide-mediated depletion of GSH through inhibition of glutathione reductase (GR) in rat embryos [169]. GR catalyzes the reduction of GSSG to GSH and thus maintains intracellular GSH levels in the embryo that are critical for cell viability and normal growth [170]. GSH depletion by exogenous compounds has been reported not only to enhance the embryolethal, teratogenic, and growth-retarding properties of these chemicals but also to cause an increase in the number of dead and malformed rat embryos *in vivo* [171]. Recent reports further demonstrate that the preimplantation stage of mouse embryo development appears to be more sensitive to GSH depletion since the embryo cannot synthesize GSH at this stage [172]. As a result, GR plays a major role in the preimplantation stage by recovering GSH *via* reduction of GSSG [173].

Mechanism

Previous studies with the antitumorigenic *N*-nitrosourea, *N, N'*-bis(2-chloroethyl)-*N*-nitrosourea (BCNU) (Fig. **22**) have revealed that its GR inhibitory properties are derived from the decomposition of the nitrosourea moiety to the reactive 2-(chloroethyl)isocyanate (**112**) intermediate that carbamoylates an active site cysteine residue in GR [174]. The structural similarity between BCNU and tolbutamide led Guan and coworkers to evaluate tolbutamide's potential to form *n*-butylisocyanate (**113**) that eventually inactivates GR in a manner similar to that observed with BCNU [175]. LC/MS/MS analysis of rat bile following administration of tolbutamide led to the identification of *S*-(n-butylcarbamoyl)glutathione (SBuG, **114**) (Fig. **22**), a GSH conjugate derived from the reaction of GSH with **113**. Incubation of synthetic **114** with yeast GR in the presence of NADPH led to a time- and concentration-dependent loss of enzyme activity. Furthermore, **113** was a more potent inactivator of enzyme activity than **114** and tolbutamide did not inhibit GR under these conditions. Biochemical mechanism(s) for the decomposition of sulfonylureas to the corresponding isocyanates remains unclear at the present time, since sulfonylureas such as sulofenur and tolbutamide have been found to be stable towards decomposition at physiological pH and increasing temperature. Likewise, no GSH-isocyanate conjugate formation is discernible in incubations of sulofenur and GSH in glutathione-*S*- transferase. Overall, these results indicate that a potential cause for the teratogenic effects of tolbutamide may be its *in vivo* metabolism to the reactive metabolite **113** that can carbamoylate active site residues in GR resulting in its inhibition. Alternately, GSH conjugation with **113** leads to the *S*-linked carbamoylating agent, **114**, which although less reactive, does possess the ability to carbamoylate GR. The enzyme(s) responsible for the degradation of tolbutamide to **113** remain unidentified. Although it may be argued whether the amount of **114** formed is significant enough to produce any biological effects, it is noteworthy to point out that the amount collected in bile may only represent the "unreacted" portion of the metabolite. *In vivo* studies on the bioactivation of sulofenur in rats also revealed the formation of the GSH conjugate of **111**, presumably derived from the decomposition of sulofenur.

Structure/Bioactivation Relationships

Biochemical mechanisms for the decomposition of sulfonylureas to the corresponding isocyanates are unknown. It is likely that conversion of the urea motif to a cyclic imidazoline group may prevent isocyanate formation. The chemical reactivity of aliphatic and aromatic isocyanates towards GSH is well established.

Thioureas

Occurrence and Frequency

Thioureas (also referred to in the literature as thiocarbamides) are widely used as insecticides, as therapeutic agents, and in the manufacture of plastics. The toxicological effects of thioureas include thyroid depression, pulmonary edema, and liver necrosis [176]. The teratogenic properties of thioureas are also well characterized. For instance, administration of 1-methyl- and 1-ethylthiourea to rats on day 12 of pregnancy resulted in high incidences of fetal toxicity/malformations; in contrast, no teratogenic effects were observed when 1-methylurea and 1-ethylurea were administered [177].

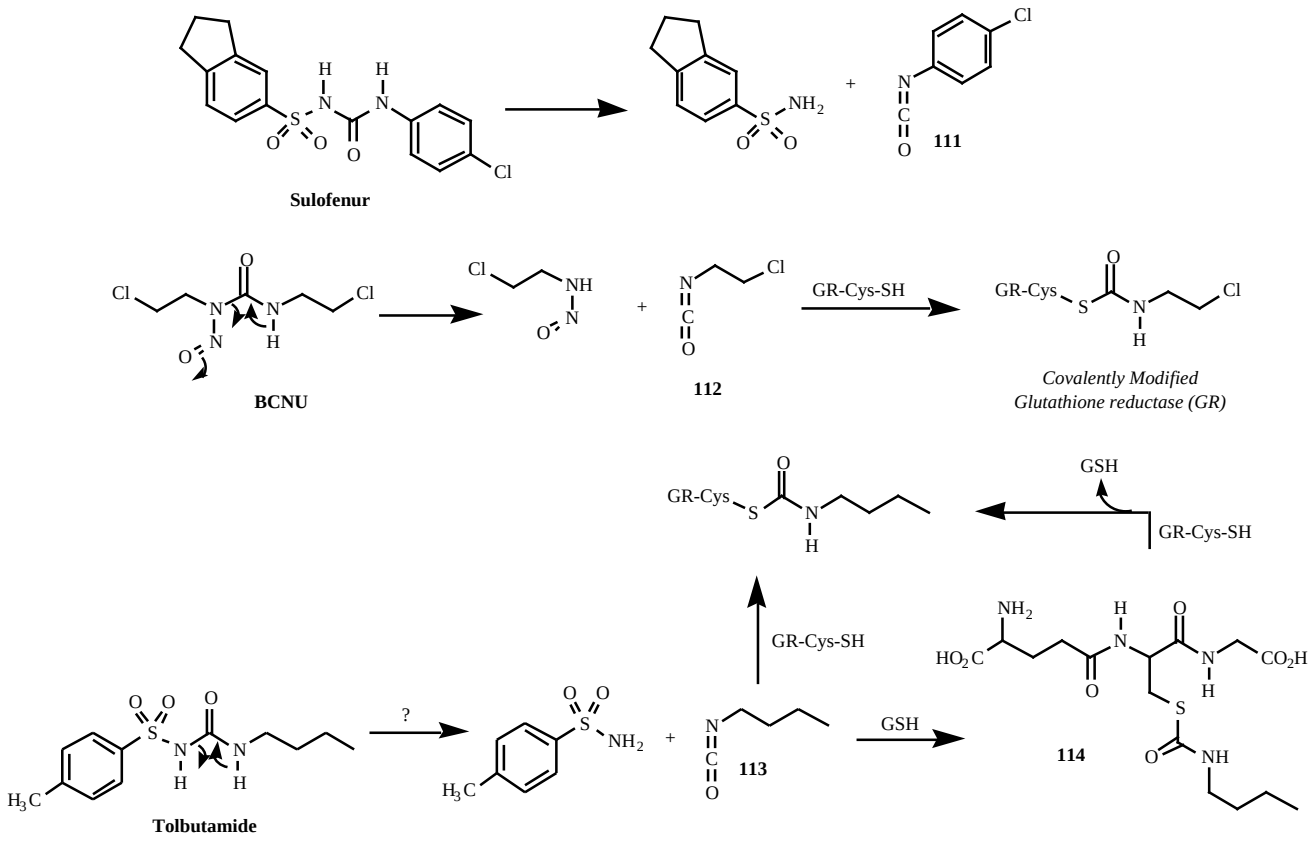


Fig. (22). Bioactivation of Sulfonylureas to Reactive Isocyanate Intermediates.

Mechanism

The adverse effects of thioureas are attributed to the formation of *S*-oxide intermediates catalyzed primarily by P450s and FMOs [178]. Initial oxidation results in the formation of electrophilic sulfenic acids (*S*-oxide), which can be further oxidized to form sulfinic (*S*-dioxide) and sulfonic (*S*-trioxide) acids. The resulting *S*-oxide can also be hydrolyzed to the corresponding urea (detoxification pathway). Many of the *S*-oxide intermediates are extremely labile and difficult to isolate and identify. Based on their reactivity, the sulfur oxidation products are believed to be the reactive intermediates responsible for thiourea-induced toxicity. Metabolic activation of thioureas result in reactive intermediates capable of protein binding and enzyme inhibition [179]. In rat hepatic microsomes, ethylenethiourea was shown to bind to microsomal protein and inactivate P450, a process that was completely prevented by the addition of GSH. In the presence of GSH, GSSG is formed and the *S*-oxides are reduced to the parent thiourea [180]. These results are supported by reports of increased biliary GSSG efflux from rat livers perfused with thiourea and phenylthiourea [180]. The futile cycling may result in rapid depletion of free GSH and ultimately of NADPH. The depletion of cellular GSH and protein adduct formation may be partly responsible for thiourea-induced liver necrosis.

An example of the reactivity of a pharmacologically active compound containing a thiourea moiety is illustrated with *N*-(5-chloro-2-methylphenyl)-*N'*-(2-methylpropyl)thiourea (SDZ HDL 376), a drug under development for atherosclerosis (Fig. **23**) [181]. SDZ HDL 376 undergoes extensive metabolism in rat, monkey, dog and human liver microsomes. Although, P450 mediated hydroxylation on the phenyl ring or on the isopropyl carbon constituted the major metabolic pathways in human and monkey liver microsomes, *S*-oxidation was the major metabolic fate in rat liver microsomes. Furthermore, the NADPH-dependent oxidation of SDZ HDL 376 in rat liver microsomes also resulted in the covalent binding to microsomal protein. Addition of GSH to the incubations decreased protein binding in a concentration-dependent manner and also resulted in the formation of a novel GSH-SDZ HDL 796 adduct **115**. Furthermore, inhibition of P450 *via* pretreatment with 1-aminobenzotriazole resulted in a 95% decrease in adduct formation. Interestingly, incubations performed with ^{35}S-GSH resulted in a [^{35}S]SDZ HDL 376-GSH adduct, demonstrating that the sulfur was derived from GSH. The novelty of **115** is evident from the observations that structurally unrelated thioureas such as phenylthiourea and methimazole do not form such adducts in liver microsomes supplemented with GSH.

Structure/Bioactivation Relationships

Although specific SAR studies have not been conducted, it is conceivable that all thioureas, which have the ability to undergo *S*-oxidation as a metabolic fate *in vitro* and *in vivo*, can be potentially toxic. This is illustrated in the SAR for

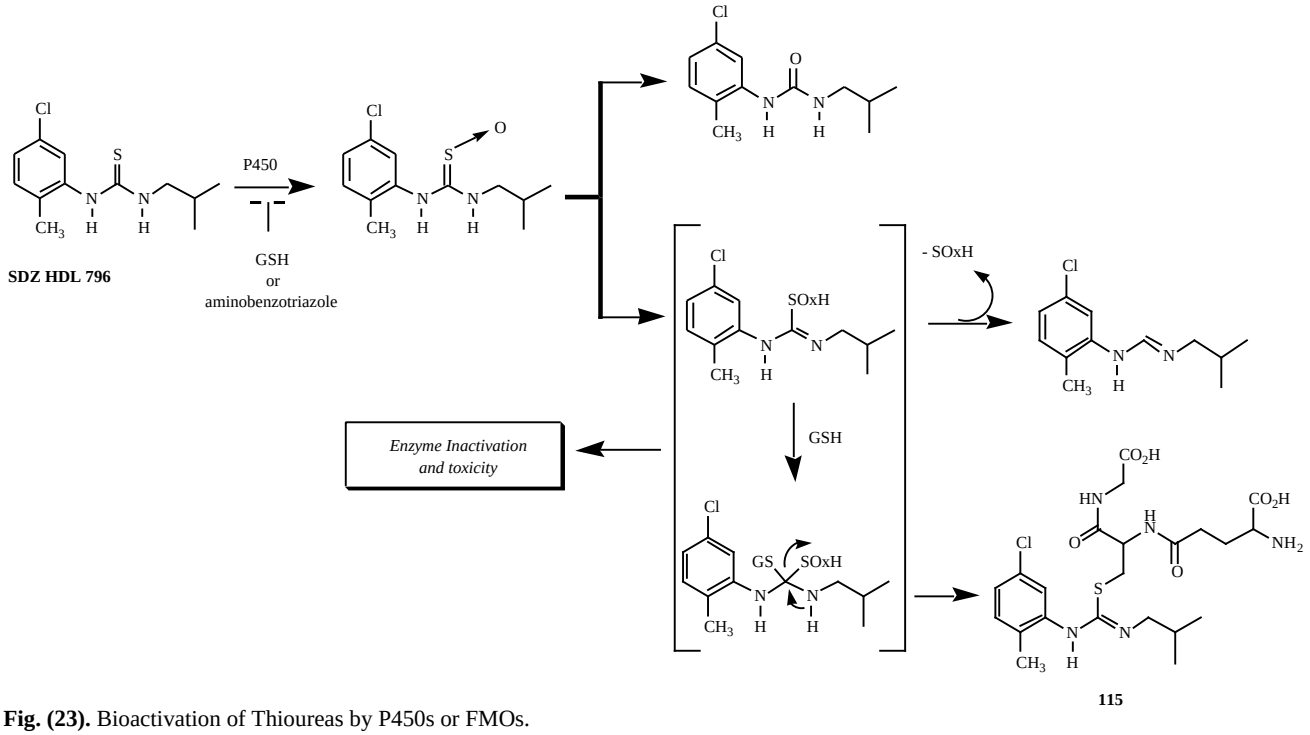


Fig. (23). Bioactivation of Thioureas by P450s or FMOs.

teratogenicity of structurally diverse thioureas in rats (simple dialkyl, diaryl, or arylalkyl). In contrast, the corresponding dialkyl, diaryl or arylalkyl ureas are not teratogenic in rats [177].

Reduced Thiols & Disulfides

Occurrence and Frequency

Drugs that contain a free thiol group react with cysteine-disulfide residues in proteins to form a new disulfide link with the concomitant release of a free cysteine residue within the protein. No metabolic activation is required for the covalent binding of these drugs. In addition, thiols are readily oxidized to sulfenic acids, which react with cysteinyl residues and reduced glutathione to form mixed disulfides. Methimazole (Fig. **24**) is an example of a reduced thiol that is used widely in the treatment of hyperthyroidism. The adverse effects caused by this drug include dermal and gastrointestinal disorders, lupoid-like syndrome, and bone marrow depression, with agranulocytosis as the most serious complication [182]. Hepatotoxicity is another ADR that occurs during therapy, although its frequency is considered low. In most cases, methimazole-induced liver injury, cholestatic jaundice, and anicteric hepatocellular damage has been reported [183, 184]. Studies in preclinical species have shown that methimazole causes severe liver damage in mice depleted of GSH by pretreatment with inhibitors of GSH synthesis. These results are consistent with previous findings that indicate the protective effect of GSH against protein binding, enzyme inactivation, and organ toxicity of thiols and thioureas (see preceding sections). Penicillamine (Fig. **24**) is a copper-chelating agent used in the treatment of rheumatoid arthritis and copper toxicity. Its use in humans is linked with unusual autoimmune reactions including a lupus-like syndrome and aplastic anaemia [185]. A similar lupus-like syndrome is also seen when Brown Norway rats are treated with penicillamine [186, 187]. This animal model of penicillamine-induced lupus is itself idiosyncratic as it only occurs in Brown Norway rats and not in Lewis or Sprague-Dawley strains. Even within a group of penicillamine treated Brown Norway rats, not all of them will develop the lupus-like syndrome (~ 60% incidence). The animal model of penicillamine-induced lupus has been used in an attempt to gain a more fundamental understanding of the mechanisms by which drugs can cause idiosyncratic adverse autoimmune reactions [188-190]. Captopril (Fig. **24**) was the first angiotensin converting enzyme (ACE) inhibitor. Because of captopril's relatively high incidence of ADRs including bone marrow toxicity and skin rashes [191], other ACE inhibitors have replaced it. It must however be stressed that some of the adverse events can be clearly traced back to the thiol group while others are due to its pharmacological activity as an ACE inhibitor. The biochemical reaction of captopril with protein cysteinyl residues leading to mixed disulfides is well established. It is interesting to note that enalapril (Fig. **24**) that lacks the free thiol does not lead to many of the ADRs associated with captopril including skin rashes and the life-threatening neutropenia.

Mechanism

As mentioned earlier, free thiol-containing agents covalently modify proteins by reacting with cysteinyl-disulfide linkages within the proteins to form a new disulfide with the drug (Fig. **25**, panel A). Alternately, thiols can also undergo enzymatic oxidation to the reactive sulfenic acids (R-SOH), a mechanism for which is shown in Fig. **25**, panel B. When FMO enzymes are involved, the pathway proceeds *via* direct oxygenation (Pathway a), whereas P450 is believed to

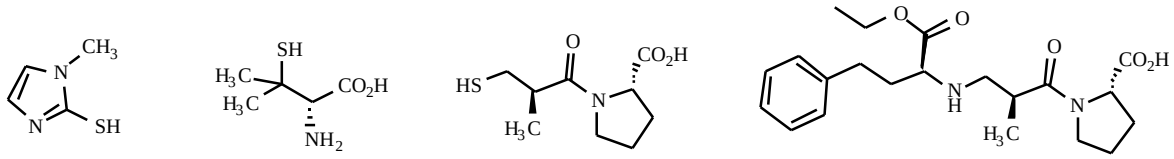


Fig. (24). Examples of Reduced Thiol-Containing Drugs.

mediate a single electron oxidation at sulfur yielding the corresponding thiyl radical, followed by oxygen transfer (Pathways b and c). The total loss of hepatotoxicity observed by methylation of the reduced thiol group in methimazole clearly indicates that the thiol substituent in methimazole is responsible for its toxicological consequences [192]. The potential sulfenic acid and sulfinic acid metabolites of methimazole have been proposed as the reactive intermediates (Fig. **25**, Panel C), although no evidence for their formation exists to date. The recent findings on the detection of the *N*-methylthiourea metabolite of methimazole in the urine of mice [192] suggests an additional pathway for the generation of reactive metabolites derived from the further *S*-oxidation of the *N*-methylthiourea metabolite (see section on thioureas). The proposed mechanism for the formation of *N*-methylthiourea from methimazole presumably involves the initial epoxidation of the 4, 5-double in methimazole followed by hydrolysis to the corresponding diol intermediate. Ring scission of the diol intermediate then leads to *N*-methylthiourea and glyoxal (Fig. **25**, Panel C). The detection of the electrophilic glyoxal metabolite in the urine of mice suggests that this compound may also be responsible for the hepatotoxic side effects of methimazole. Consistent with this hypothesis, saturation of the C4, 5-double bond in methimazole results in complete loss of hepatotoxicity [192]. Although, the role of sulfenic and sulfinic acid metabolites remains unclear, it is noteworthy to point out that sulfenic acids generated from thioureas such as *N*-methylthiourea oxidize GSH to GSSG nonenzymatically. Thus, GSH can be involved in the detoxification of thioureas as a cellular reductant for their reactive intermediates and its depletion may lead to the observed hepatotoxic effects of methimazole. The possibility that methimazole hepatotoxicity is also mediated by the reactive dialdehyde glyoxal also cannot be ruled out.

Structure/Bioactivation Relationships

A variety of thiols have been studied *in vitro* as FMO substrates, the product of the reaction being the symmetrical disulfide (since under typical *in vitro* conditions, the reactive sulfenic acid tends to react with excess thiol substrate) [193]. In general, preferred thiols that undergo FMO mediated oxygenation are those that bear a negative charge on the sulfur (a very nucleophilic sulfur atom).

Quinones

Occurrence and Frequency

Quinones are Michael acceptors that alkylate cellular proteins and DNA resulting in cellular damage and DNA adduct formation. The cytotoxicity and DNA adduct formation of the parent *p*-benzoquinone has been well studied [194]. Alternately, they are highly redox active compounds that can redox cycle with their corresponding hydroquinone and semiquinone radical forms, leading to the formation of reactive oxygen species (ROS) including superoxide, hydrogen peroxide and hydroxyl radical. These reactive species in turn lead to oxidative stress and the formation of oxidized proteins. Fig. **26** provides examples of the bioactivation of various therapeutic agents through the formation of a quinone metabolite. Each compound is known to exhibit toxicities ranging from skin sensitization and liver injury (cholestasis or hepatocellular injury) to carcinogenesis. The quinone-containing anti-cancer agents adriamycin and daunorubicin (Fig. **26**) exert their therapeutic effect *via* quinone-hydroquinone redox cycling, leading to ROS generation [195]. These ROS help destroy the cancer cells, although selectivity is not absolute. The catecholamines, α-methyldopamine and dopamine, are implicated in 3, 4-(methylenedioxy)amphetamine toxicity, given their ability to undergo oxidation to the corresponding quinones [196]. In addition to these compounds, naturally occurring estrogens, such as estrone and estradiol, have been shown to form quinone metabolites that are believed to contribute to the carcinogenicity of estrogens beyond that attributed to the better established receptor-mediated tumor promotion process [197, 198]. Xenobiotics and other pharmaceutical products like diethylstilbestrol and hexestrol [197, 199] represent a more unpredictable class of quinone-forming chemical entities that, through their complexity and structural diversity, help make prediction of potential quinone-related toxicity unrealistic and unreliable. While few, if any, pharmaceutical agents developed today contain quinonoid motifs; it remains a possibility that bioactivation processes may lead to quinone formation for a variety of these chemical entities.

Mechanism

With the exception of anti-cancer agents adriamycin and daunorubicin, quinones are typically generated from seemingly innocuous aromatic compounds *via* enzymatic oxidative processes (Fig. **27**). Monoalkoxy phenols, like eugenol, and dialkoxy substituted aromatic systems, where substituents are located in an *ortho* or *para* arrangement, can undergo P450-mediated oxidative *O*-dealkylation reactions which serve to "unmask" catechols that are then readily oxidized by a number of enzymatic and potentially non-enzymatic systems to form quinones [200]. Alternatively, in the case of benzene and other polyaromatic hydrocarbons, it is possible that quinones exist in an even less predicable

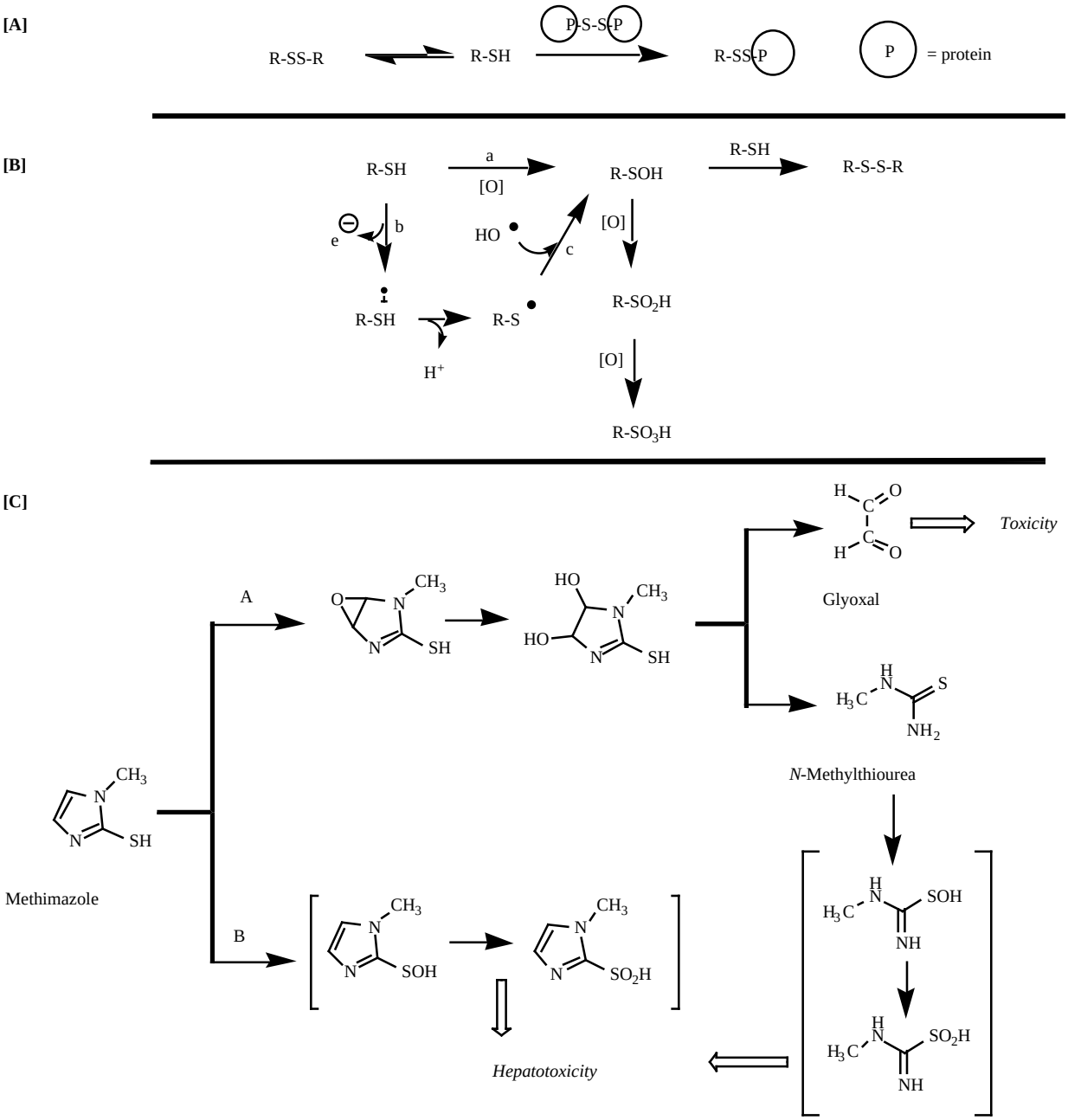


Fig. (25). Pathways of Thiol Reactivity

more "latent" form like a phenyl substituent. Sequential P450-mediated aromatic hydroxylation events in an *ortho* or *para* arrangement also result in catechol formation and the likelihood of further oxidation to quinones. *O*-Methylation of the catechol metabolites by catechol-*O*-methyl transferase (COMT) constitutes a detoxification pathway. The electrophilic nature of quinones is cited as the most likely explanation for their toxicity. Nucleophiles presented by DNA bases and proteins can adduct the aromatic ring by means of a 1, 4 Michael-type addition reaction. GSH also may react with quinone metabolites, and this activity is often viewed as a potential detoxification pathway. Once adducted, the conjugates typically rearomatize and, where feasible, may be oxidized yet again and react further. Quinone metabolites that react with DNA have been implicated in mechanisms of carcinogenesis [197], while those that react with cellular proteins to form haptens may be responsible for immune-related toxicities [200]. Necrotic cell death as a result of protein binding may also be a factor in certain toxic events.

In the redox environment of biological systems, quinones may also cause toxicity through the formation of ROS (Fig. **28**). Superoxide anion radicals ($O_2^{\cdot-}$) undergo spontaneous or enzymatic dismutation to generate hydroxy radicals ($HO^{\cdot}$),

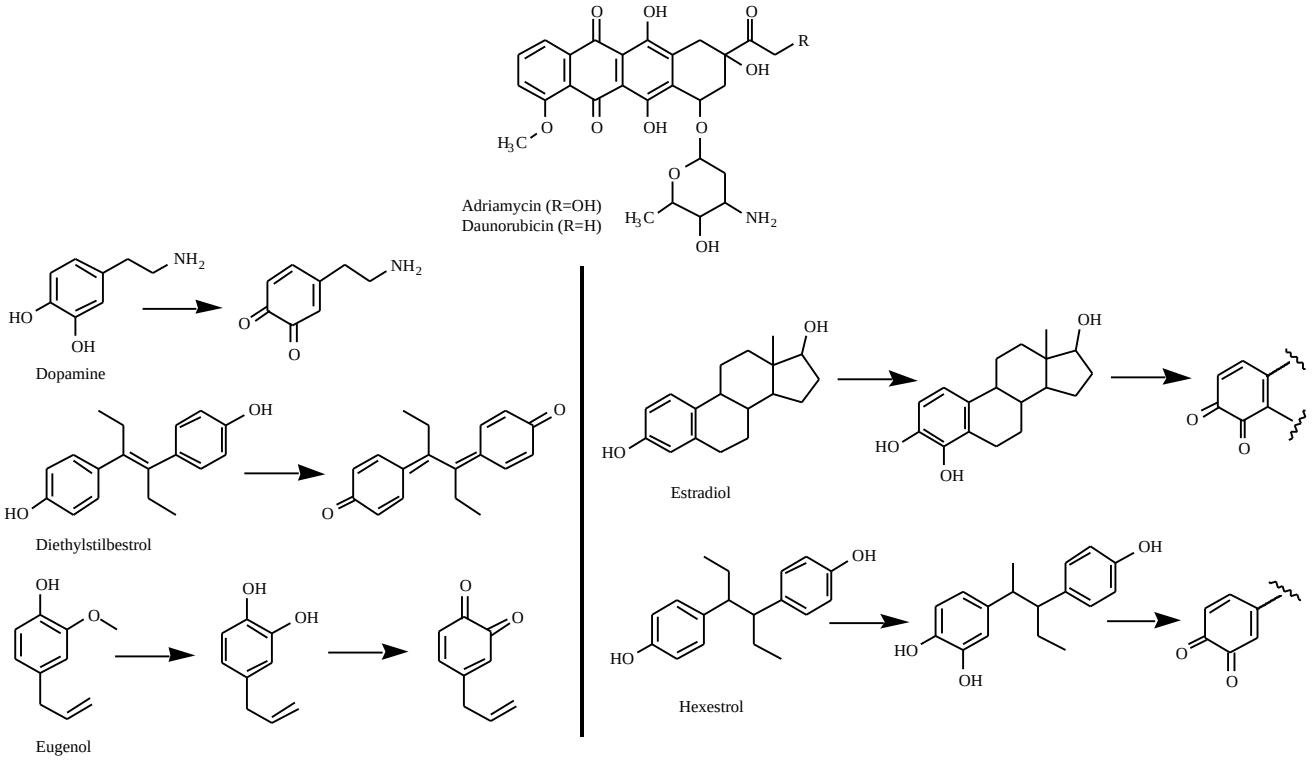


Fig. (26). Selected quinone-containing anti-cancer agents and other quinone-forming endobiotics and xenobiotics.

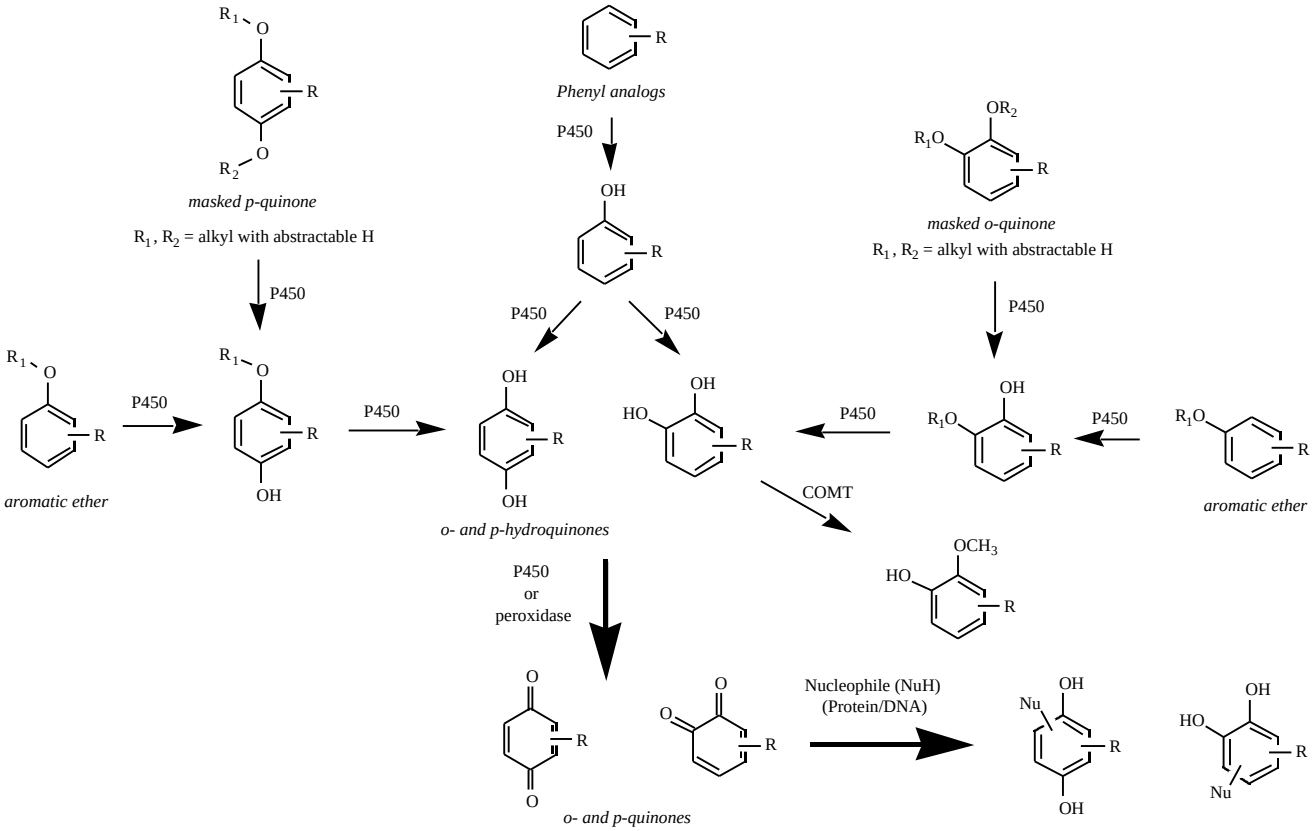


Fig. (27). Bioactivation of Benzene and Phenolic Compounds to Quinones.

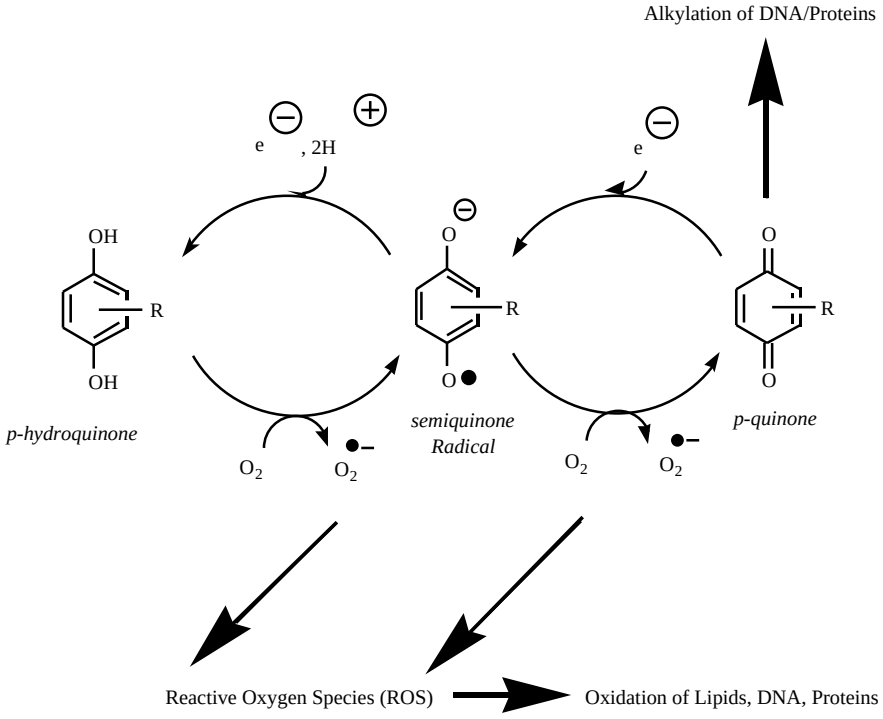


Fig. (28). Redox Cycling of Quinone Metabolites to ROS.

which alter protein structure *via* oxidation of essential aminoacid residues such as cysteine and effect cellular lipids through oxidative processes that involve lipid hydroperoxides [201]. Peroxidated lipids have been linked to malondialdehyde-DNA adducts [202], and have led to the use of 8-oxo-2'deoxyguanosine production as a biomarker for DNA damage related to ROS [203].

Structure/Bioactivation Relationships

Considering the theoretical possibility that phenols and even phenyl rings can undergo bioactivation to quinones, specific SAR studies around the potential for quinone generation are not possible. However, compounds that have *ortho*- or *para*-alkoxy substituents on an aromatic ring system or that have the potential to undergo hydroxylation at these positions should be considered suspect. It should be noted that haloarenes (particularly chlorophenyl and bromophenyl) may undergo P450-mediated *ipso* substitution to the corresponding phenol followed by further oxidation to quinone [204].

Quinone-Methides

Occurrence and Frequency

Quinone-methide formation occurs naturally during the lignin biosynthetic pathway, and many flavonoids produced as part of this pathway, like quercetin, are further able to undergo bioactivation to quinone-methides [205, 206]. Aside from natural processes that are responsible for the formation of quinone methides, many xenobiotics also possess the appropriate structural architecture conducive to bioactivation to quinone-methide intermediates. The synthetic antiestrogen tamoxifen, butylated hydroxytoluene, 2, 6-dimethoxy-4-allyl phenol, eugenol, *p*-cresol, the anti-bacterial agent trimethoprim, and more recently, the anti-diabetic drug troglitazone, have all been shown to undergo conversion to reactive quinone-methide intermediates (Fig. **29**) [200, 207-211]. The potassium channel opener, maxipost (BMS-204352) also undergoes P450 mediated bioactivation in the rat to generate a *o*-quinone-methide intermediate **116**, which covalently binds to albumin *in vivo* [212]. Acidic hydrolysis of plasma collected after intravenous administration of [^{14}C]-BMS-204352 to rats led to the characterization of a lysine conjugate of des-fluoro des-*O*-methyl BMS-204352 **117** (see Fig. **29**). Whether a similar bioactivation pathway occurs in human hepatic tissue remains unclear. Toxicity that arises as a result of quinone-methide exposure is generally the result of binding of the electrophilic metabolite to DNA and protein cellular macromolecules [198, 213], much like that observed with quinones. Sensitization reactions, hepatotoxicity, and cancer are among the major toxic effects linked to some of the above-mentioned compounds.

Mechanism

Tamoxifen serves as a good example in providing mechanistic aspects and diversity with which quinone methides may be formed [214] (Fig. **30**). In one case (Fig. **30**, pathway A), the *N*, *N*-dimethylethylamine group is oxidatively *O*-dealkylated by P450 to generate the corresponding phenol **119** that upon further oxidation affords quinone-methide **120**, which, like a quinone, is a good Michael acceptor. In an alternate scenario (Fig. **30**, pathway B), P450-mediated hydroxylation occurs on the aromatic ring geminal to the phenyl ether to afford phenol **121**, which upon further oxidation

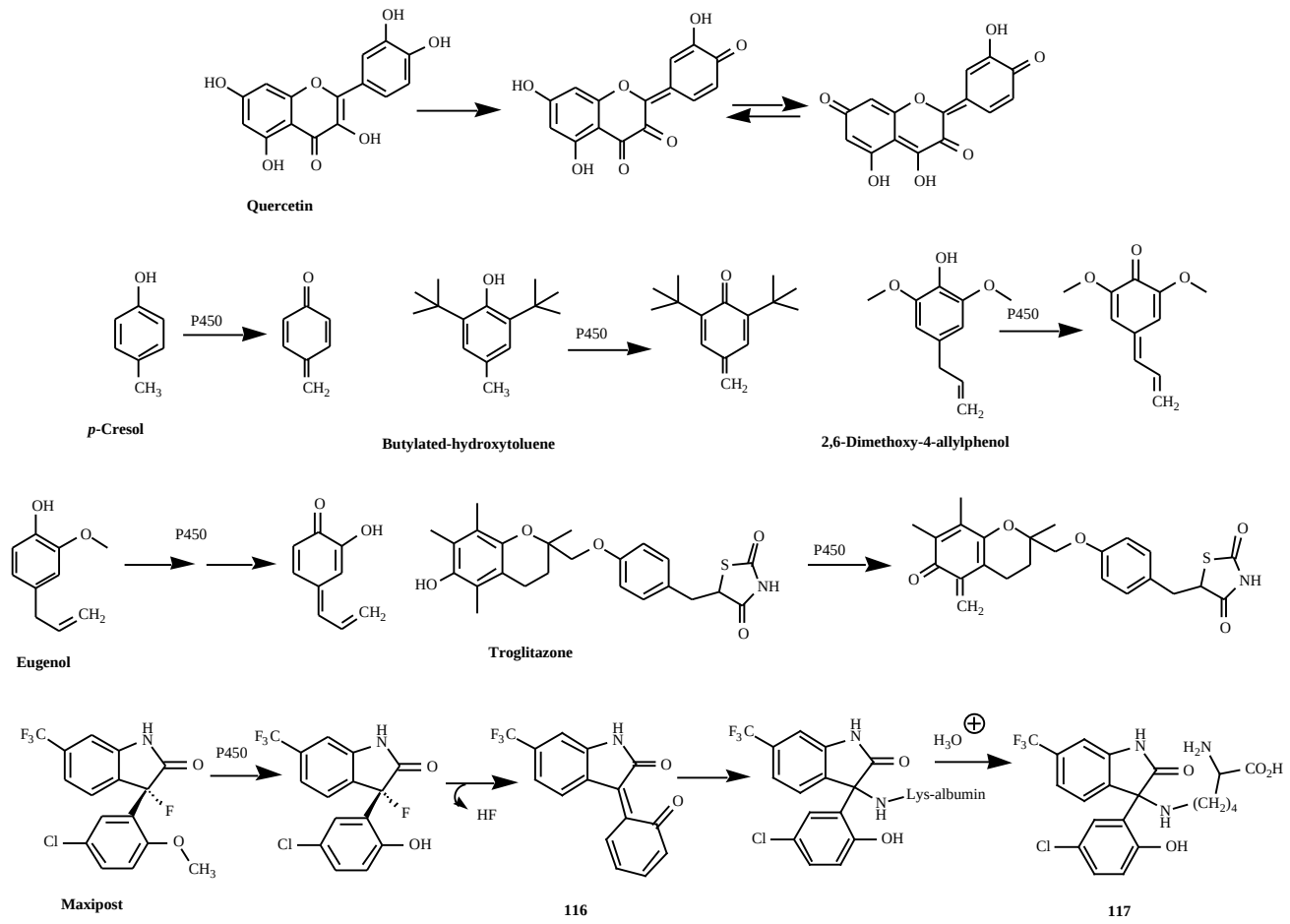


Fig. (29). Examples of Xenobiotics That Are Bioactivated to Quinone-Methides.

generates quinone-methide **122** [215]. The structures of GSH and DNA adducts of tamoxifen are consistent with the hypothesis that both bioactivation pathways occur to some degree [207, 216].

Trimethoprim is also bioactivated to the quinone-methide **123** [217] as shown in Fig. **31**, panel A. Unlike most quinone-methides in which nucleophilic attack by the trapping agent is almost exclusively on the exocylic methylene carbon, in the case of trimethoprim, GSH preferentially adds to the pyrimidine ring. A potential reason for this phenomenon is that the pyrimidine ring is more electron-deficient compared to the exocyclic methylene group. Overall, the bioactivation pathway leading to quinone methide formation is similar to that proposed for quinones and quinone-imines, with the exception that an alkyl group is involved in quinone-methide formation instead of a second hydroxyl (quinone) or amine (quinone-imine) group.

Recent studies on the microsomal metabolism of a pyrazinone-containing thrombin inhibitor MRL-B in NADPH and GSH supplemented rat and human liver microsomes [218] have revealed a bioactivation process involving either direct two electron oxidation of the parent compound or dehydration of the hydroxymethyl metabolite to the reactive imine-methide intermediate, amenable to trapping with GSH (Fig. **31**, panel B).

Structure/Bioactivation Relationships

Similar to quinones, no specific SAR consensus exists to help predict the likelihood of quinone methide formation. Again, the challenge here is that even with the necessary functionality present in a given molecule, it is possible that a quinone-methide will not be formed. With that caveat, basic requirements are an aromatic system where an alkyl and a hydroxy substituent are separated in either an *ortho* or *para* arrangement. The alkyl group must contain either a hydrogen atom or suitable leaving group on the carbon atom adjacent to the aromatic ring. As demonstrated with tamoxifen, the hydroxy group can be masked as an ether or added directly by means of CYP activity.

Methylenedioxyphenyl (1, 3-Benzodioxole) Derivatives

Occurrence and Frequency

The methylenedioxyphenyl (1, 3-benzodioxole) group is a common substituent in xenobiotics. It is found in natural products such as terpenes, safrole and isosafrole and alkaloids including eupaverine (Fig. **32**). In addition to its

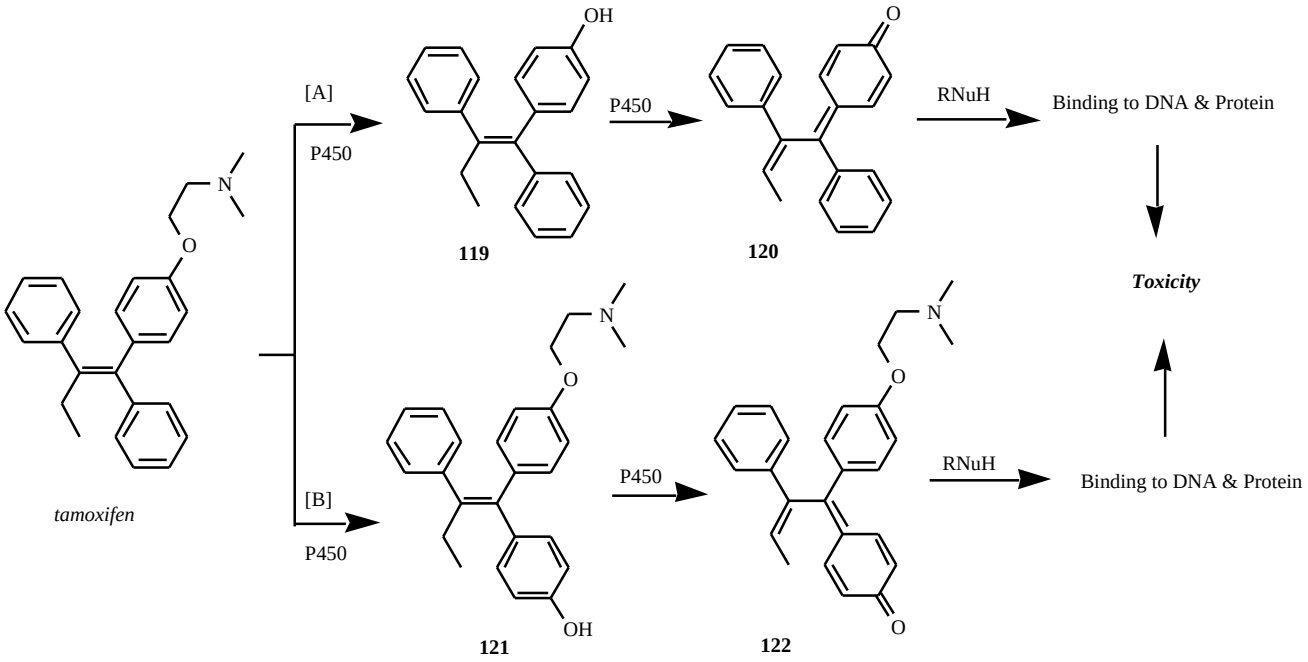


Fig. (30). Bioactivation of Tamoxifen.

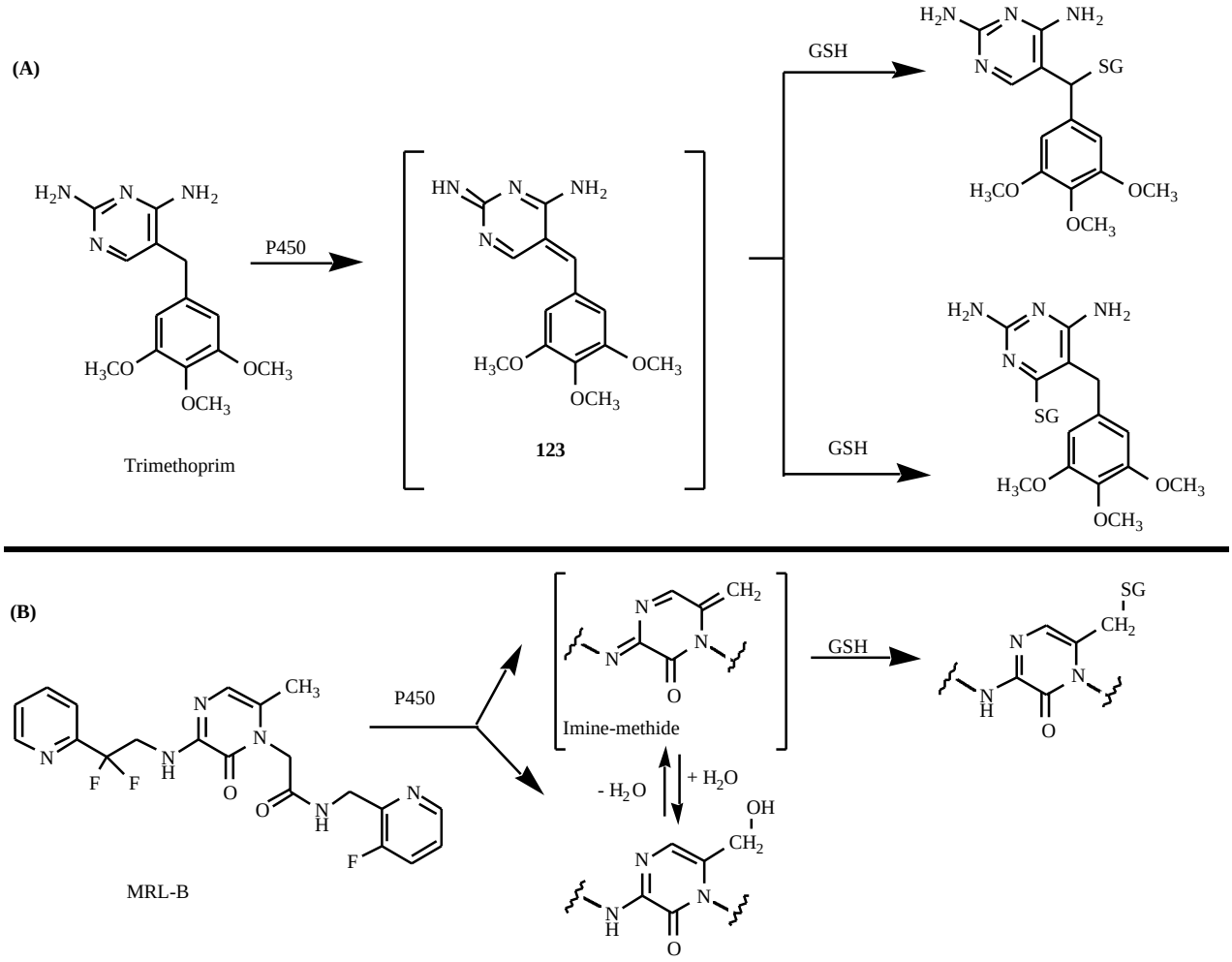


Fig. (31). Bioactivation of Trimethoprim and the Pyrazinone-Containing Thrombin Inhibitor MRL-B.

occurrence in natural products, the methylenedioxyphenyl group is also found in synthetic medicinal agents. The substituent is often incorporated as an alternative to 1, 2-alkoxyphenyl analogs, and like the alkoxy group, provides an electron rich aromatic ring that is more resistant to oxidation than the corresponding catechol motif. The methylenedioxyphenyl derivatives are less polar than the corresponding catechols and can penetrate the brain more readily. The anticonvulsant stiripentol, the M3 muscarinic receptor antagonist zamifenacin, the antidepressant paroxetine and the hallucinogen methylenedioxyamphetamine and its N-methyl derivative methylenedioxymethamphetamine (see Fig. 32) serve as representative examples of biologically active synthetic agents that contain the methylenedioxyphenyl group. The methylenedioxyphenyl group is a cyclic acetal of formaldehyde and catechol, which is susceptible to hydrolysis under acidic conditions, and it can also be oxidized by hydroxyl radical generating systems. Furthermore, methylenedioxyphenyl substituents are often substrates/ inhibitors of P450 enzymes [219]. The metabolism of methylenedioxyphenyl substituents by P450 leading to reactive catechols and formate has been well characterized [220-222]. Altogether, metabolic processing of this substituent by P450 leading to autoinhibition of the P450 enzyme and subsequent formation of reactive metabolites have been proposed as mechanism(s) to account for IADRs and clinical drug-drug interactions (DDIs). For instance, zamifenacin, which was intended for the symptomatic treatment of irritable bowel syndrome was suspended from clinical development due to occasional increases in hepatic enzyme levels [223]. Metabolism was the major route of clearance of zamifenacin in preclinical species and human, with the primary metabolic step resulting in opening of the methylenedioxy ring to yield the catechol **124** [224]. In humans, **124** is the major metabolite of zamifenacin that is excreted from the circulation primarily as the corresponding glucuronide, whereas in animals, it was primarily metabolized by COMT to afford the corresponding guaiacol isomers **125** and **126** (Fig. **33**). Subsequent studies in primary hepatocytes from preclinical species indicated a delayed but substantial cytotoxic response to zamifenacin, suggestive of bioactivation to a reactive metabolite(s) [223]. The finding that the synthetic guaiacol metabolites induce rapid cytotoxicity to the cells as evident from the rapid increases in hepatic enzyme levels [223], further support a link between methylenedioxyphenyl ring scission and the observed ADRs in humans. The selective serotonin reuptake inhibitor paroxetine demonstrates non-linear pharmacokinetics in humans, consistent with its properties as a substrate [225] and time-dependent inactivator of CYP2D6 [226, 227]. The inactivation of CYP2D6 by paroxetine also translates into clinical DDIs with other CYP2D6 substrates such as desipramine and metoprolol [228, 229]. Experimental evidence suggests that CYP2D6 inactivation by paroxetine occurs in concert with the methylenedioxy ring opening to the reactive catechol metabolite **127** (Fig. **34**) [227]. In humans, **127** undergoes metabolism by COMT to afford isomeric guaiacol metabolites **128** and **129** that are excreted as the corresponding glucuronide and sulfate conjugates (see Fig. **34**) [230, 231].

Mechanism

Time-dependent inhibition of P450 by the methylenedioxyphenyl group occurs *via* the oxidation of the methylenedioxy ring carbon to a carbene intermediate (Fig. **35**) [232]. The carbene intermediate can form a metabolite-inhibitor (MI) complex with the P450 heme prosthetic group resulting in the inhibition of P450 catalysis [233]. This MI complex exhibits a characteristic absorption band at 456 nm, with a secondary peak at 430 nm (referred to as type 3 binding spectra) as has been demonstrated with many methylenedioxyphenyl compounds including paroxetine [227] and stiripentol [234]. A similar binding spectrum was observed for methylenedioxyamphetamine, however a nitroso-heme ligand was instead implicated, analogous to those formed

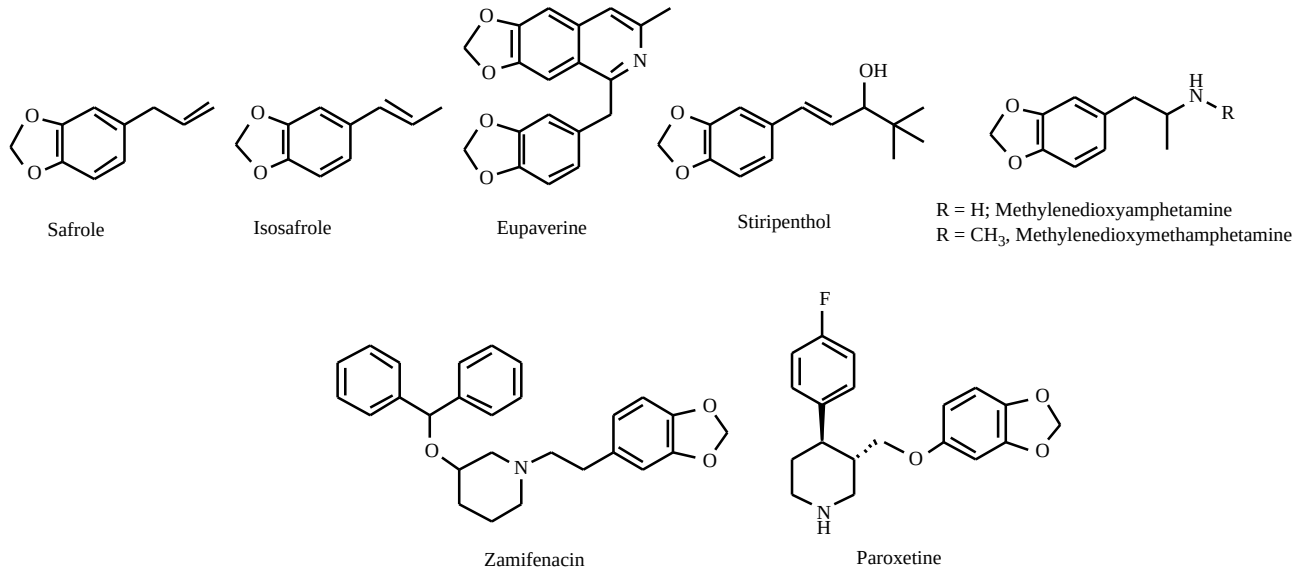


Fig. (32). Examples of Xenobiotics That Contain the Methylenedioxyphenyl Substituent.

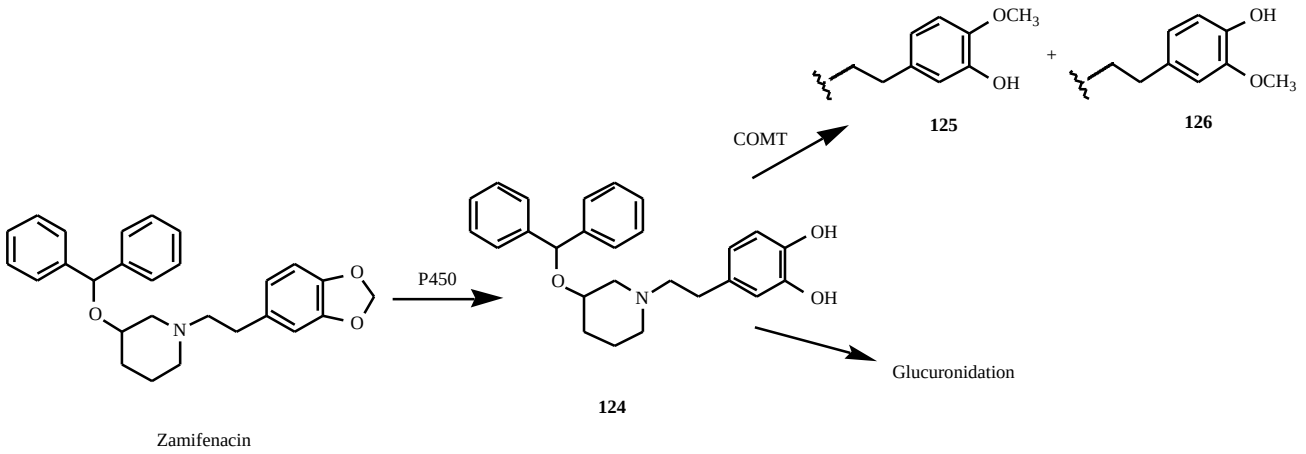


Fig. (33). P450 Catalyzed Bioactivation of the Muscarinic Receptor Antagonist Zamifenacin.

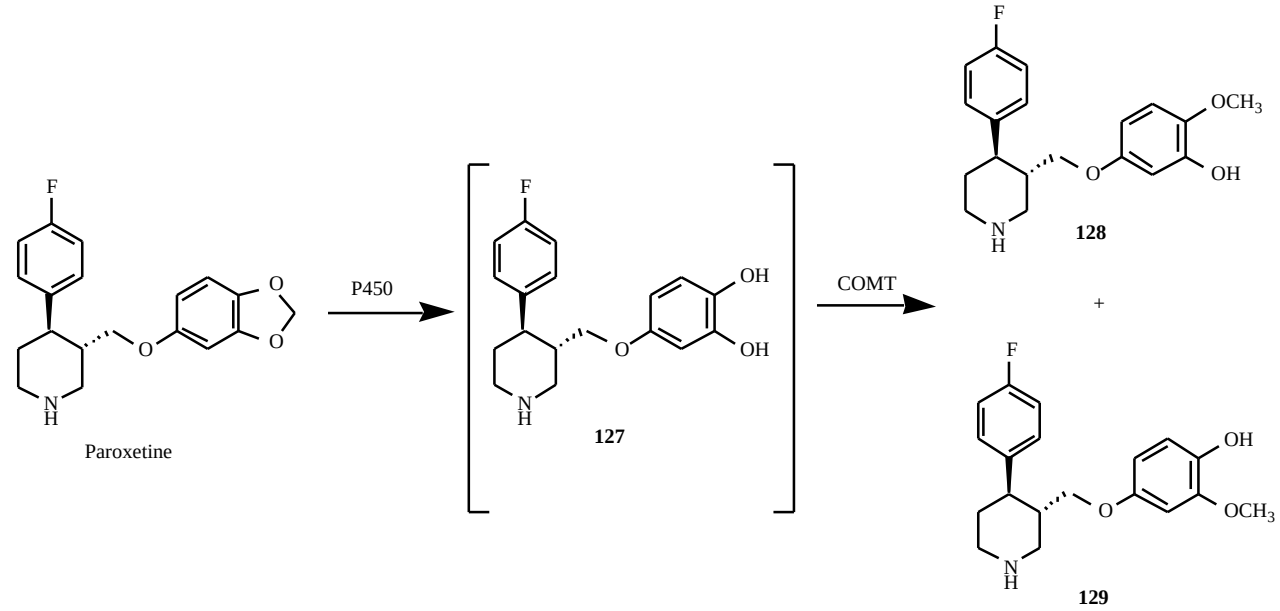


Fig. (34). Methylenedioxy ring opening in the Selective Serotonin Reuptake Inhibitor Paroxetine.

from other primary amines [235-238]. Carbene intermediates have not been implicated in binding to other macromolecules besides P450.

In general, P450 oxidizes the methylene carbon of the methylenedioxyphenyl group to generate catechol and formate. The generation of formate can be rationalized by the initial hydroxylation of the methylene carbon followed by ring opening to the phenolic formate ester and then to the catechol (see Fig. **35**). The formation of formate in liver microsomal incubations containing [^{14}C]-methylenedioxybenzene is consistent with this pathway [222] as are the deuterium isotope labeling studies on the P450 mediated cleavage of methylenedioxybenzene [236]. The catechol has been identified as the major metabolite of safrole and isosafrole [239], methylenedioxyamphetamine [235] and methylenedioxymethamphetamine [240]. In accordance with the known susceptibility of catechols to undergo further oxidation to reactive *o*-quinones, corresponding GSH conjugates have been observed for isosafrole and methylenedioxyamphetamine [241, 242].

Structure/Bioactivation Relationships

No specific SAR studies exist to help predict the likelihood of methylenedioxy ring scission. The replacement of the methlyenedioxyphenyl group with an indane or dihydrobenzofuran should circumvent the issue.

5-Alkoxyindoles

Occurrence and Frequency

Indomethacin, oxypertine, melatonin, ibogaine are examples of 5-alkoxyindoles. Of these, there are only two examples of 5-alkoxyindole bioactivation to reactive quinone-imine intermediates namely oxypertine [243] and indomethacin [244]. There are no examples of 5-amino- or 5-alkylindole bioactivation. Nevertheless, the lack of examples of

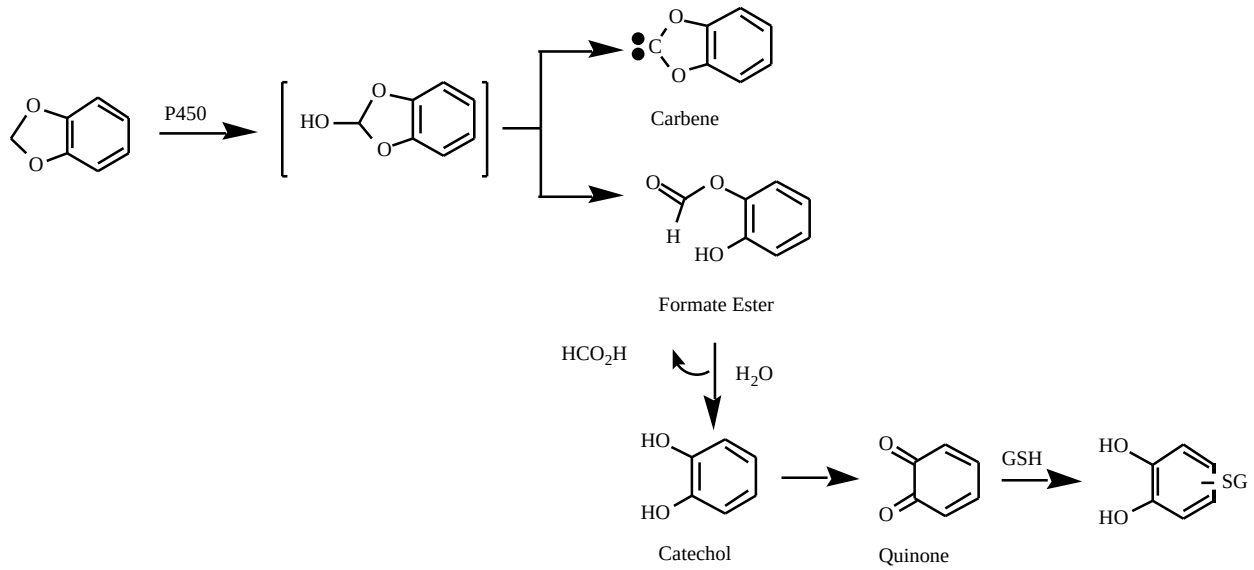


Fig. (35). Proposed Mechanisms for the Bioactivation of Methylenedioxy Groups.

bioactivation for the latter two classes does not indicate the absence of such a pathway. Indomethacin has been implicated in numerous cases of aplastic anemia, agranulocytosis and death. Indomethacin represents the only 5-alkoxyindole that has seen widespread use.

Mechanism

The mechanism of bioactivation of indomethacin requires initial amide bond hydrolysis and *O*-dealkylation on the 5-methoxy substituent leading to the 5-hydroxyindole metabolite **130** (Fig. **36**) [244]. 5-Hydroxyindoles such as **130** are bioactivated by the MPO/HOCl system to the electrophilic quinone-imine **132** *via* the intermediate *N*-chloroindole **131**. The quinone-imine derived from this bioactivation reaction has been trapped with *N*-acetylcysteine to afford conjugate **133**. While this specific mechanism was demonstrated for leukocyte peroxidases, and modeled using HOCl in an attempt to explain the specific blood toxicity observed for indomethacin, there is no rationale precluding the possibility of this type of oxidation occurring *via* other peroxidases in other tissues.

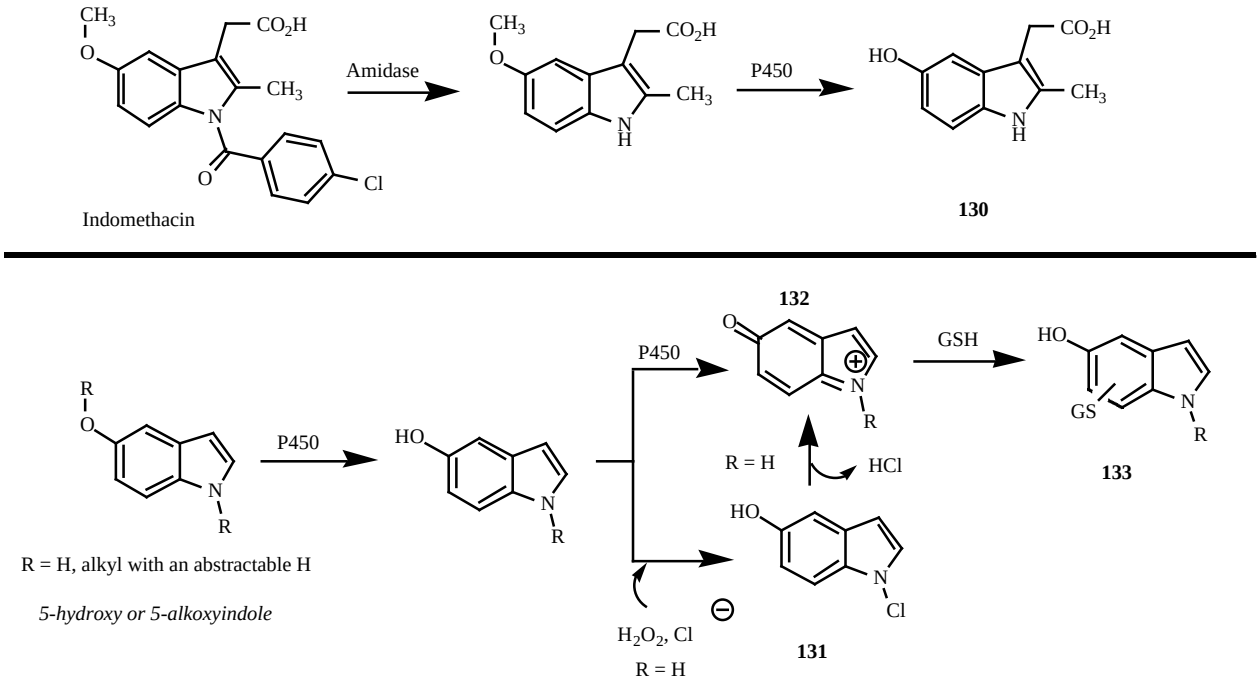


Fig. (36). Bioactivation of Indomethacin.

Structure/Bioactivation Relationships

There have been no formalized structure-bioactivation relationship studies done on 5-alkoxyindoles to address their bioactivation. Nevertheless, intuitively, preventing the initial *O*-dealkylation reaction would preclude this metabolic cascade from occurring. Standard substituent modifications that prevent *O*-dealkylation, such as removing all abstractable hydrogens by replacement with *t*-butyl or slowing the rate by addition of electron withdrawing groups on the alkyl or 4- or 6-positions of the indole could be applied. For the chloroperoxidase mechanism, addition of an alkyl group on the indole nitrogen should prevent this mechanism, however oxidation to the electrophilic quinone-imine could still occur. It should be kept in mind that 3- and 7-alkoxyindoles could be similarly reactive.

3-Methylindole Derivatives

Occurrence and Frequency

The parent compound, 3-methylindole, is a ruminal degradation product of the essential amino acid tryptophan, and is selectively toxic to pulmonary tissues following bioactivation by P450, COX or peroxidases [245]. It is also formed as the pyrolysis product of tryptophan in cigarette smoke [246]. 3-Methylindole circulates systemically to the pulmonary tissues where it is bioactivated to reactive intermediates that alkylate biomacromolecules initiating a cascade of toxic events including acute pulmonary edema in cattle [247]. 3-Methylindole toxicity has not been fully evaluated in humans, but the compound is a substrate for several human P450 enzymes including CYP2F1, CYP2A6, CYP3A4 and CYP2C8 [248, 249], some of which are expressed selectively in lung tissue. Although the bioactivation of 3-methylindole has been well characterized, additional studies with analogs have not been conducted.

Mechanism

The toxicity of 3-methylindole has been predominantly attributed to the action of 3-methyleneindolenine (**134**) obtained by the selective CYP2F1 mediated dehydrogenation of 3-methylindole [247, 248]. The mechanism of dehydrogenation proceeds *via* an initial hydrogen atom abstraction from the 3-methyl group followed by a one-electron oxidation that affords the imine-methide **134** (Fig. **37**, panel A). Adducts of **134** have been observed with thiol nucleophiles [250] and nucleosides [251], upon incubation of 3-methylindole with microsomes and either thiol trapping agents, nucleosides, or DNA. Additionally, nucleoside adducts have been observed after incubation of 3-methylindole with rat hepatocytes, followed by isolation and digestion of the DNA. These findings suggest that 3-methylindole and related analogs have the potential to cause tissue damage as well as genetic injury.

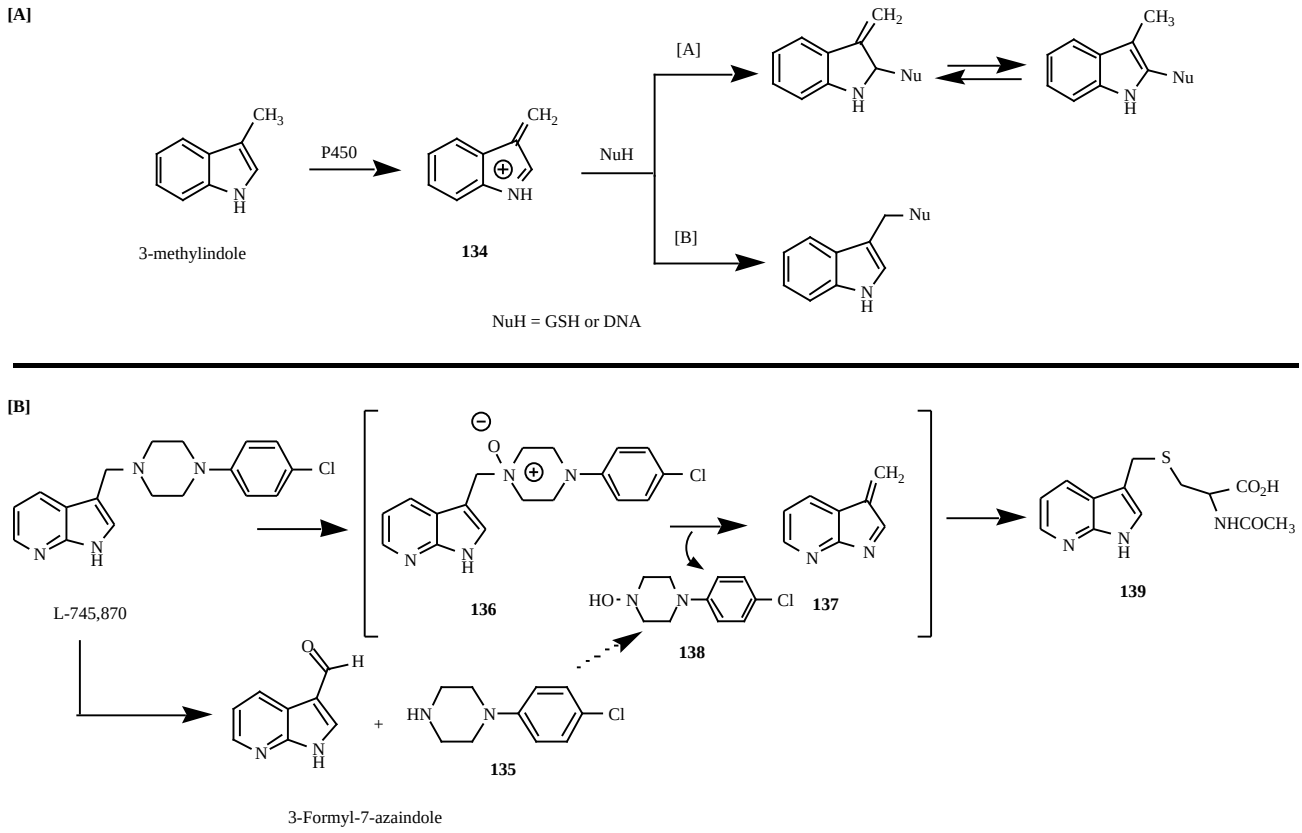


Fig. (37). Bioactivation of the Pneumotoxin 3-Methylindole and a Related 3-Methyleneazaindole Derivative to Reactive Imine-Methide Intermediates.

Of additional interest in this aspect, is the report on the 3-methylindole-like bioactivation of a 3-substituted-7-azaindole derivative L-745,870, intended for treatment of schizophrenia [252]. Analysis of the urinary metabolites of L-745,870 in preclinical species and human revealed substantial amounts of the *N*-dealkylated metabolite **135**, the mercapturic acid conjugate **139** and 3-formyl-7-azaindole (Fig. **37**, panel B). The mechanism of formation of **139** has been speculated to occur *via* the formation of the reactive imine-methide **137**. Thus initial *N*-oxygenation of L-745, 870 would afford the positively charged *N*-oxide metabolite **136**, β-elimination would then lead to **137** and *N*-hydroxy-4-chlorophenylpiperazine (**138**). Nucleophilic addition of GSH across **137** followed by peptide cleavage of GSH and acetylation would generate **139**. The potential link between reactive metabolite formation and ADRs in this example remain unknown, since the drug was suspended from clinical development due to lack of efficacy in phase II trials [252].

Structure/Bioactivation Relationships

Although no structure/bioactivation studies have been conducted to address structural determinants required for this bioactivation process, structural modification expected to avoid this pathway can potentially include removal of the hydrogen atoms on the 3-alkyl group to prevent the reaction, or introduction of electron withdrawing groups on the indole nitrogen to attempt slowing down this reaction.

Furans

Occurrence and Frequency

Furan is an industrial chemical that has also been detected as an environmental contaminant in smog, tobacco smoke, coffee and canned foods. Furan is a hepatic and renal toxicant and hepatocarcinogen in rodent species [253, 254] and has been classified as a potential human carcinogen. Bioactivation plays a central role in furan toxicity [255, 256]. Furan is converted into the toxic *cis*-2-butene-1, 4-dial metabolite **140** in a reaction catalyzed by CYP2E1 (Fig. **38**) [257-259]. The proposed mechanism of furan-induced carcinogenesis involves reaction of **140** with proteins to elicit a toxic response. Adduction to important cellular constituents is the rate-limiting step towards tumor formation [253]. Although furan is inactive in the Ames assay [260], **140** is mutagenic in this assay [261], suggesting that this metabolite contributes to the carcinogenic activity of furan by reacting with DNA to form mutagenic adducts. Consistent with this observation, **140** alkylates nucleosides (2'-

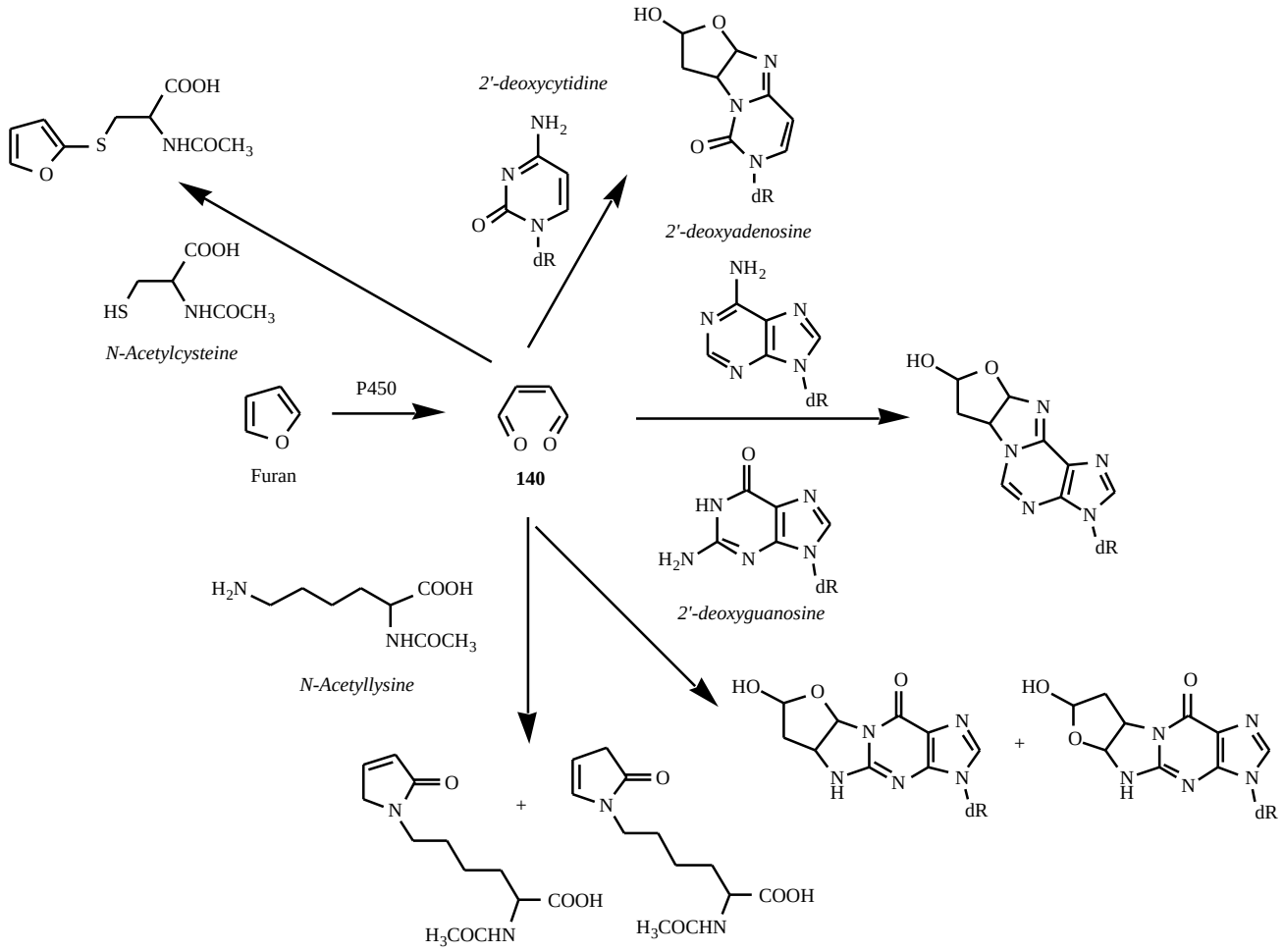


Fig. (38). Bioactivation of Furan.

deoxycytidine, 2'-deoxyguanosine and 2'-deoxyadenosine), amino acids such as N^{α}-acetyl-L-lysine and thiols such *N*-acetylcysteine and GSH (see Fig. **38**) [258, 262].

Hepatocellular carcinoma is one of the major causes of premature death in certain regions of Africa and Asia. Although the exact molecular mechanism has not been determined, infection by hepatitis B and exposure to the furan ring-containing natural product aflatoxin B_1 (AFB_1) (Fig. **39**) are known risk factors [263-265]. AFB_1 is a mycotoxin that is produced by the fungi *Aspergillus Flavus* and *A. parasiticus* under conditions of high heat and humidity. Exposure to AFB_1 occurs primarily through ingestion of mold-contaminated foodstuffs (e.g. peanuts and corn). Several other furan-containing compounds (see Fig. **39**) such as ipomeanol [266-268], menthofuran [269-271] and furosemide [272-274] also cause hepatic and renal necrosis in mice and humans or develop potentially lethal pulmonary lesions in rat.

Mechanism

The predominant biotransformation pathway of furans involves ring scission to α, β-unsaturated dicarbonyl or γ-ketocarboxylic acid metabolites *via* the initial furan-2, 3-epoxide intermediate **141** [259, 275-277]. Evidence for epoxidation of the furan ring has been shown in the metabolism studies on *R*-(+)-pulegone and *R*-(+)-menthofuran by human liver P450s using $^{18}O_2$ and $H_2{}^{18}O$ and C-2 deuterium-labeled menthofuran [270]. Indirect evidence for epoxidation has also been reported for furosemide. Incubation of furosemide with mouse liver microsomes in the presence of epoxide hydrolyase inhibitor, 1,2-epoxy-3,3,3-trichloropropane, results in a 2-fold increase in covalent binding [272]. This increase in reactivity is attributed to the higher epoxide concentrations that result from inhibition of epoxide hydrolase activity in liver microsomes. Epoxide **141** directly rearranges to hydroxyfuran **142**, which predominantly exits as the lactone tautomer **143**. Hydrolysis of **143** affords the γ-ketocarboxylic acid **144** as shown in Fig. **40** (pathway a) [278, 279]. Alternately, ring opening of **141** can also lead to the formation of the γ-ketoenal **145** (Fig. **40**, pathway b) [270]. These electrophilic α, β-unsaturated dicarbonyl metabolites react with biological macromolecules *via* a 1,4-Michael addition across the α, β-unsaturated dicarbonyl moiety or *via* nucleophilic 1, 2-addition to the aldehyde [258, 270]. Alternatively, **141** can also undergo a nucleophilic attack by various macromolecules. This may depend on the stability of the epoxide intermediate and the substituents on furan ring.

The carcinogenicity of AFB_1 is due to its metabolic conversion to the *exo*-8, 9-epoxide (**146**), which subsequently

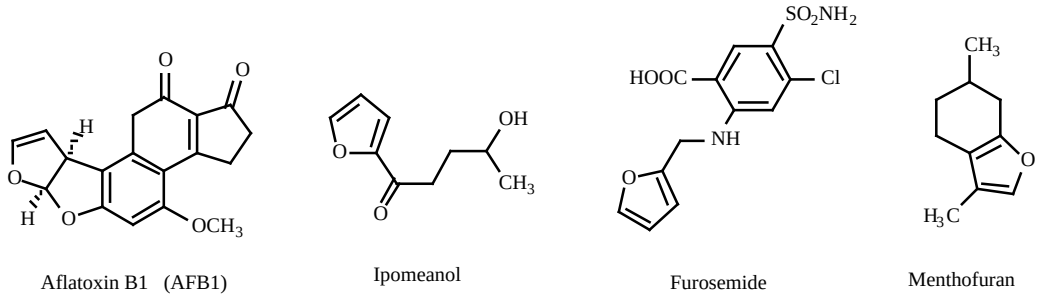


Fig. (39). Examples of Furan-Containing Xenobiotics.

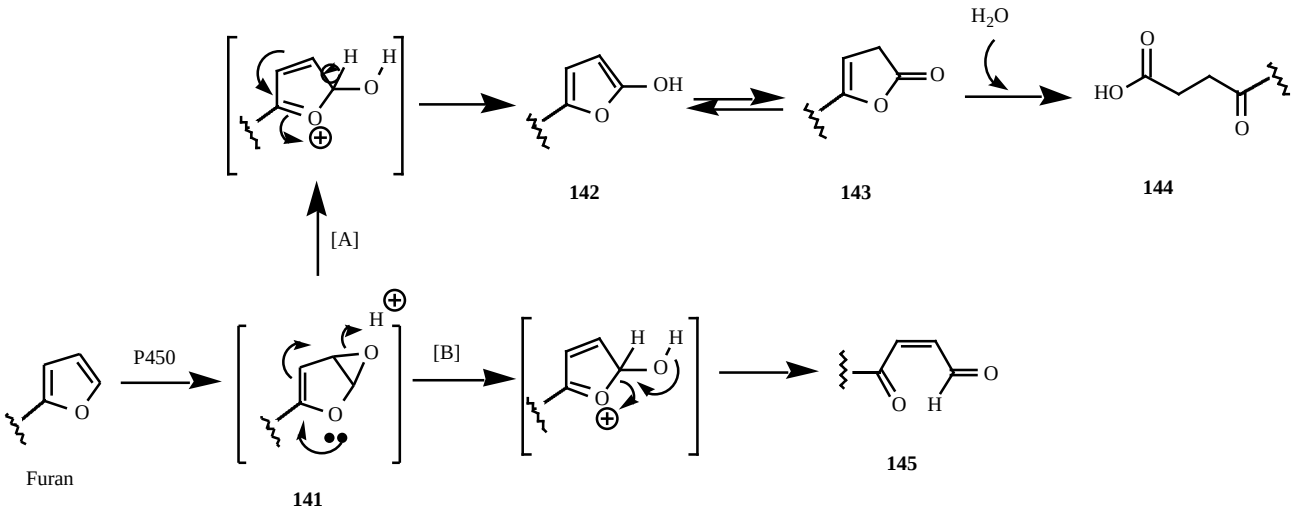


Fig. (40). Mechanism of Bioactivation of the Furan Ring.

irreversibly alkylates DNA (Fig. **41**). The metabolic activation of AFB_1 is a P450 dependent process. Immunoinhibition and chemical inhibition experiments identified CYP3A4 and CYP1A2 [280] as the major enzymes responsible for the bioactivation of AFB_1. Other P450 enzymes such as CYP1A1, 1B1 and 2A6 [281] and human liver lipoxygenase [282] have also been shown to activate AFB_1, but to a lesser extent. Once formed **146** reacts almost exclusively with the N7 nitrogen of guanine [283]. Epoxide **146**, which has a half-life of ~ 1 second in aqueous medium, is believed to first intercalate on the 5' side of the target guanine residue in DNA. The intercalation is proposed to provide a kinetic and entropic advantage for reaction with DNA rather than hydrolysis. *In vitro*, **146** forms a reversible complex with calf thymus DNA and reacts with guanine with a rate of 35 s^{-1} [284]. Essigmann and coworkers have shown that blocking the intercalation of AFB_1, by placing a covalently linked benzofuran in the 5' intercalation site, greatly reduces the formation of the N7 guanine adduct [285]. Epoxide **146** is also susceptible to enzymatic or non-enzymatic hydrolysis to yield AFB_1-dihydrodiol (**147**). The role of the AFB_1-dihydrodiol in the overall toxicological consequence of AFB_1 is not well established, but at physiological conditions it rearranges to a dialdehyde, which may also lead to protein alkylation. Aldo-keto reductases are proposed to play a protective role in this pathway [286], but further studies are needed to shed light on this issue. The GST mediated detoxification of **146** plays a major protective role in the liver [287]. In mice, GST activity towards AFB_1 is 52-fold higher than in rats, which accounts for the resistance of mice to AFB_1 mediated hepatotoxicity [288].

Structure/Bioactivation Relationships

The possibility that bulky substituents on the furan ring could sterically hinder epoxidation remains to be explored. Furthermore, aflatoxin G_1 (AFG_1) has reduced genotoxicity when compared to AFB_1. AFG_1 has a δ-lactone ring in place of the cyclopentanone ring in AFB_1 and this modification leads to a reduction in DNA binding affinity by approximately an order of magnitude [289]. It is believed that this reduction in DNA binding affinity is due to the loss of rigidity of the ring system and results in less DNA alkylation. Thus, any modifications to reduce the rigidity of compounds in this class may reduce reactivity. To address the possibility of furan ring bioactivation, metabolism studies in liver microsomes in the presence of various nucleophiles such as GSH, methoxylamine or semicarbazide, which could trap the ring-opened metabolites of furan rings, should be contemplated. Studies with the lipooxygenase inhibitor L-739,010 and an experimental anti-HIV agent L-754, 394 have demonstrated the formation of *O*-methyloxime derivatives **148** and **149**, respectively, following incubation of these furan derivatives with liver microsomes in the presence of methoxylamine (Fig. **42**) [275, 290].

Thiophenes

Occurrence and Frequency

Thiophene rings have proven to be an attractive isosteric replacement in the quest for improved pharmacological potency. The removal of some of the thiophene-containing drugs from the market due to ADRs has led to an increased

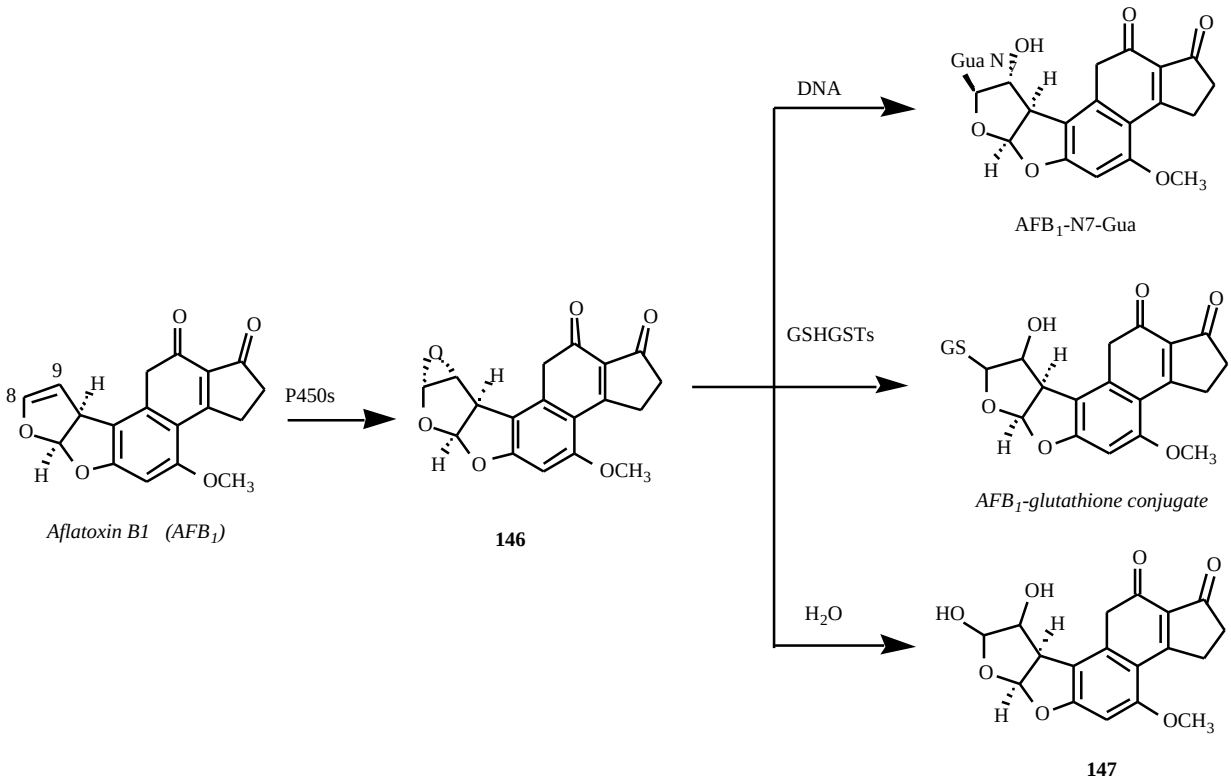


Fig. (41). Mechanism of Bioactivation of AFB_1.

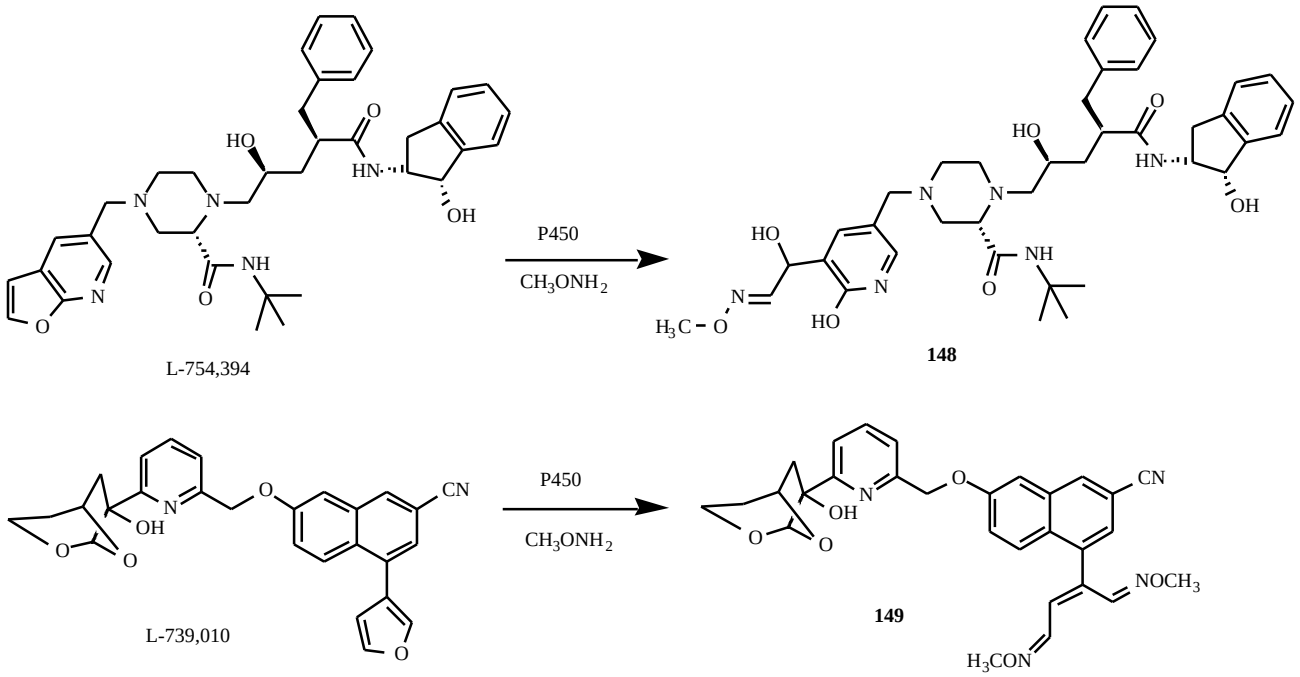


Fig. (42). Trapping Electrophilic γ-Ketoenals with Amines.

effort in understanding the cause of these ADRs particularly in relation to their bioactivation potential. Unsubstituted thiophene-containing compounds removed recently from the market include the diuretic tienilic acid and the NSAIDs tenoxicam and suprofen (Fig. **43**). Although the thiophene-containing drug ticlopidine (see Fig. **43**) is still utilized clinically as a inhibitor of platelet aggregation, its use is associated with a relatively high incidence of agranulocytosis, aplastic anaemia and thrombocytopenia [291, 292]. In the case of tienilic acid, autoimmune mediated hepatotoxicity was observed at a frequency of 0.01 to 0.07% [293]. The autoimmune response in these subjects was characterized by selective covalent binding of tienilic acid to CYP2C9 since antibodies directed to the acylated CYP2C9 hapten were found circulating in the serum of these individuals [294]. In contrast, suprofen, a structurally-related 2-aroylthiophene-containing NSAID was removed from the market due to acute renal failure [295]. Epidemiological studies with this compound suggested the nephrotoxicity observed with suprofen was distinctly different from the toxicity usually associated with other propionic acid NSAIDS [296]. The condition, referred to as acute flank pain syndrome has also been observed in individuals using tienilic acid [297]. The manifestation of the same clinical syndrome by both suprofen and tienilic acid suggest perhaps a similar bioactivation process. Although the formation of a reactive acyl glucuronide has been implicated as the causative factor in the acute nephrotoxicity caused by suprofen [298], recent studies have revealed the CYP2C9 mediated bioactivation of the thiophene ring in a manner similar to that observed with tienilic acid [299]. Like tienilic acid, the bioactivation of the thiophene ring in suprofen is accompanied by the mechanism-based inactivation of CYP2C9 [299].

Mechanism

Like, furans the metabolism of thiophenes is mediated by P450. Thiophenes undergo hydroxylation adjacent to the sulfur forming 2- and 5-hydroxythiophene metabolites.

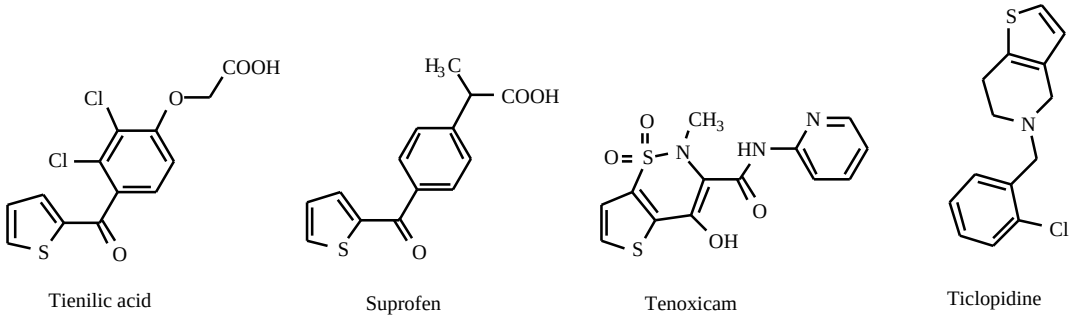


Fig. (43). Examples of Thiophene-Containing Drugs.

Thiophenes can also undergo P450 mediated oxidation to the unstable and reactive thiophene-*S*-oxide metabolite **150** (Fig. **44**) [300, 301]. The detection of isomeric thiophene-*S*-oxide dimers (see Fig. **44**) provided evidence for thiophene *S*-oxidation in *in vitro* and *in vivo* studies [302]. A mercapturic acid conjugate of dihydrothiophene-*S*-oxide **151** (see Fig. **44**) has been identified in urine of rats treated with radiolabeled thiophene. This conjugate is the result of a 1, 4-Michael-type addition of thiol on thiophene-*S*-oxide.

It has been proposed that tienilic acid also undergoes sulfoxidation to a reactive electrophilic *S*-oxide intermediate in a reaction catalyzed by CYP2C9, which also results in the suicide inactivation of the enzyme [303]. The tienilic acid-*S*-oxide is also thought to be responsible for the formation of 5-hydroxytienilic acid, in addition to inactivating the enzyme. It is important to note that the formation tienilic acid sulfoxide has not been demonstrated, and as noted earlier, the only evidence for this pathway is the characterization of the sulfoxide dimers of thiophene [302]. The tienilic acid-*S*-oxide is then thought to react with various nucleophiles in the active site of the enzyme by a Michael-type 1, 4-addition resulting in covalent modification at the active site of CYP2C9 [300]. Alternately, reaction of the *S*-oxide with water yields 5-hydroxytienilic acid [304-307]. The hypothesis for the formation of 5-hydroxytienilic acid metabolites *via* the *S*-oxidation pathway has been further investigated using stable isotopes [308]. Microsomal incubation of tienilic acid and its 3-aroyl isomer in the presence of NADPH and under $^{18}O_2$ atmosphere results in the incorporation of ^{18}O in the 5-hydroxythiophene metabolite. This finding suggests that the oxygen in 5-hydroxythiophene metabolites is exclusively derived from molecular oxygen and not from water as proposed previously. Similar results have been obtained in NADPH-supplemented liver microsomal incubations of suprofen [299] and a probe substrate 2-(4-methoxybenzoyl)thiophene with $^{18}O_2$ in our laboratories (O'Donnell, J.P. unpublished observations). Overall, these results provide indirect evidence that 5-hydroxythiophene metabolites can also be formed *via* an epoxide pathway in addition to the sulfoxidation pathway, unless the oxygen of thiophene-*S*-oxide rearranges to afford 5-hydroxythiophene. Further evidence for the epoxidation pathway includes the trapping of the pyridazine derivative in microsomal incubations of suprofen in the presence of NADPH and semicarbazide [299]. The formation of the pyridazine conjugate of suprofen can be rationalized by a pathway involving initial epoxidation of the thiophene ring to the unstable epoxide metabolite, spontaneous ring opening of which, would generate the reactive γ-thioketo- α, β-unsaturated aldehyde intermediate. Condensation of this aldehyde intermediate with semicarbazide will lead to the pyridazine adduct. The formation of the γ-thioketo-α, β-unsaturated aldehyde intermediate is analogous to the ring opening bioactivation pathways established for furans. A summary of the proposed mechanisms of thiophene ring bioactivation is depicted in Fig. **45** using 2-aroylthiophenes as model compounds.

Structure/Bioactivation Relationships

Unsubstituted thiophenes are particularly prone to P450 mediated oxidation. The effect of substitution at each position of the thiophene ring has not been studied formally. Some studies in-house suggest substitution at the thiophene ring carbons with halo or alkyl groups reduces metabolic oxidation of the thiophene ring. This finding seems to be more associated with the reduced affinity (sterics and/or electronic effects) of these compounds towards the P450 enzyme that metabolizes the unsubstituted thiophene. However, given the possible role for a reactive *S*-oxide, substitution at one or two positions may not circumvent the bioactivation potential of the thiophene ring system.

Thiazoles

Occurrence and Frequency

The predominant fate of the thiazole ring is its oxidative ring scission catalyzed by P450 enzymes resulting in the

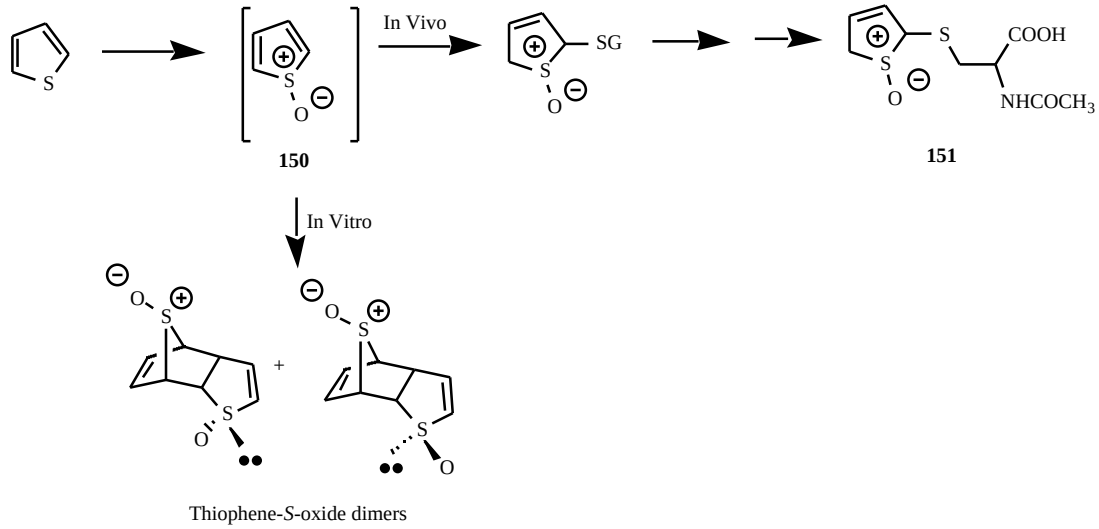


Fig. (44). Metabolism of Thiophene to *S*-Oxide Metabolites.

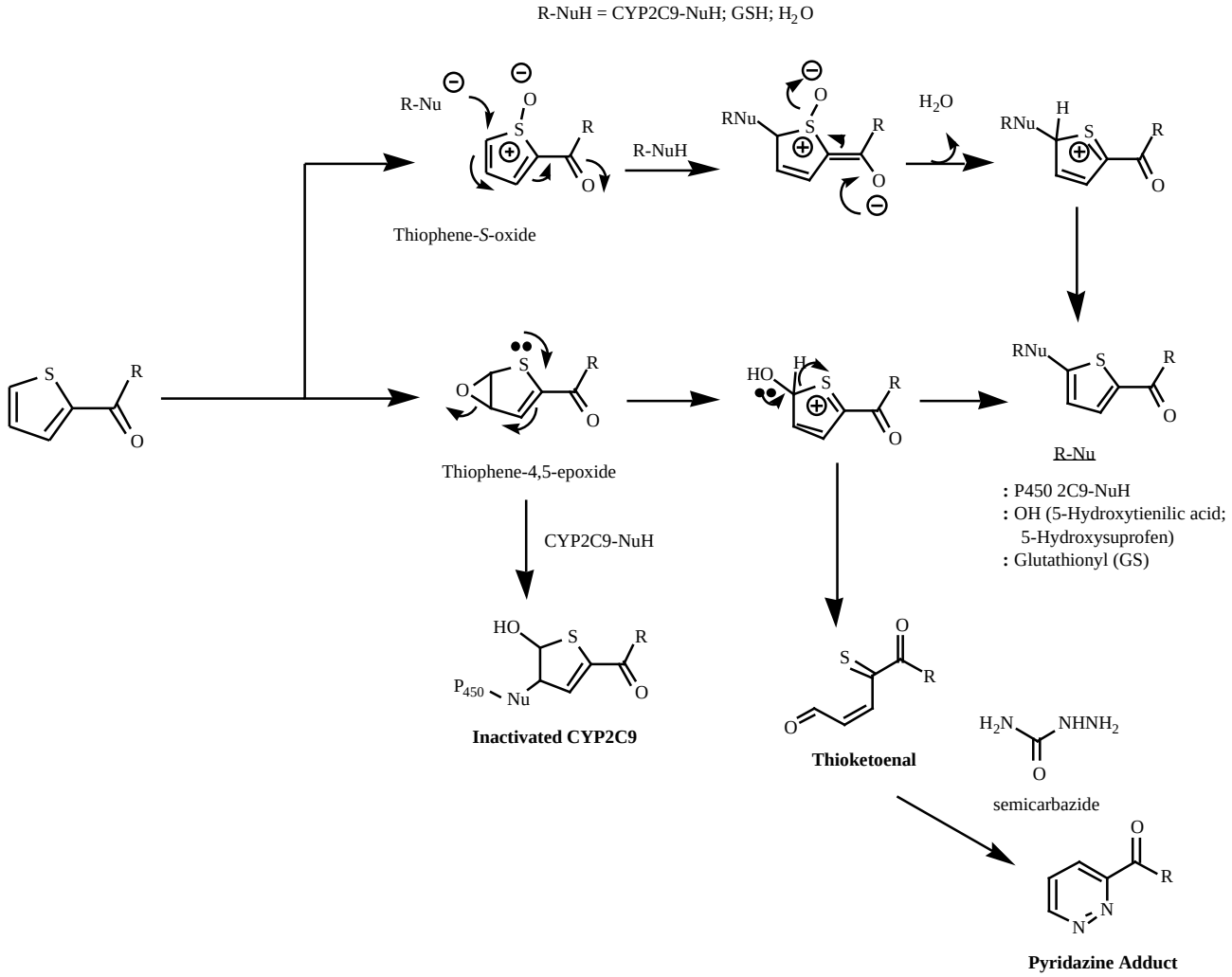


Fig. (45). Proposed Bioactivation Pathways of Thiophene Derivatives.

formation of the corresponding α-dicarbonyl metabolites and thioamide derivatives [309, 310]. The well-established toxicity associated with thioamides and thioureas has led to the speculation that thiazole toxicity is attributed to ring scission yielding the corresponding thioamide metabolite. The finding that treatment of rodents with P450 inhibitors attenuates thiazole toxicity provides a crucial link between thiazole bioactivation and toxicity [311]. Studies on the elucidation of the biochemical mechanism of toxicity of thiabendazole (Fig. **46**), a fungicide and anthelmintic, have demonstrated that the compound is also oxidatively metabolized to thioformamide (**152**) and benzimidazol-2-ylglyoxal (**153**) metabolites (see Fig. **46**) in preclinical species and humans [312]. The thioformamide thus formed is believed to be the proximate toxicant derived from thiabendazole [313]. Additional examples of biologically-active thiazoles that undergo ring opening (see Figure 49) include the immunomodulatory agent SM-8849 [314], the NSAID sudoxicam [315], anti-inflammatory 2-acetylaminothiazoles [316] and the hepatoprotective agent YH-439 [317]. The NSAID sudoxicam has been withdrawn from the market due to hepatotoxic consequences, a phenomenon that has been linked to the thiazole ring scission observed during the metabolism of this compound. 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 HIV protease inhibitor ritonavir [318-320]. This proposal is consistent with the observations that the HIV protease inhibitors indinavir and saquinavir (see Fig. **46**), which do not possess the thiazole ring, are devoid of CYP3A4 inactivation properties [319, 321].

Mechanism

The mechanism of thiazole ring scission is illustrated in Fig. **47**. Like imidazoles and oxazoles, epoxidation of the double bond between the 4, 5-position followed by hydrolysis of the corresponding epoxide yields the dihydrodiol derivative **154**. Subsequent rearrangement of the diol intermediate affords the corresponding α-dicarbonyl and thioamide metabolites. Oxidation of the thiazole ring can also generate an electrophilic *S*-oxide, which can covalently bind to biomacromolecules or exogenously added thiol nucleophiles. This has been demonstrated by incubation of L-766, 112 a potent and selective inhibitor of human COX-2, with rat and rhesus monkey hepatic microsomal fractions under oxidative conditions that results in the formation of the

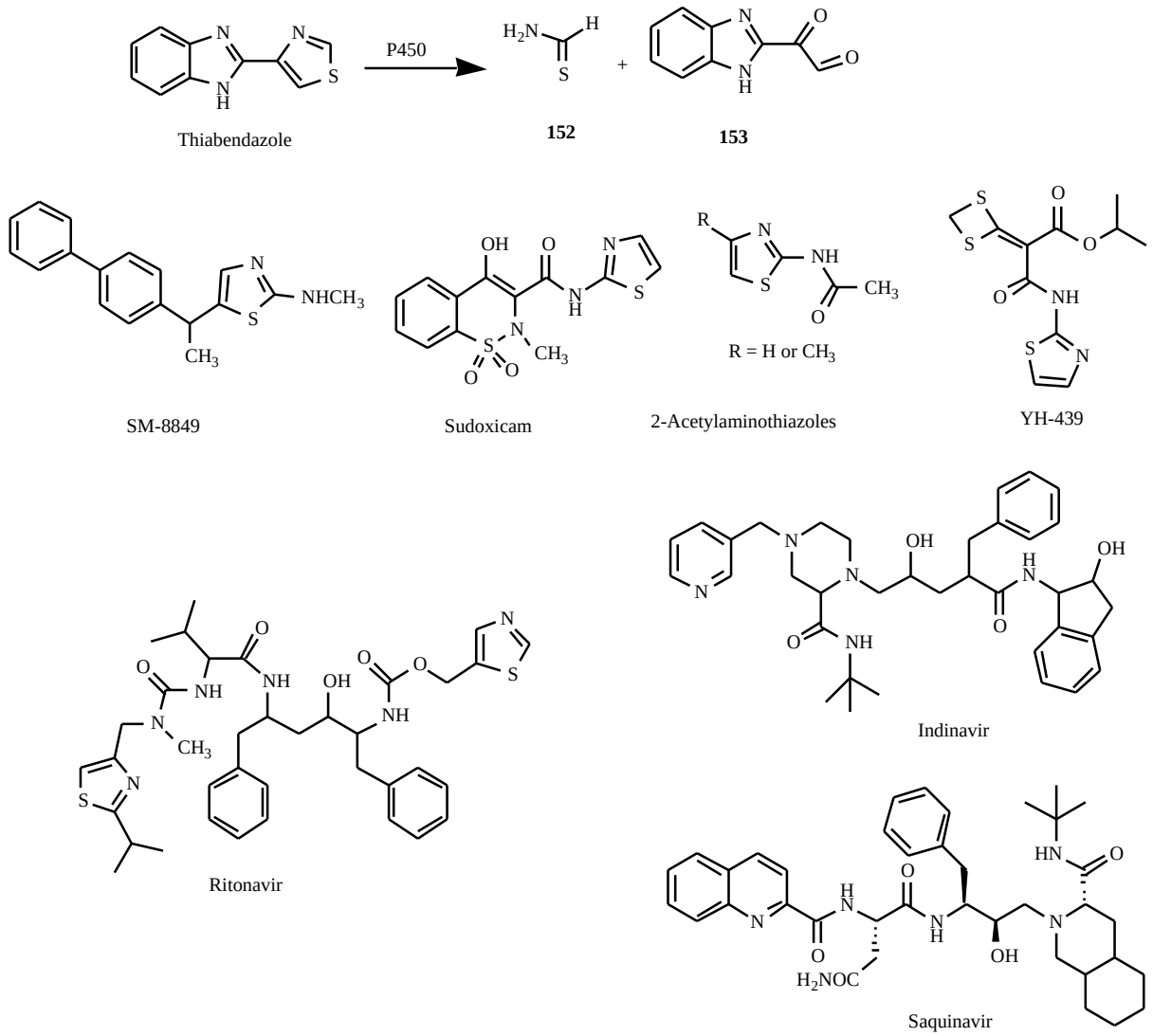


Fig. (46). Oxidative Thiazole Ring Opening in Xenobiotics.

electrophilic thiazole-*S*-oxide metabolite **155** that can be trapped by GSH (see Fig. **47**) [322]. In other examples, the non-enzymatic nucleophilic displacement of substituents at the C-2 position of the thiazole ring has also been reported [323, 324] as shown with several C-2 substituted benzothiazole analogs in Fig. **48**. The enhanced electrophilicity at the C-2 imine carbon coupled with the presence of a good leaving group at this position undoubtedly contributes towards the occurrence of this pathway.

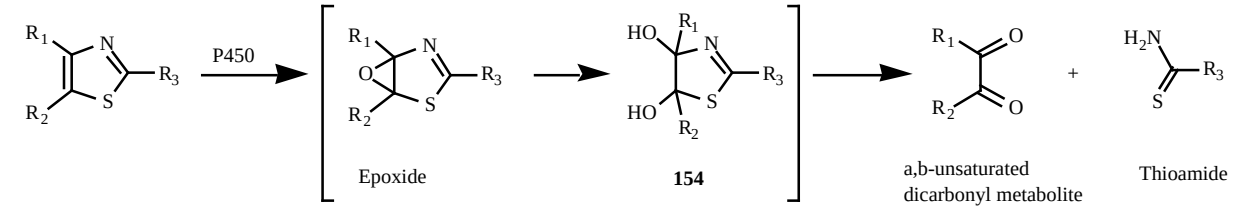
Structure/Bioactivation Relationships

Metabolic switching and resistance to ring opening has been observed upon incorporation of substituents in the 5-position of the thiazole ring (Fig. **49**). A comparison of the biotransformation pathways of the two oxicam derivatives, sudoxicam and meloxicam indicates that sudoxicam is metabolized to the thiohydantoic acid and thiourea metabolites. Incorporation of a methyl group at the 5-position of the thiazole ring in meloxicam results in extensive oxidation of the methyl group resulting in an alcohol metabolite that undergoes further oxidation to carboxylic acid analog [325]. Little to no oxidative ring opening of the thiazole moiety in meloxicam is observed in humans.

Thiazolidinediones

Occurrence and Frequency

The thiazolidinedione ring system is frequently exploited as a template in medicinal organic chemistry. Troglitazone, rosiglitazone and pioglitazone (Fig. **50**) are orally active antidiabetic agents for the treatment of type 2 diabetes. These drugs bind to the nuclear peroxisome proliferators-activated receptors in tissues, resulting in the increased expression of genes encoding proteins that are involved in glucose and lipid metabolism. However, despite its effectiveness as an antidiabetic agent, troglitazone therapy has been associated with severe hepatotoxicity, which, in some cases, has led to hepatic failure and death. As a consequence of safety concerns over the use of this agent, troglitazone was withdrawn from the U.S. market in March 2000 [326]. The



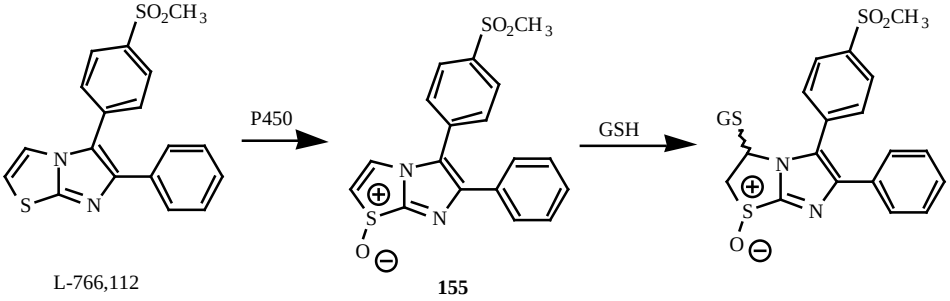


Fig. (47). P450 Catalyzed Bioactivation of the Thiazole Ring System.

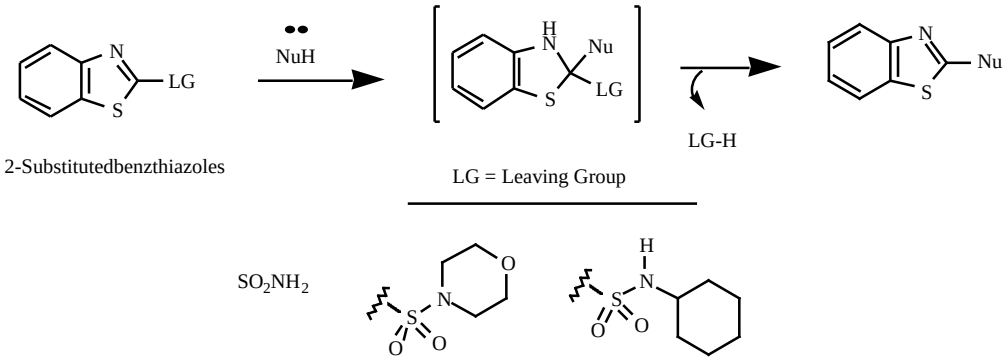


Fig. (48). Non-enzymatic Nucleophilic Displacement in C-2 Substituted Benzothiazoles.

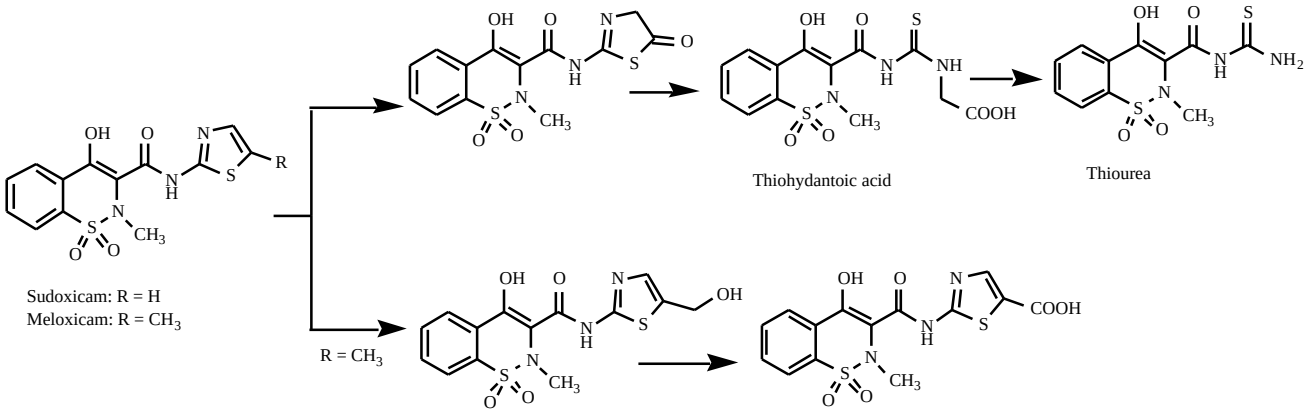


Fig. (49). Comparison of the Metabolism of COX inhibitors Sudoxicam and Meloxicam.

incidence of liver transplant or fatalities due to liver failure was approximately 1 in 100,000. After being on the market for 17 months the FDA received 560 reports of hepatotoxicity and 24 cases of acute liver failure. On average patients were on drug therapy for 116 days and 69% were taking 400 mg/day, 20% were taking 200 mg/day and 11% were taking 600 mg/day. This reflects the therapeutic dose distribution and does not suggest a dose-related effect. In controlled clinical trials a >3 fold elevation in liver enzymes (ALT) was observed (1.9% vs. 0.6% in placebo) [327]. Bioactivation of both the chromane and the thiazolidinedione ring systems to reactive metabolites that bind to liver microsomal protein and to GSH have been proposed as possible mechanisms in initiating drug-induced liver injury [210, 328, 329]. Comparison of the hepatotoxic and nephrotoxic potential of imidazolinedione, oxazolidinedione and thiazolidinedione analogs of the nephrotoxic fungicide, *N*-(3, 5-dichlorophenyl)succinimide (Fig. **50**) in rats after *in vivo* administration indicated that only *N*-(3, 5-dichlorophenyl)thiazolidinedione caused hepatic damage. It is conceivable that bioactivation of the thiazolidinedione ring to electrophilic intermediates is responsible for the observed hepatotoxicity in rats [330]. An alternate theory to account for troglitazone-induced hepatic cholestasis in humans is that the major metabolite of troglitazone in humans, troglitazone sulfate, which is derived *via* sulfation of the phenolic hydroxyl group, interferes with the hepatobiliary export of bile acids by inhibiting the canalicular bile salt export pump protein, as has been observed in the rat [331, 332]. It is interesting to note that no ALT elevation has been reported with either rosiglitazone or pioglitazone that also contain the thiazolidinedione ring and there were only 3 reports of acute hepatotoxicity with rosiglitazone and none with pioglitazone [327]. A plausible explanation for this discrepancy is that the efficacious dose of troglitazone is higher (200-400 mg QD) than that of rosiglitazone and pioglitazone (< 10 mg QD) and therefore, the liver is exposed to a lesser concentration of reactive metabolite(s) with the latter drugs. Recent studies with an investigational peroxisome proliferators-activated receptor agonist MK-0767 have revealed that the thiazolidinedione ring in the compound is also prone to oxidative scission leading to similar electrophilic intermediates as observed with troglitazone [333, 334].

Mechanism

The reason(s) for troglitazone-mediated hepatotoxicity is presently unknown. *In vitro* and *in vivo* characterization of GSH conjugates were used to identify possible metabolic pathways/intermediates responsible for the bioactivation of troglitazone. Following incubations with human microsomes and rCYPs in the presence of GSH, 5 GSH conjugates were identified and characterized [210]. Based on these structures of the GSH conjugates the two proposed pathways for the bioactivation of troglitazone include metabolism of the chromane ring to the quinone or *ortho*-quinonemethide (Fig. **51**, panel A) and oxidative cleavage of thiazolidenedione ring (Fig. **51**, panel B). The mechanism of thiazolidinone ring opening is thought to involve oxidation of the sulfur atom affording a sulfoxide intermediate **156**, which undergoes spontaneous ring opening to a highly electrophilic α-ketoisocyanate derivative **157**. Hydrolysis of the isocyanate followed by decarboxylation to the amide, accompanied by attack of GSH on the reactive sulfenic acid, would afford GSH conjugate **158**. Alternatively, conjugation of the isocyanate with GSH, followed by oxidation to the corresponding sulfinic acid derivative, provides a route for the second GSH conjugate **159**. Dehydration of the sulfinic acid intermediate is thought to lead to the formation of third sulfine-containing GSH conjugate **160** (Fig. **51**, panel B). Direct attack of GSH on either the sulfur atom or the adjacent carbonyl group of the putative thiazolidinedione sulfoxide also would produce the first two GSH conjugates without the intermediacy of the ring-opened isocyanate. It is noteworthy that the three GSH conjugates, which were generated *in vitro* using either native human liver microsomes or expressed CYP3A4, were detected in the bile of troglitazone-treated rats, providing evidence for the existence of the bioactivation pathway *in vivo*. The second route for the formation of reactive metabolites from troglitazone involves oxidation of the substituted chromane moiety. Thus, cytochrome P450-mediated one-electron oxidation of the phenolic hydroxyl group would result in the phenoxy radical **161**, a resonance form of which (the carbon-centered radical) undergoes oxygen rebound at the *ipso* position to form an unstable hemiketal **162**. Spontaneous ring opening of this latter species would afford the known quinone metabolite

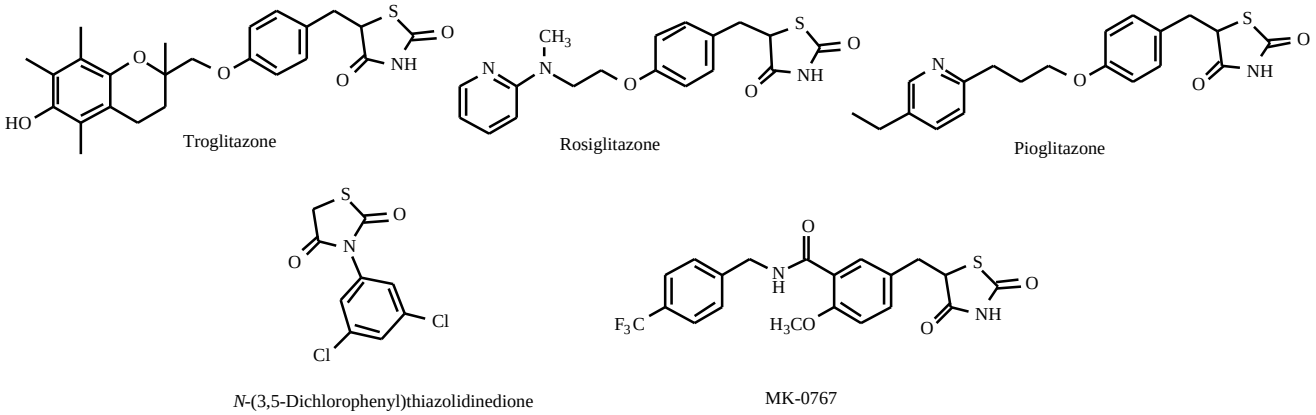


Fig. (50). Structures of Thiazolidinedione-Containing Drugs and Xenobiotics.

163 of troglitazone. Once formed, **163** appears to undergo thiazolidinedione ring scission, in a fashion similar to that followed by the parent compound, leading ultimately to GSH conjugate **164**. The formation of both the quinone metabolite of troglitazone and corresponding GSH conjugate was found to be dependent on CYP3A enzymes. An alternative fate for the phenoxy radical intermediate is that it undergoes P450-mediated hydrogen atom abstraction from the 5-methyl group of the substituted chromane ring, leading to the *o*-quinone-methide derivative **165** that is trapped by GSH to afford **166**. Unlike the metabolites derived through bioactivation of the thiazolidinedione ring the formation of the *o*-quinone-methide is not specifically catalyzed by CYP3A4 enzymes, although it was found to be NADPH-dependent. The role of CYP3A4 in the toxicity of troglitazone, however, remains unknown [210]. The observed toxicity may be due to protein adduct formation or enhanced redox cycling. Experiments to evaluate the toxicity of the quinone intermediate in rat hepatocytes and HepG2 cells determined that the troglitazone quinone is less toxic than the parent drug [335]. Although, no information exists on the P450 mediated metabolism of the thiazolidinedione ring in rosiglitazone and pioglitazone, it is interesting to note that the thiazolidinedione ring is the major site of metabolism in MK-0767 in preclinical species and human hepatic tissue as shown in Fig. 52 [333, 334]. It is quite likely that reactive metabolites are also generated during the oxidative cleavage of the thiazolidinedione ring in MK-0767.

Benzene Rings

Occurrence and Frequency

Many drug molecules and xenobiotics that contain a phenyl ring have the potential to undergo enzymatic epoxidation to electrophilic arene oxide species en route to the stable phenol metabolites. For a number of drugs and xenobiotics (Fig. **53**), the arene oxide metabolite has been proposed to be responsible for covalent binding to cellular macromolecules and subsequent toxicity. Carbamazepine, phenytoin and phenobarbital cause hypersensitivity with skin rash, hepatic failure and hematological disorders as side-effects (reviewed in [336, 337]). With carbamazepine adverse reactions have been reported in 33-50% of patients, among these reactions 5% can be classified as idiosyncratic in nature. The antiepileptic drug lamotrigine causes toxic epidermal necrosis and an arene oxide-GSH conjugate has

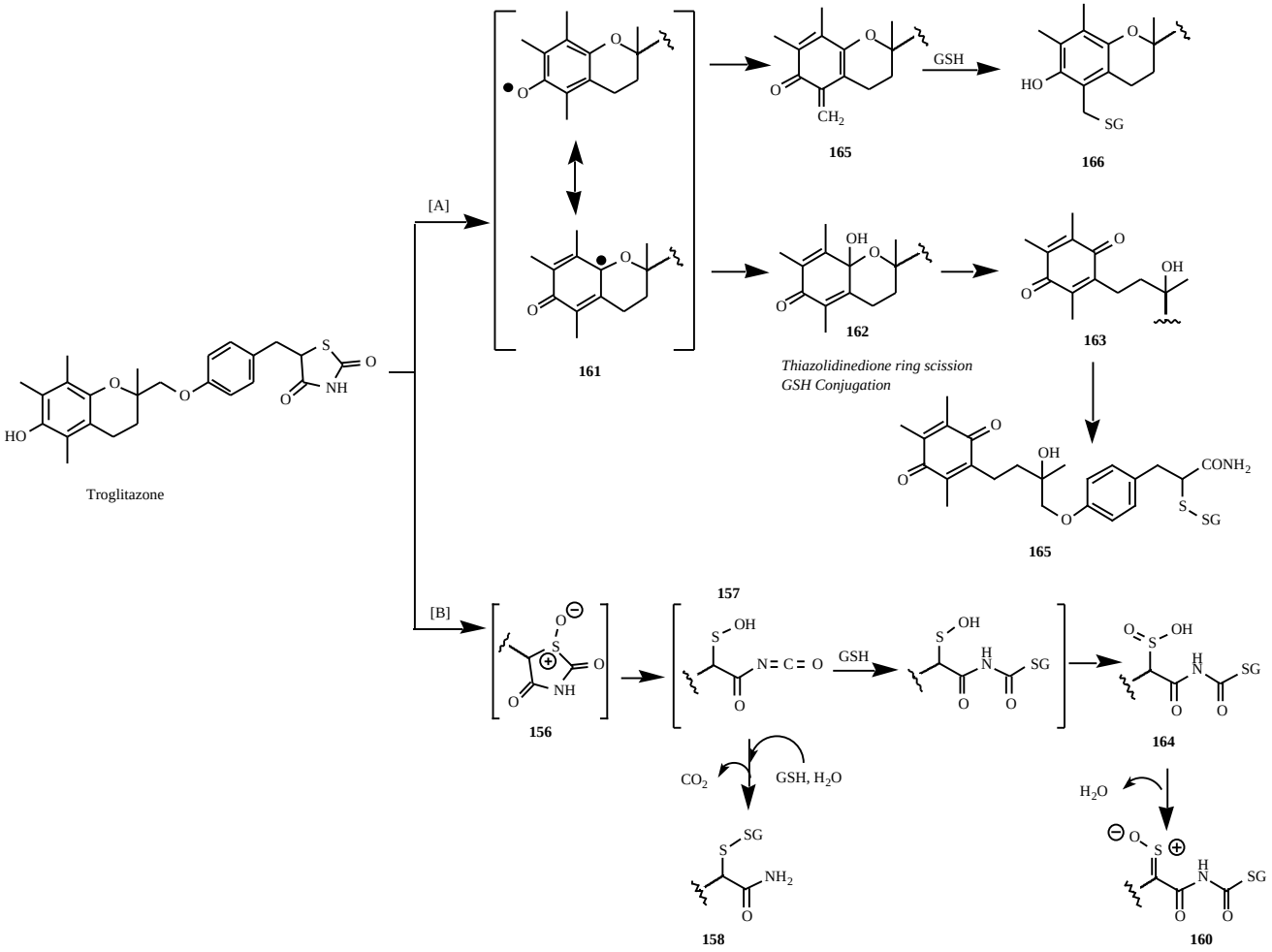


Fig. (51). Proposed Mechanisms of CYP3A4 Mediated Troglitazone Bioactivation.

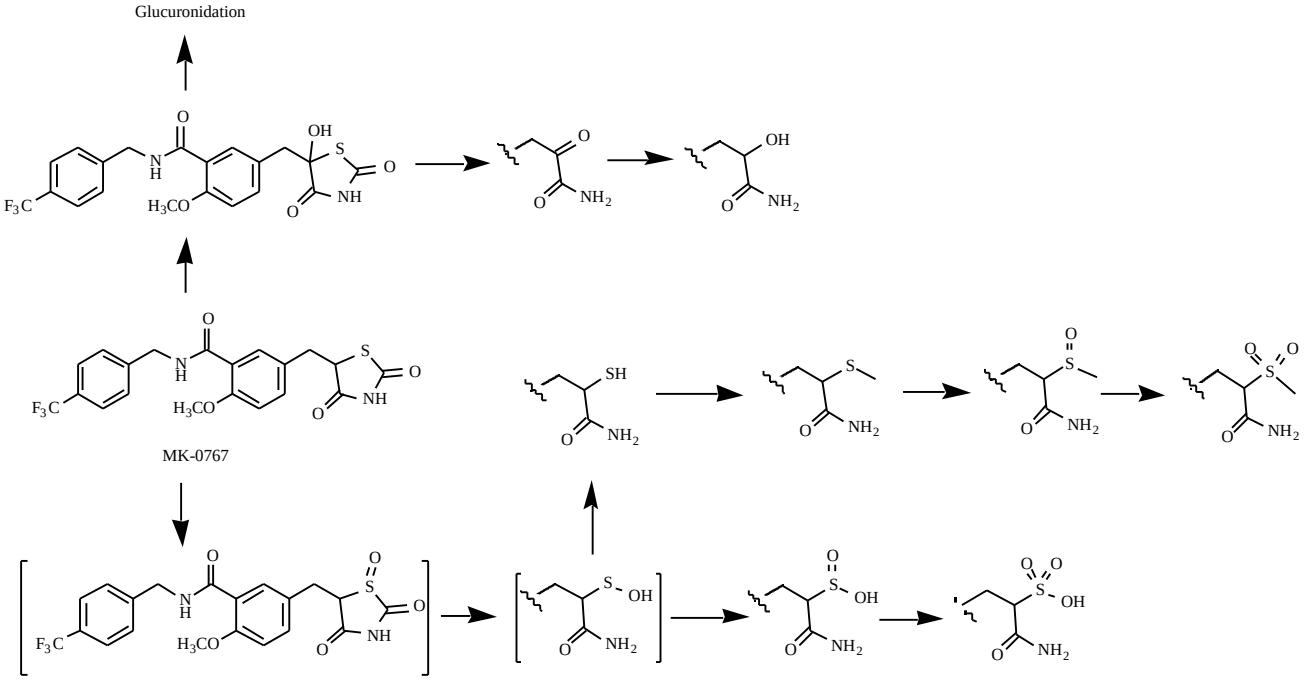


Fig. (52). Proposed Bioatransformation Pathways of MK-0767 in Preclinical Species and Human Hepatic Tissue.

been observed in rats treated with the drug [338, 339]. Raloxifene is bioactivated to a reactive arene oxide that can be trapped with glutathione [340]. It has been proposed that this reactive intermediate irreversibly inhibits CYP3A4 [340]. Carcinogenesis following exposure to polycyclicaromatichydrocarbons (PAH) such as benzo[a]pyrene is thought to arise from arene oxide formation and smaller PAH compounds such as naphthalene and 2-methylnaphthalene [341-344] have been shown to form dihydrodiol metabolites and/or to covalently bind to proteins. However, other compounds such as propranolol and imipramine are proposed to be metabolized to arene oxides but cause little toxicity in man. For both compounds GSH conjugate formation and covalent binding have been demonstrated [345-347].

Mechanism

The isolation of a stable arene oxide metabolite of naphthalene in liver microsomal incubations provided evidence for the P450 catalyzed epoxidation of phenyl rings [333, 334]. Arene oxides are usually unstable and undergo ring opening by mechanism of general acid catalysis, ultimately leading to phenols (Fig. **54**) [348, 349]. Such a reaction can

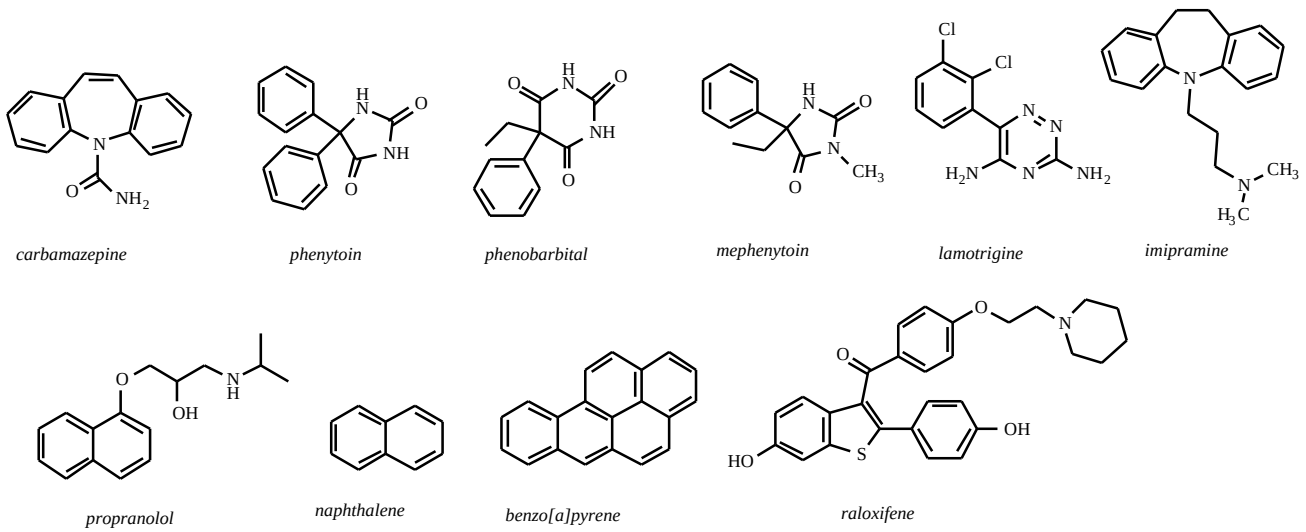


Fig. (53). Examples of compounds metabolized to an arene oxide.

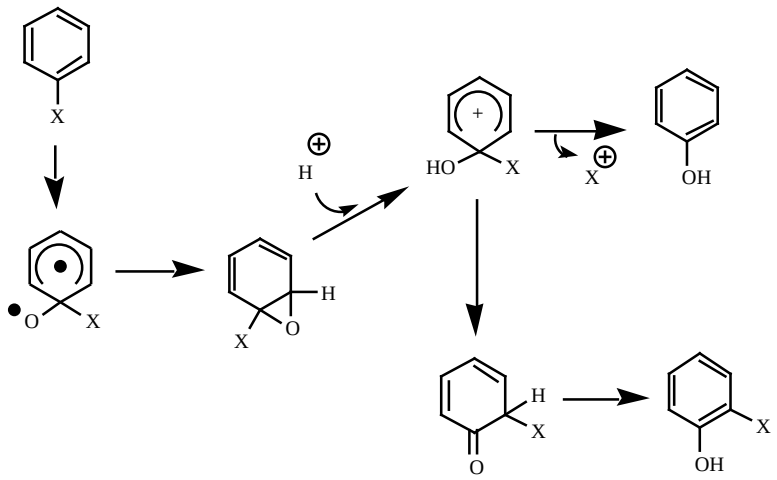


Fig. (54). P450 Catalyzed Epoxidation of Phenyl Rings.

proceed *via* two pathways, namely a direct and an indirect aromatic hydroxylation [350]. The direct pathway involves a complete loss of the geminal group while the indirect pathway involves the complete shift of the geminal group upon ketone formation.

Typical metabolic pathways to an arene oxide metabolite are illustrated using carbamazepine (Fig. **55**) and benzo[a] pyrene (Fig. **56**) as examples. Carbamazepine is metabolized to the reactive arene oxide **167** by P450. Hydrolysis of the arene oxide by epoxide hydrolase resulting in the formation of the dihydrodiol **168** or reaction with GSH to afford the corresponding dihydrohydroxyglutathione conjugate **169** constitute the further metabolic fate of an arene oxide intermediate [351]. Dehydration of the dihydroxyglutathione conjugate provides a stable rearomatized GSH adduct **170**. An alternative bioactivation pathway of carbamazapine through a quinone-imine intermediate **171** has also been proposed [352].

The bioactivation of benzo[a]pyrene is complex (Fig. **56**). The compound is metabolized to an arene oxide **172** followed by epoxide hydrolase mediated hydrolysis yielding the 7, 8-dihydrodiol metabolite **173**. The dihydrodiol under-

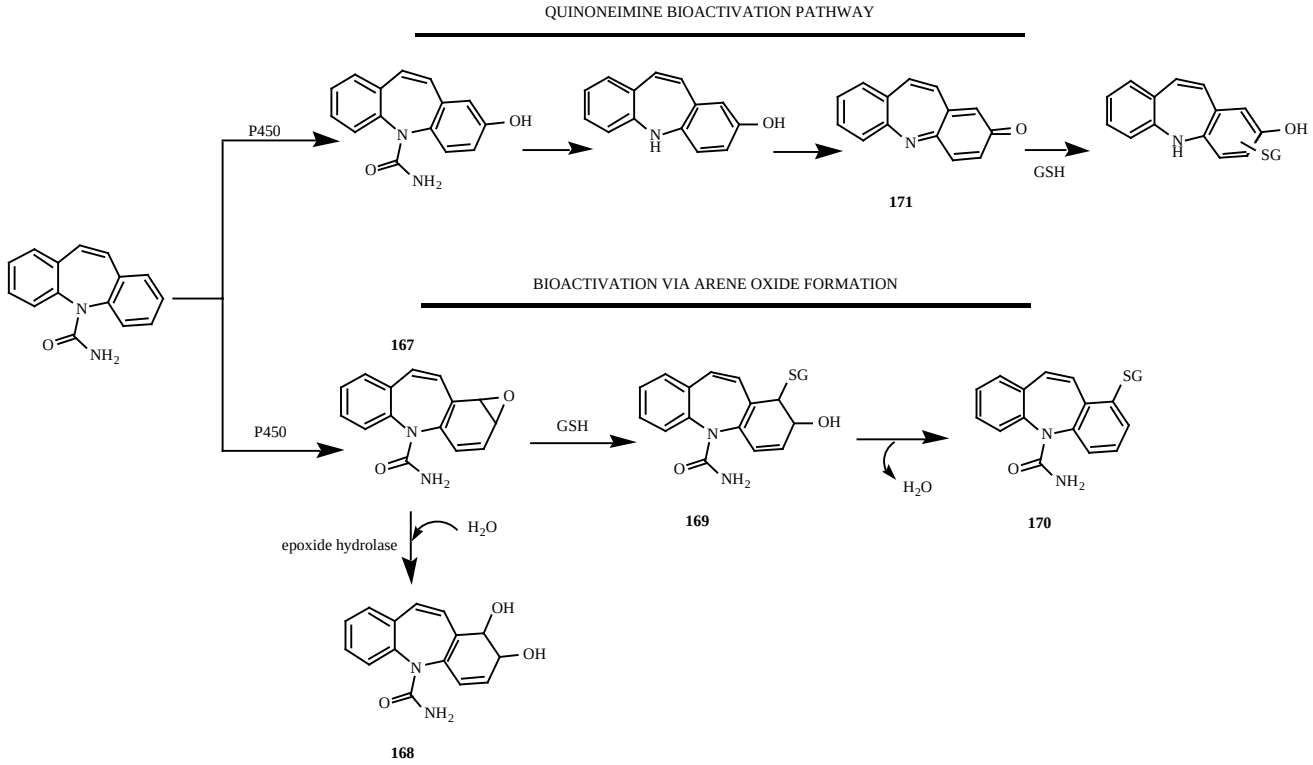


Fig. (55). Metabolism of Carbamazepine.

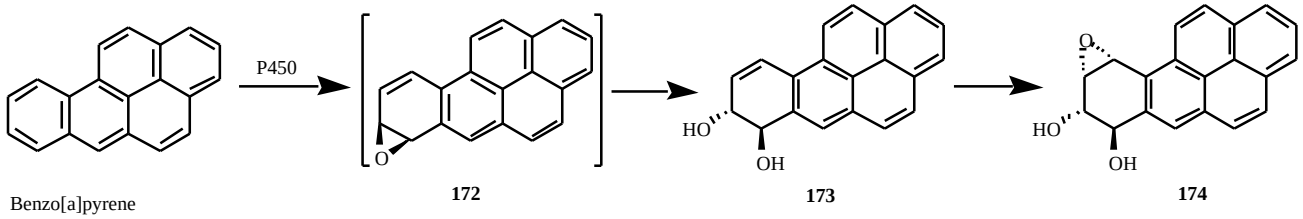


Fig. (56). Metabolism of Benzo[a]pyrene.

goes a second P450 catalyzed epoxidation to **174**, which is the proximate carcinogen. The generation of the proximate carcinogen is stereoselective with the major carcinogenic species being [+]-benzo[a]pyrene-7, 8-diol-9, 10-epoxide [(+)-**174**] [353].

Structure/Bioactivation Relationships

Potentially any phenyl ring can give rise to an arene oxide metabolite. Obviously the vast majority of drugs containing phenyl rings are safe and this should be borne in mind. Similarly compounds that are hydroxylated on the aromatic ring *via* an arene oxide are not of concern, if only the phenol metabolite is observed. However, if dihydrodiol formation or GSH/*N*-acetylcysteine conjugates are identified in *in vitro* or *in vivo* samples then further studies would be warranted. A further complication in assessing the role of arene oxides in drug toxicity is that in a number of cases where arene oxide formation has been suggested to lead to toxicity, other pathways leading to reactive metabolites may also be involved. For instance, a quinone-imine metabolite of carbamazepine has been proposed as a candidate for the major reactive metabolite [352] and the involvement of an arene oxide in lamotrigine toxicity has been questioned [38]. Phenytoin is bioactivated to reactive metabolites other than the arene oxide as detailed earlier. Raloxifene as well as forming an arene oxide can be bioactivated to an extended quinone species, which could give rise to the same GSH conjugates [340]. These observations raise the question of whether arene oxides are the reactive species responsible for the production of idiosyncratic toxicity of these compounds in humans.

Bromobenzene Derivatives

Occurrence and Frequency

Although the aryl bromide moiety is found in various therapeutic agents, such as the NSAID bromofenac, the majority of investigations to determine its bioactivation to reactive metabolites have been studied with the organic solvent bromobenzene [354]. Bioactivation is required for bromobenzene to exert its hepato- and nephrotoxicities. Such hepatotoxicity is a result of a sustained elevation of cytosolic Ca^{2+} levels, which is believed to occur by covalent modification (and subsequent inhibition) of Ca^{2+}-ATPase in cell membrane and/or endoplasmic reticulum [355]. The potential for bromobenzene metabolites to modify cellular constituents is supported further by the identification of numerous rat hepatocellular proteins as targets of reactive bromobenzene metabolites [356-359]. Interestingly, the covalent binding of reactive aryl bromide metabolites to cellular proteins only becomes significant after depletion of cellular GSH, which attests to the excellent electrophilicity and reactivity of the P450-generated reactive metabolites.

Mechanism

The mechanism of aryl bromide toxicity is best exemplified by bromobenzene, which is oxidized by hepatic P450 enzymes to bromobenzene-3, 4-oxide (BBO) followed by its oxidation to quinone *via* the intermediate bromohydroquinone (BHQ) [360]. GSH then conjugates the quinone to afford 2-bromo-bis(glutathione-S-yl)hydroquinone and three positional isomers of 2-bromo-(glutathione-S-yl)hydroquinone (Fig. **57**). The former conjugate is ~ 1,000-fold more potent than bromobenzene at causing nephrotoxicity [355]. A sequence of pathways for the renal disposition of these GSH conjugates has been proposed along with the identification of possible toxic reactive intermediates [360], but the mechanisms by which these compounds generate proximal tubular cell death remain to be elucidated [355]. In regards to the mechanism of toxicity, although much of the net bromobenzene-related covalent binding originates from BHQ metabolites more recent evidence suggests that bromobenzene-3, 4-oxide (BBO), which arylates protein side chains to a lesser extent, is the most significant species toxicologically [358].

Structure/Bioactivation Relationships

The conversion of aryl bromides to electrophiles ripe for endogenous nucleophilic species is due to the electron rich nature of the aromatic ring accompanied by the inductive effect of the highly electronegative bromine. The easiest way to hinder such susceptibility is to remove the halogen itself, the acceptability of which will be dictated by pharmacological SAR studies. A possible alternative to removal of the bromine would be to incorporate steric bulk at the phenyl carbons, preferentially *meta*- or *para*- to bromine, which may prevent 3, 4-epoxide formation and/or sterically hinder nucleophilic attack by protein side chains if a BBO-like intermediate is formed.

Alkynes

Occurrence and Frequency

Drugs that contain the alkyne group include 17α-ethynylsteroids (e.g. norethindrone, 17α-ethynylestradiol) used as estrogenic components in oral contraceptives (Fig. **58**). The antiprogestin agent mifepristone that is used for abortion in the first trimester of pregnancy and the reverse transcriptase inhibitor efavirenz (see Fig. **58**) are other examples of alkyne-containing drugs. Although the antifungal

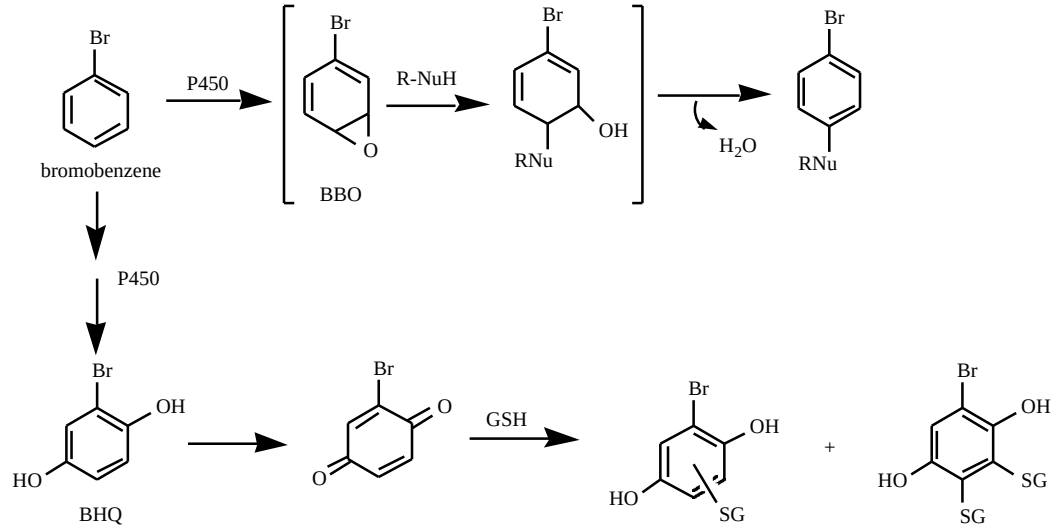


Fig. (57). P450-Mediated Bioactivation of Bromobenzene.

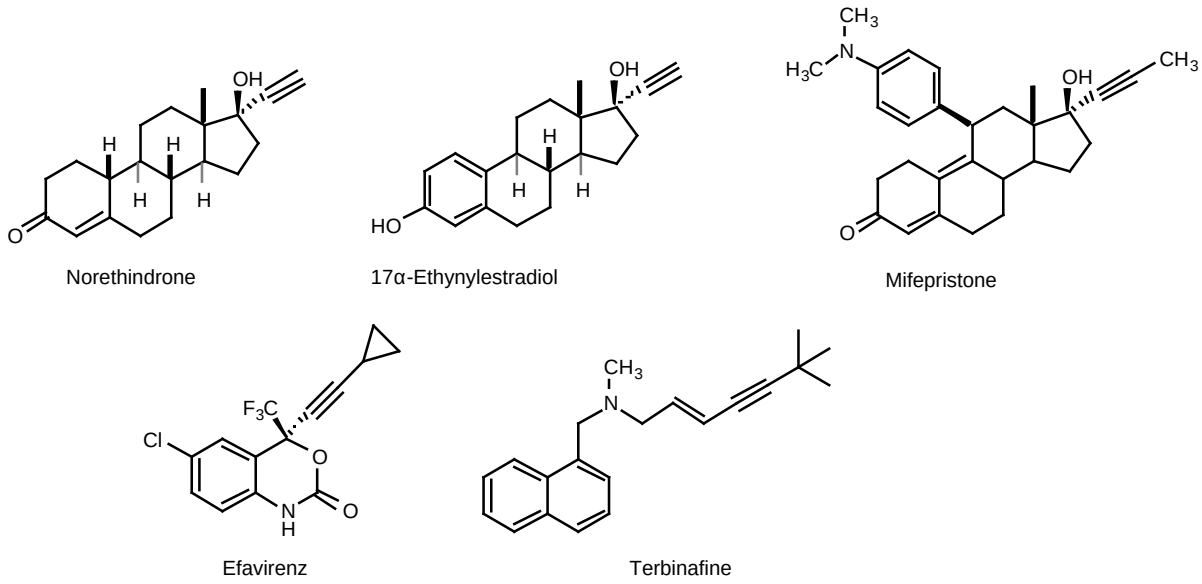


Fig. (58). Examples of Drugs That Contain Alkyne Groups.

agent terbinafine (see Fig. **58**) also serves as an additional example of an alkyne-containing drug, the substituent is not subject to oxidative metabolism. Efavirenz produces renal tubular epithelial cell necrosis in rats but not in cynomolgus monkeys or humans. This species selectivity in nephrotoxicity is thought to result from a species-specific metabolism/bioactivation of efavirenz, since detailed comparison of efavirenz metabolites produced by rats, monkeys, and humans indicates that rats produce a unique GSH adduct derived from the bioactivation of the acetylenic group within the drug [361]. In general, metabolism of the alkyne group in structurally diverse xenobiotics [46], catalyzed by P450 enzymes results in the formation of reactive metabolites that covalently modify the protein resulting in their inactivation. For instance, 17α-ethynylestradiol metabolism by P450 results in the irreversible inactivation of the responsible enzymes *via* mechanisms involving covalent adduction of reactive acetylenic intermediates to the apoprotein [362] and destruction of the heme group *via* alkylation of the porphyrin ring nitrogens [363-366]. The formation of green pigments in incubations of alkynes and alkenes with P450 is consistent with *N*-alkylation of porphyrin ring nitrogen [364, 365]. In humans, 17α-ethynylestradiol is metabolized by CYP3A4, a process that results in the inactivation of this enzyme [367-369]. The mechanism-based inactivation of CYP3A4 by the antiprogestin drug mifepristone has also been recently demonstrated; the compound alkylates apoprotein but not the heme prosthetic group [370, 371]. It is interesting to note that despite the generation of reactive metabolites leading to CYP3A4 inactivation, there have been no cases of idiosyncratic toxicity with 17α-ethynylestradiol, a phenomenon that may be explained by the very low dose (< 0.035 mg) of this agent.

Mechanism

The mechanism of alkyne metabolism by P450 is shown in Fig. **59**. In the case of terminal acetylenes, oxygenation on the terminal carbon followed by a 1,2-hydrogen shift of the acetylenic carbon is thought to generate the reactive ketene, which can react with GSH and/or apoprotein to afford corresponding adducts or undergo hydrolysis to afford an acetic acid derivative, the final product of terminal acetylene metabolism [372, 373]. With internal alkynes, oxidation on the internal carbon is followed by rearrangement to the oxirene, which then reacts with the enzyme or exogenous nucleophiles [374, 375].

Structure/Bioactivation Relationships

The magnitude of structural diversity of alkynes that have been used as tools in studying mechanism-based inactivation of P450 enzymes is enormous. Sterically hindered carbon-carbon triple bonds in internal alkynes have often proven a safer alternative to terminal alkynes as P450 inactivators, at least in preclinical species [376]. Complete saturation of the triple bond in terminal or internal alkynes, however, is the most effective way to prevent bioactivation and irreversible P450 inhibition.

α,β-Unsaturated Carbonyl Compounds (Michael Acceptors)

Occurrence and Frequency

α,β-unsaturated carbonyl derivatives are highly reactive species and many compounds containing such moieties are toxic, thus are generally avoided in drug discovery. An example of a drug candidate that bears an α,β-unsaturated carbonyl motif is shown in Fig. **60** with the epidermal growth factor receptor (erbB tyrosine kinase) inhibitor, CI-1033, a clinically promising agent for the treatment of breast and other cancers that is active against all four members of the erbB receptor tyrosine kinase family [377-379]. The erbB family of cell surface receptor proteins plays a role in the development and growth of the normal breast. The activity of this signaling pathway is normally tightly controlled, and dysregulation has been shown to play a significant role in the pathogenesis and progression of breast and other cancers [380]. The mechanism of CI-1033 inhibition of the erbB receptors involves chemical reaction of the acrylamide motif in CI-1033 with a cysteine residue (see Fig. **60**) in the ATP binding pocket in these enzymes, which results in the irreversible inhibition of these kinases, and thereby provides for prolonged suppression of erbB receptor-mediated signaling [381-383]. Clinically, it has been shown to have an acceptable side effect profile at potentially therapeutic doses and schedules. Besides the erbB inhibitors, there are a few examples of drugs that upon bioactivation yield α,β-unsaturated carbonyl intermediate(s), which are believed to be responsible for the observed toxicity associated with these drugs.

Mechanism

Cyclophosphamide is an oxazophosphorine antitumor agent and immunosuppressant used widely in the United States. A dose-limiting bladder toxicity (hemorrhagic cystitis) occurs in most patients receiving the drug [384]. The Michael acceptor acrolein, which is formed *via* a β-elimination reaction following an initial P450 mediated hydroxylation of cyclophosphamide has been implicated as the metabolite responsible for bladder damage (Fig. **61**) [385, 386]. Acrolein is highly electrophilic and reacts with GSH to form the acrolein-GSH adduct **175** that is capable of undergoing further metabolism (oxidation/reduction). The characterization of a hydroxypropyl mercapturic acid conjugate **176** (see Fig. **61**) in the urine of animals and humans

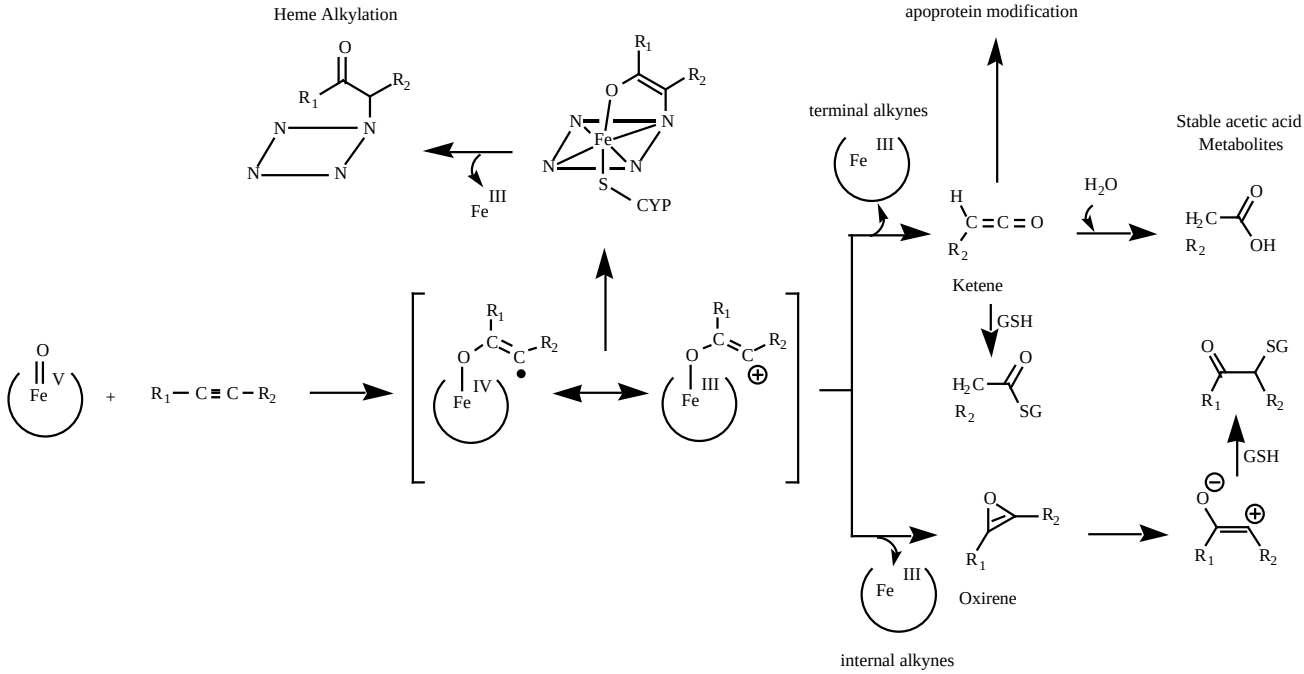


Fig. (59). P450 Mediated Bioactivation of Alkynes.

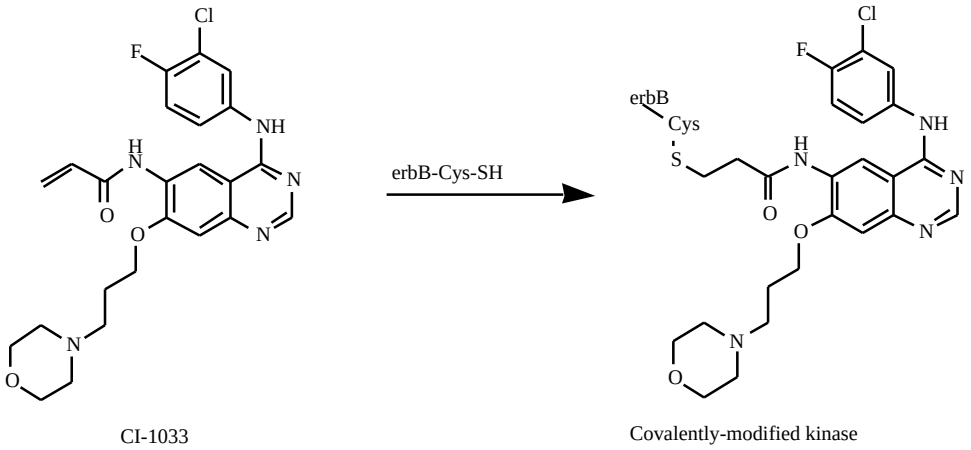


Fig. (60). Affinity Labeling of erbB receptors by the Michael Acceptor and Antitumor Agent CI-1033.

treated with cyclophosphamide is consistent with the notion that this bioactivation pathway operates *in vivo* [387, 388]. The protective effects against bladder injury associated with cyclophosphamide with cysteine and *N*-acetylcysteine [389, 390], depletion of cellular GSH levels [391] and the loss of protein thiols from murine bladder [392] are consistent with the presence of an electrophilic metabolite. It has been speculated that the reactivity of acrolein appears to preclude the movement of the corresponding thiol conjugates from the main site of formation (liver) to the target organs for toxicity (bladder and lung) followed by re-release of acrolein by a β-elimination process (see Fig. **61**) [393, 394].

Within the first year of introduction of the antiepileptic drug felbamate, an unexpected association between felbamate therapy and reported cases of aplastic anemia and hepatotoxicity were realized [395-399]. In all, 34 cases of

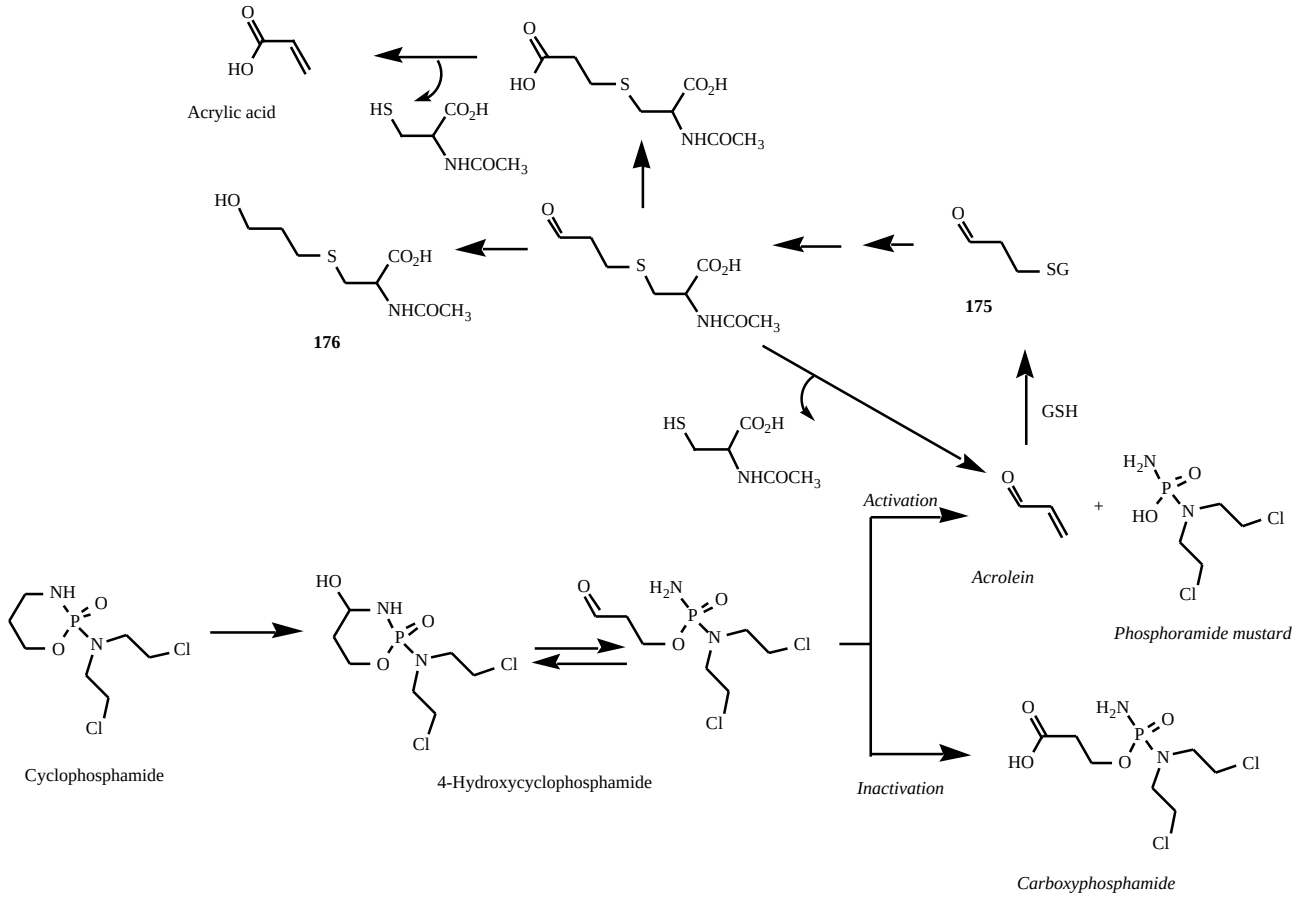


Fig. (61). Bioactivation of the Antitumor Agent Cyclophosphamide.

aplastic anemia, resulting in 13 deaths, and 23 cases of hepatic failure, resulting in 5 deaths, were reported to the manufacturer. The toxicities associated with felbamate resulted in a black box label warning by the FDA, which severely curtailed its use. Since felbamate offers relief to refractory patients, the FDA did not withdraw its use, but rather limited its use to cases where the benefit of therapy outweighs the risks of untreated seizures. An estimated 12, 000 patients remain on felbamate therapy within the United States today. Besides the well-characterized felbamate metabolites 2- and *p*-hydroxyfelbamate (compounds **177** and **178**, respectively), 2-phenyl-1, 3-propanediol-monocarbamate (**179**) and 3-carbamoyl-2-phenylpropionic acid (**180**) [400, 401], a bioactivation pathway was also recently elucidated and involved the conversion of felbamate to the highly reactive α, β-unsaturated carbonyl derivative atropaldehyde (2-phenylpropenal, **181**) (Fig. **62**) [402-405]. The first evidence for the *in vivo* formation of **181** was demonstrated in rats dosed with felbamate by the identification of the expected mercapturates (compounds **182** and **183**) (see Fig. **62**) derived from the reaction of GSH with **181** in urine followed by further downstream metabolism [406]. A subsequent single administration of 600 mg to a healthy male volunteer allowed for the identification of the mercapturates as urinary excreted metabolites, thus providing evidence for the *in vivo* formation of atropaldehyde as a consequence of human metabolism [407]. The coupling of **181** with GSH to generate the non-reactive GSH conjugates *in vivo* is considered a protective metabolic route, unless this results in a GSH-depleted state.

Terbinafine (Lamisil) (Fig. **63**) represents a new class of antifungal agents, and is widely used for the treatment of superficial fungal infections of the toe and fingernails. Hepatobiliary dysfunction is a rare adverse reaction of terbinafine. Such cases are usually asymptomatic and reversible; however, symptomatic cholestatic injury occurs in 1 of

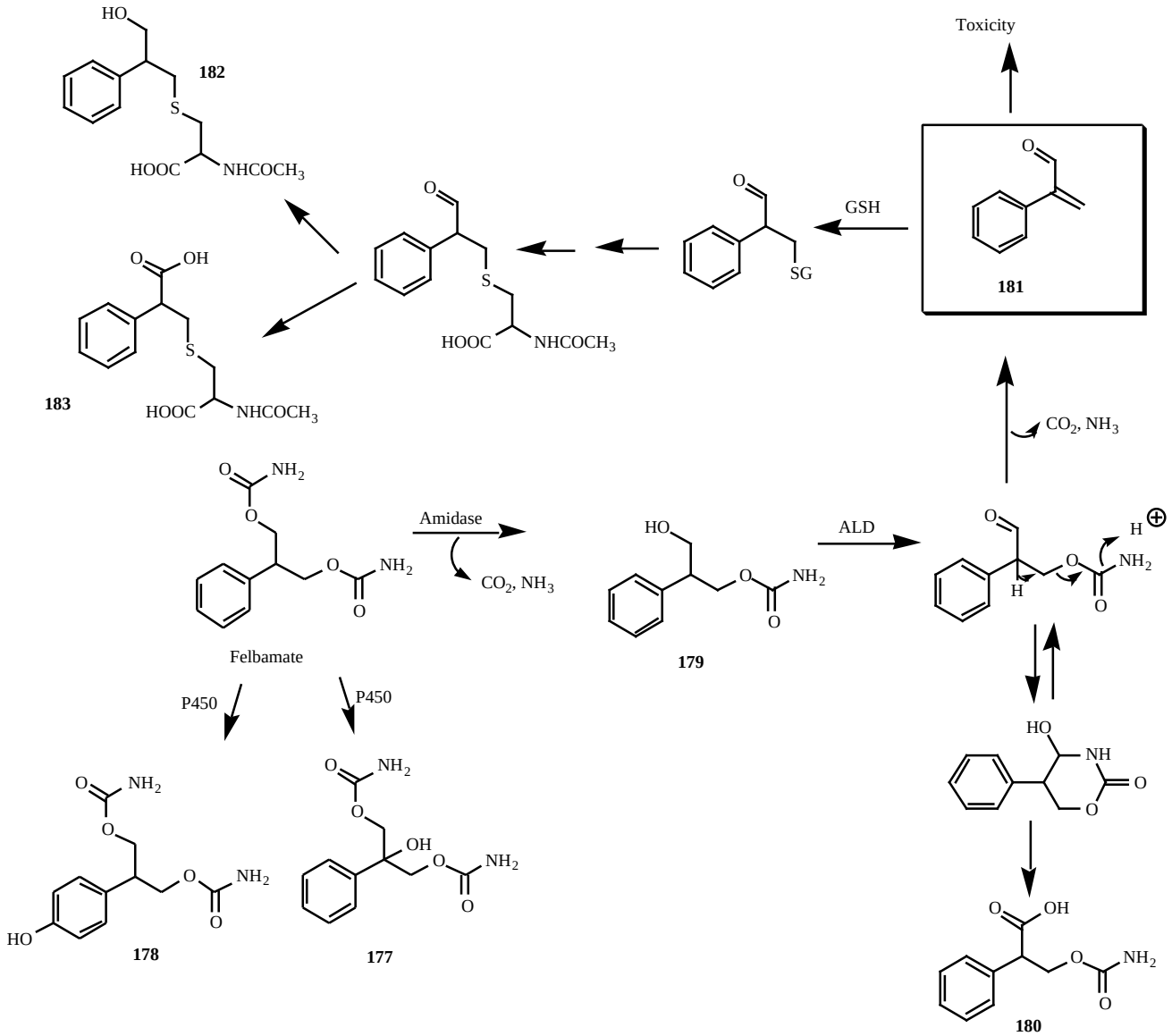


Fig. (62). Bioactivation of the Antiepileptic Drug Felbamate to a Michael Acceptor.

45000-54000 exposed patients [408, 409]. Recent studies by Uetrecht *et al.* [410] have resulted in the characterization of an α, β, γ, δ-unsaturated aldehyde metabolite **184** of terbinafine in NADPH-supplemented rat and human liver microsomal incubations. This aldeyhde metabolite corresponds to the *N*-dealkylation product of terbinafine and can be conveniently trapped in a 1, 4- and 1, 6-Michael fashion with GSH yielding adducts **185** and **186**, respectively, as shown in Fig. **63**. It is interesting to note that there is a second reactive domain in the 1,6-GSH conjugate **186**, which can bind to a second molecule of GSH or to cellular proteins *via* a 1,4-Michael addition. Considering that the formation of these GSH conjugates was shown to be reversible, it has been speculated that the allylic aldehyde metabolite, formed in the liver and conjugated with GSH, would be actively transported across the canicular membrane of heptatocytes and concentrated in the bile. The 1, 6-GSH conjugate **186**, which is still intrinsically electrophilic, could bind to hepatobiliary proteins and lead to toxicity.

The reverse transcriptase inhibitor, abacavir (ziagen®), causes a potentially life-threatening hypersensitivity syndrome in about 5% of patients [411, 412]. Rechallenge of patients after a hypersensitivity reaction typically results in recurrence of symptoms within hours that is suggestive of an immune mediated reaction [413]. A strong association between presence of the HLA-B*5701, HLA-DR7 and HLA-DQ3 haplotype and hypersentivity to abacavir has been noted suggesting that there is a genetic component to this adverse reaction [414, 415]. Abacavir is bioactivated to a reactive aldehyde by human cytosol and by the αα and γ2γ2 human isoforms of alcohol dehydrogenase (Fig. **64**). The

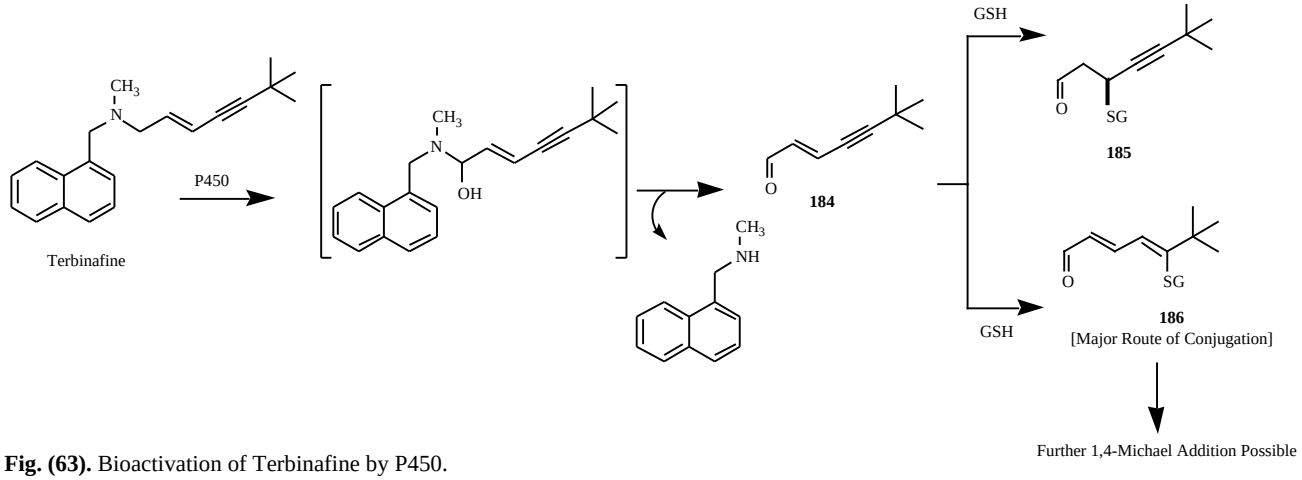


Fig. (63). Bioactivation of Terbinafine by P450.

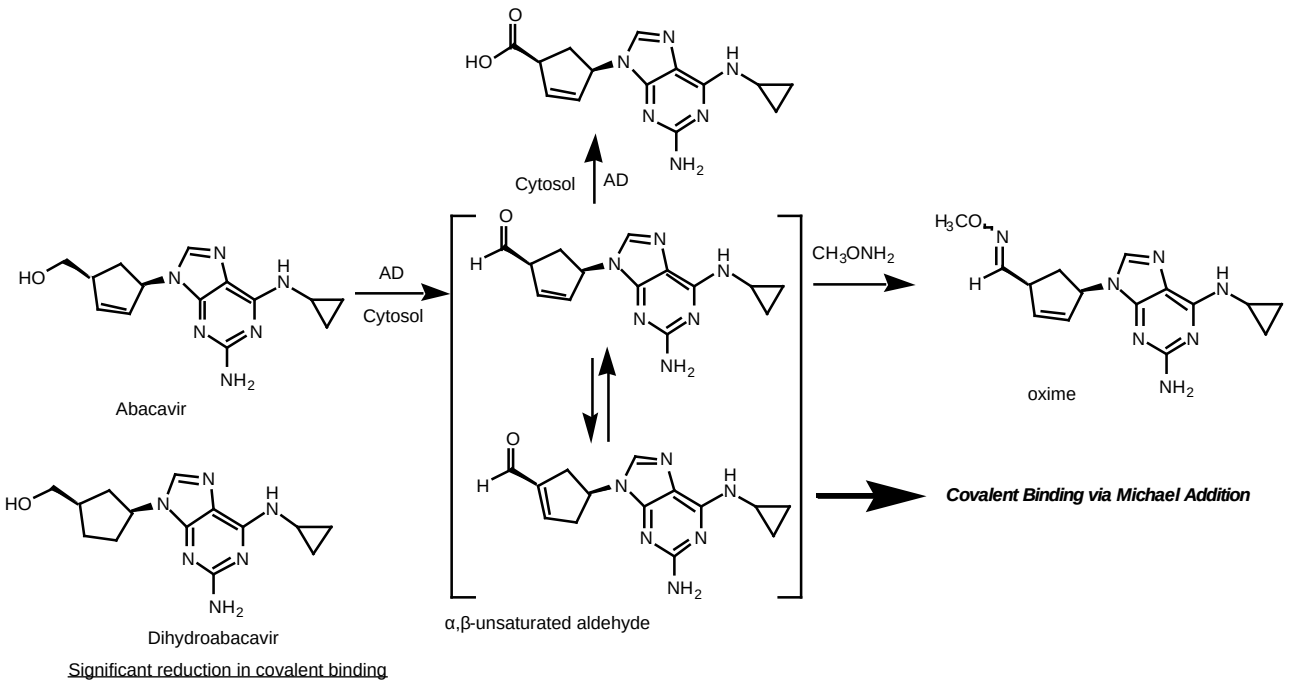


Fig. (64). Proposed Bioactivation Pathway of the Reverse Transcriptase Inhibitor Abacavir in Human Liver Cytosol.

aldehyde was shown to covalently bind to protein *in vitro* and was amenable to trapping as an oxime derivative [416]. Covalent binding of an abacavir analogue in which the double bond was removed was markedly reduced suggesting that the mechanism of covalent binding may be through double bond migration and 1, 4-addition (see Fig. **64**) [416].

Another example worth mentioning in this context is the industrial chemical 1, 3-butadiene, which is a known rodent carcinogen and a probable human carcinogen [417]. The compound undergoes metabolism to several potential toxic intermediates; one of them is hydroxymethylvinyl ketone (HMVK), a Michael acceptor [418]. 1, 3-butadiene is initially metabolized to butadiene monoxide by P450 enzymes and myeloperoxidase (Fig. **65**), followed by epoxide ring opening to diol and further oxidation to HMVK [419-423]. HMVK has been shown to react with biomacromolecules including DNA bases [424]. The existence of this bioactivation pathway *in vivo* is evident from the characterization of mercapturic acid conjugates of HMVK in the urine of rodents exposed to 1, 3-butadiene [425].

Structure/Bioactivation Relationships

Saturation of the double bond in the α, β-unsaturated carbonyl compound is an obvious method for reducing intrinsic reactivity towards thiols. Furthermore, incorporation of bulky functionalities on the double bond (on the α and β-positions) may reduce reactivity towards nucleophiles.

Alkanoic Acid Bioactivation

Occurrence and Frequency

Valproic Acid (VPA) (Fig. **66**) is a classical example of a drug in this class. Two clinical forms of VPA-mediated hepatotoxicity have been characterized: an asyptomatic dose-dependent increase in serum amino-transferase, which occurs in 44% of patients; and, a more serious dose-independent microvesicular steatosis, which results in liver failure in 1/32,000 to 1/100,000 patients [426-428]. The histopathological lesion is similar to those found in Reyes Syndrome and Jamaican Vomiting Sickness. VPA is also a potent teratogen in humans and mice (spina bifida), which is thought to be mediated by parent drug [429].

Mechanism

VPA hepatotoxicity is believed to be due to the inhibition of mitochondrial β-oxidation. This inhibition may be due to the alkylation of enzymes by the reactive metabolites or the depletion of necessary cofactors. VPA and its metabolites (Fig. **66**) have been shown to inhibit mitochondrial β-oxidation both *in vitro* and *in vivo* [429-431]. It is believed that the 4-ene VPA metabolite [432], is further metabolized to reactive α,β-unsaturated carbonyl intermediates that may directly impair mitochondrial β-oxidation. These reactive intermediates have been trapped by GSH and *N*-acetyl-cysteine, and were subsequently identified in urine and bile

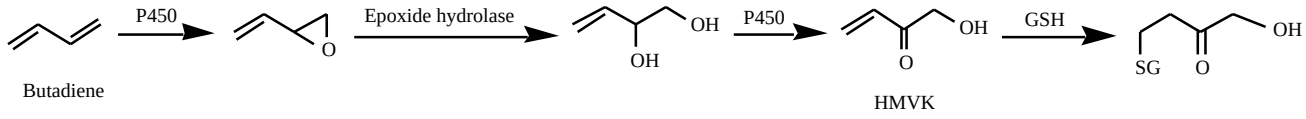


Fig. (65). Bioactivation of the 1, 3-Conjugated Diene Butadiene to a Reactive α, β-Unsaturated Carbonyl Metabolite.

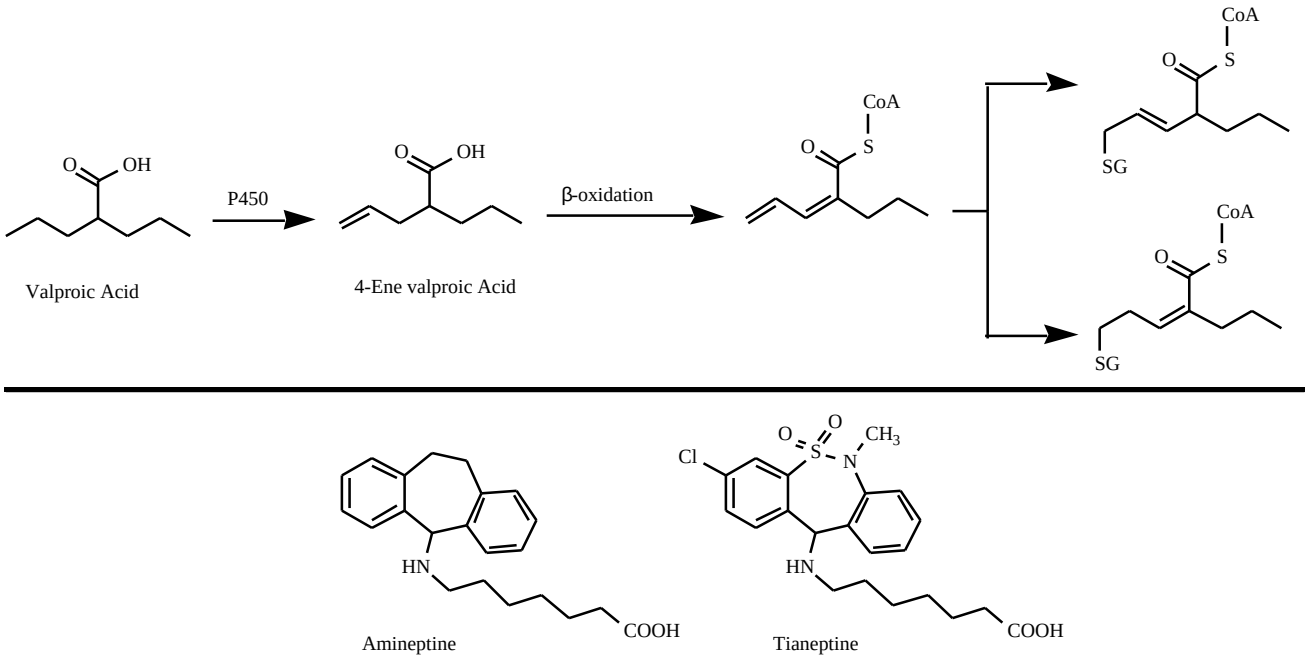


Fig. (66). Bioactivation of Valproic Acid.

from preclinical species and urine from humans [433, 434]. Additionally, VPA has been shown to decrease serum and liver carnitine and deplete CoA, which can also lead to decreased mitochondrial β-oxidation [435].

The antidepressants tianeptine, amineptine and their metabolites have also been shown to block fatty acid oxidation [436, 437]. Both compounds undergo extensive β-oxidation on the aminohepatonic acid chain [437, 438]. It has been proposed that β-oxidation may result in the blockage of the oxidation of essential endogenous short/medium chain acids, which may cause hepatotoxicity following prolonged exposure [429, 439].

Structure/Bioactivation Relationships

Amineptine and tianeptine (Fig. **66**) are tricyclic antidepressants that share an identical heptanoic acid side chain. Both compounds inhibit mitochondrial β-oxidation and are thought to have the same mechanism of toxicity as valproic acid. Tianeptine appears to be extremely safe compared to amineptine, which is responsible for 80% of hepatitis caused by tricyclic antidepressants. This may be dose dependent since the recommended dose of tianeptine is 6 times lower than that for amineptine.

Carboxylic Acid Bioactivation

Occurrence and Frequency

Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most prescribed pharmaceuticals in the world. Due to their structural diversity, the incidence of hepatotoxicity within this drug class is not uniform, with the frequency of liver injury attributable to NSAIDs among current users estimated at ~ 5 per 100, 000 persons per year [440]. Furthermore, five of the twenty-five drugs withdrawn from the U.S. and British market due to severe toxicity from 1964–1983 were NSAIDs of the aryl-alkyl acid class [441]; the aryl-alkyl acids suprofen and bromofenac were also recently withdrawn for similar reasons. In two large epidemiological studies, sulindac was identified as the NSAID with the highest risk of hepatic drug reactions [440, 442].

Mechanism

The mechanism by which the majority of aryl-alkyl acid NSAIDs exert their attributed hepatotoxicities, has been proposed to involve an immune-mediated mechanism [443] or disruption of cellular function [444] *via* a reactive acyl glucuronide [445-447]. Glucuronidation is a well-known Phase II metabolic process, and has until recently been viewed as a detoxification route for carboxylic acids (Fig. **67**). UDPGT-mediated glucuronic acid conjugation of the aryl-alkyl acid affords an acyl glucuronide which may adduct proteins directly (pathway A) or after acyl migration to an aldehyde species (pathway B). Such intramolecular transesterification can occur from the 1-hydroxyl position of glucuronic acid to its 2-, 3- or 4-hydroxyl moiety to render a β-glucuronidase resistant aldehyde isomer [445]. Recent work demonstrates that the degradation rates (encompassing both intramolecular rearrangement and ester hydrolysis) of acyl glucuronides show an excellent correlation with the extent of covalent binding of the drug to albumin *in vitro* [447]. Similar irreversible protein modification occurs *in vivo* in humans with the aryl-alkyl acid NSAIDs zomepirac [448] and tolmetin [449].

Although the formation of acyl glucuronides is consistent with adverse effects associated with the majority of aryl-alkyl acids, bioactivation of other substitutents within these compounds may also account for some of the cited toxicological consequences as exemplified by the NSAIDs diclofenac and suprofen. Acyl glucuronides are responsible for the majority of diclofenac protein covalent binding in rats, but P450 oxidation is solely implicated in the formation of microsomal protein adducts [446]. The P450-generated reactive metabolite responsible for microsomal protein covalent modification has been speculated to be an arene oxide [450].

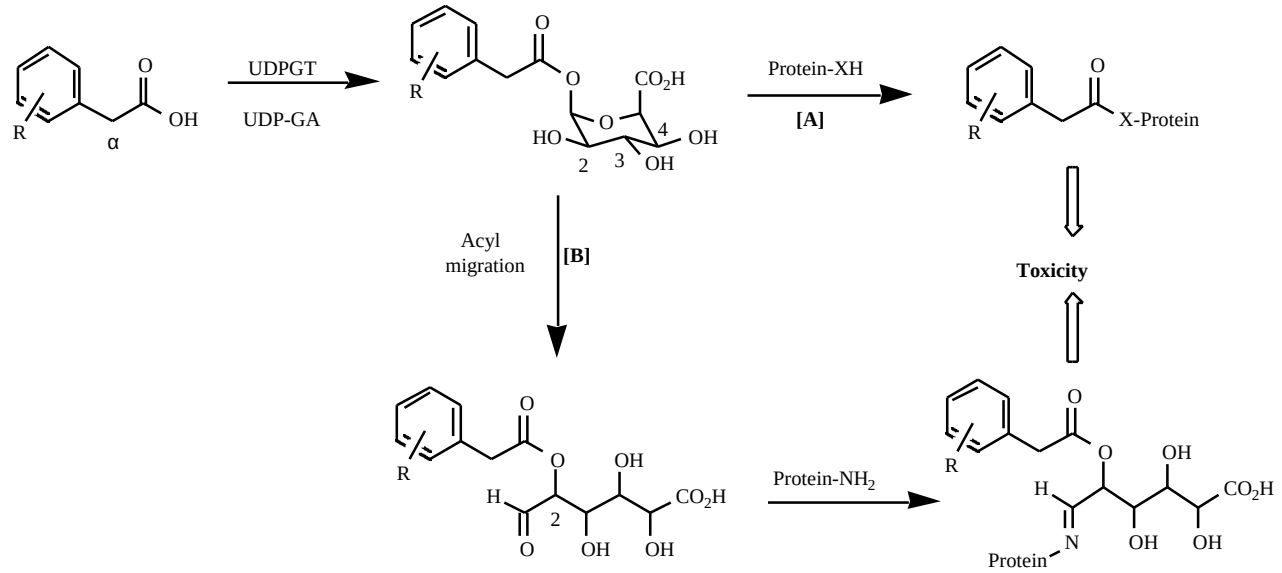


Fig. (67). Acyl Glucuronidation of Carboxylic Acid-Containing Drugs.

In humans, the reactive intermediate is thought to be a quinone imine, which could arise following the CYP2C9-catalyzed 4'-hydroxylation of diclofenac [30, 451]. Similarly, a P450-generated electrophilic sulfoxide is postulated for suprofen-related protein adduction.

Structure/Bioactivation Relationships

The majority of covalent binding which results from aryl-alkyl acids occurs subsequent to acyl glucuronide formation, and a number of studies suggest that the extent of covalent binding for carboxylic acid drugs *in vivo* is correlated to the degradation rate constant of the acyl glucuronide. It has recently been reported that the binding capacity of respective aryl-alkyl acids is predictable based on the chemical structure of the acid and depends on the degree of substitution at the carbon alpha to the carboxylic acid [447]. For example, two aryl-alkyl acids that contain unsubstituted α-carbons, tolmetin and zomepirac, exhibit the greatest degree of protein binding. The decrease in degree of protein binding seems to be directly proportional to the increase in substitution at the α-carbon with carboxylic acids fully substituted at the α-carbon (e.g. the diuretic furosemide) exhibiting the least amount of covalent adduct formation. Although some compounds, like valproic acid [452] and etodolac [453], do not fit this α-carbon substitution correlation, they do fit the degradation rate correlation. It is important to note that the magnitude of acyl glucuronide formation relative to all other metabolic pathways to which the drug is subjected must always be considered. For example, acyl glucuronide formation represents >90% of the metabolism of zomepirac, while it is only a minor pathway for tolmetin, which instead predominately undergoes benzylic hydroxylation, a metabolic option unavailable to zomepirac. To address the possibility of acyl glucuronidation the following experiments may be attempted: (a) determine whether the carboxylic acid is a substrate for glucuronidation in hepatocytes or liver microsomes supplemented with UGT cofactor and appropriate detergent [446] and (b) If an acyl glucuronide is detected, a synthetic standard may be used to determine the rate of acyl glucuronide degradation and protein binding *in vitro* with human serum albumin [447].

Alkyl Halides

Occurrence and Frequency

With the exception of the antibacterial chloramphenicol and the volatile anesthetics (e.g. halothane, isofluorane and desfluorane), there are not many drugs that contain alkyl halide substituents. One reason is the good leaving group tendency of certain halogens such as chlorine and bromine renders simple alkyl halides susceptible to enzymatic and non-enzymatic nucleophilic attack by amino acid nucleophiles. ADRs associated with chloramphenicol and volatile anesthetics appear to be dependent upon their bioactivation to reactive intermediates. Hepatotoxicity resulting from the volatile anesthetics such as halothane [454, 455], is believed to occur either by covalent modification of proteins by a halohydrin metabolite to form neo-antigens that ultimately elicit autoimmune hepatitis; and/or, generation of a free-radical species, which causes lipid peroxidation, and/or protein covalent modification. The relative importance of these two pathways to halothane-induced hepatotoxicity appears to be species dependent [355], with the incidence of iatrogenic disease in humans being 1 in 20,000 [456]. Although it may be possible for the organic solvent carbon tetrachloride to also exert its deleterious effects *via* the same routes, most evidence suggests that the tetrahaloalkyl derivatives undergoes CYP2E1-catalyzed sequential reductive dehalogenation and oxidation to afford phosgene [457].

Mechanism

The P450-mediated [458] biotransformation of halothane (Fig. **68**) to a reactive species best illustrates the different mechanistic pathways for molecular toxicity available to the majority of halogenated alkyl metabolites. Halothane may undergo P450-catalyzed oxidative dehalogenation (pathway A) to trifluoroacetylchloride (**187**), a potent acylator of cellular macromolecules, which results in hapten-like neo-antigen formation and subsequent immune response. In humans, halothane hepatotoxicity appears to result from this pathway [459-462]. Alternatively, halothane may undergo CYP2A6 and CYP3A4-catalyzed reductive dehalogenation (pathway B) to afford a carbon-centered radical **188**, which itself can undergo three fates: (a) reduction to alkane **189**, (b) reductive dehalogenation to the olefin **190**, or (c) bind to intracellular proteins and/or initiate lipid peroxidation [463]. Pathway B is the mechanism of halogen-induced hepatotoxicity in rats.

Likewise, it is hypothesized that the P450-catalyzed oxidative dehalogenation of chloramphenicol affords an oxamyl chloride, which could readily form macromolecular adducts (Fig. **69**). It is noteworthy to point out that reductive metabolism of the nitro group to the corresponding reactive nitroso metabolite has also been proposed as an alternate mechanism of bioactivation leading to toxicity [47]. Chloramphenicol has been reported to be a suicide substrate for cytochrome P450 [464]; its mechanism of inactivation may be similar to that hypothesized below.

Structure/Bioactivation Relationships

Biotransformation of halogenated alkyls to extremely reactive acylating agents is usually due to the availability of an extractable hydrogen atom on the halogenated alkyl carbon. One possible way to hinder acyl halide formation may be to replace the aliphatic hydrogen with an alkyl or cycloalkyl moiety. However, depending upon the structure of the compound, such a modification may cause a metabolic shift from oxidative dehalogenation to reductive dehalogenation, which may lead to similar toxic consequences. It is important to note that other anesthetics such as isofluorane and desfluorane, also undergo oxidative metabolism resulting in the formation of reactive acyl halides (Fig. **70**); and the degree to which the anesthetic is metabolized by this pathway correlates with the risk that the drug will cause liver toxicity [462].

CONCLUDING REMARKS

In retrospect, many of the observations on xenbiotic metabolism suggest that enzymatic bioactivation of certain functional groups to reactive metabolites may represent a rate-limiting step in the initiation of a toxic response, however there is no formal proof for this hypothesis. Without a better understanding of the mechanisms involved

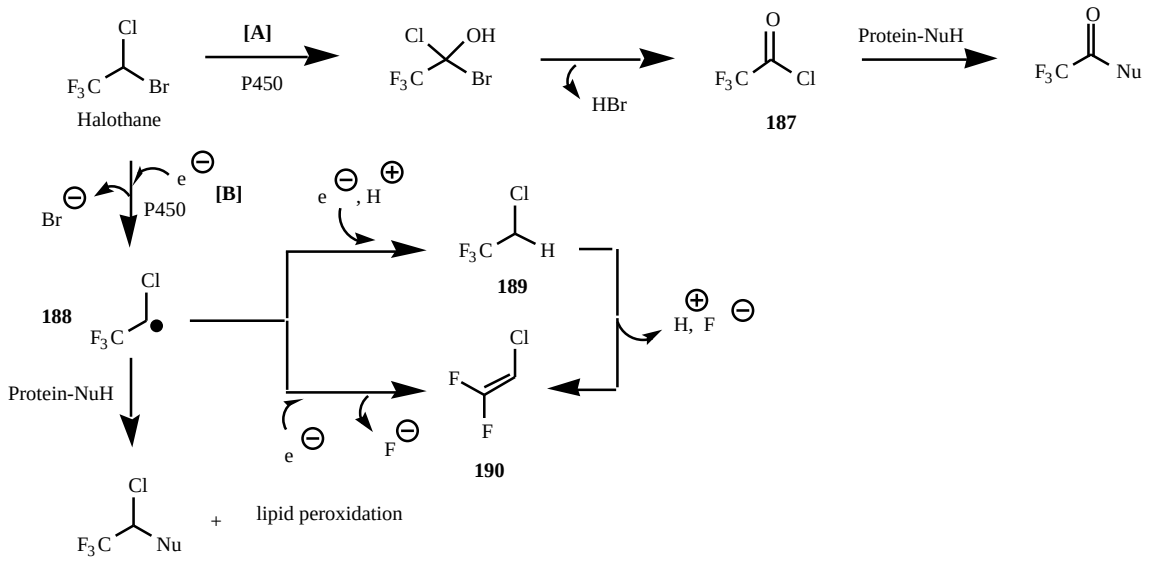


Fig. (68). Proposed Pathways of Halothane Bioactivation.

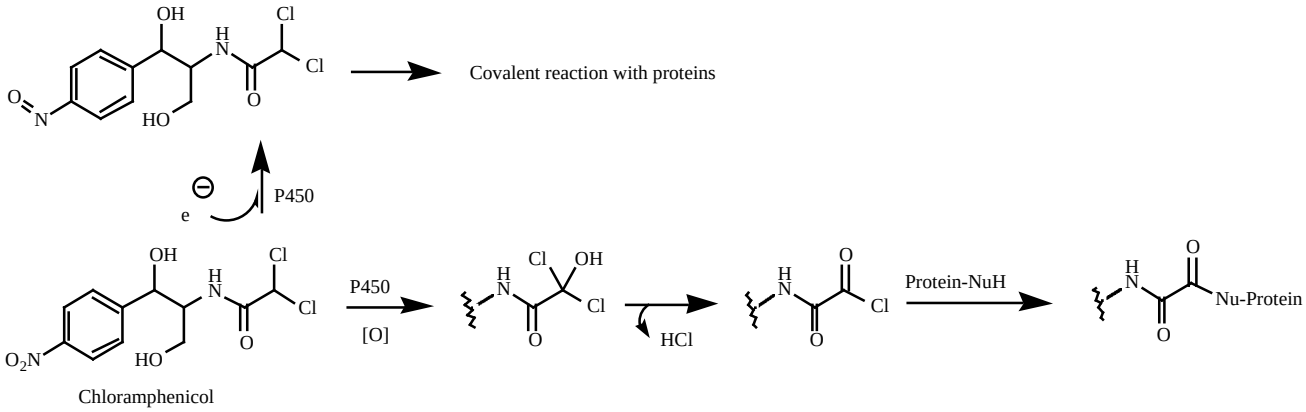


Fig. (69). Proposed Mechanism of Chloramphenicol Bioactivation by P450.

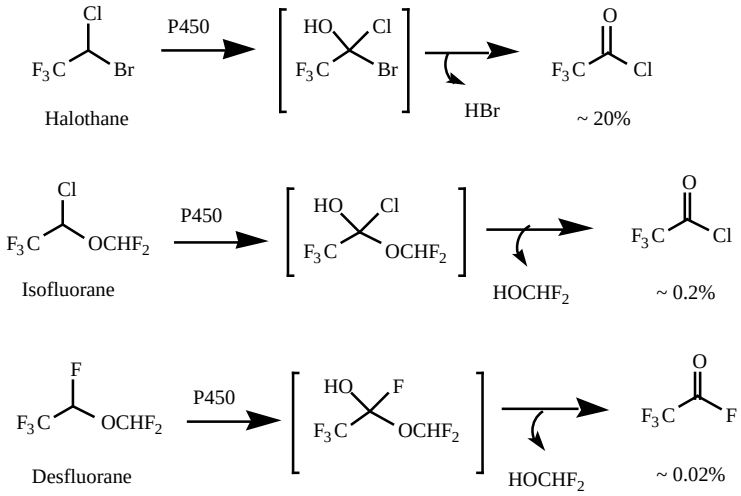


Fig. (70). Differences in the Extent of Bioactivation of Volatile Anesthetics to Reactive Acyl Halides.

in idiosyncratic drug reactions it is difficult to judge the *in vivo* significance of reactive metabolite formation *in vitro* with new drug candidates, especially when there are no animal models to predict idiosyncratic toxicity in humans. Considering the high price of failure due to withdrawal of drugs, particularly those that cause IADRs, it is beneficial to screen for reactive metabolite formation in early lead development, since the information on the mechanism of bioactivation and structure of the reactive metabolite(s) could be incorporated in subsequent medicinal chemistry efforts to circumvent the issue, while retaining pharmacology and other attractive pharmacokinetic attributes. If more than one chemical series exists for a particular target, reactive metabolite screening may help in choosing the "safer" series. As always, dose size and regimen is most important, more potent drugs will be safer drugs. Likewise, appropriate consideration needs to be given for drug candidates for potential treatment options for unmet and urgent medical need.

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