6 Drug Bioactivation and Oxidative Stress

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6.1 BIOACTIVATION AND OXIDATIVE STRESS SUMMARY

Drug metabolism generally renders drug molecules less reactive and increases their hydrophilicity, assisting the drug, and its metabolites' elimination from the body. Drug metabolism can also uncover more pharmacologically reactive groups, thereby making the metabolite more reactive than the parent compound (prodrug). Reactive metabolites (RMs) can also be created by the metabolism of certain drugs. RMs are generally short lived and are *bioinactivated* by conjugation reactions with glutathione (GSH). If cellular stores of GSH become depleted or its production has been impaired, RMs, created by this bioactivation of the parent compound, can covalently bind with cellular macromolecules and/or induce oxidative stress, which are important initiators of toxicity.

The propensity of a drug molecule to be bioactivated is a function of its chemistry. A number of functional groups have been identified that are frequently associated with bioactivation and subsequent drug-induced toxicity (Table 6.1). In some instances, these structural fragments are crucial to the potency and pharmacological selectivity of the compound. However, the mere presence of these moieties in a drug or compound

Encyclopedia of Drug Metabolism and Interactions, 6-Volume Set, First Edition.
Edited by Alexander V. Lyubimov.

TABLE 6.1 Examples of Drugs Causing Off-target Clinical Adverse Drug Reactions and the Reactive Intermediates Involved

Drug	Adverse Reaction	RM
Amodiaquine	Hepatotoxicity	Quinone imine
Paracetamol	Hepatotoxicity	Quinone Imine
Halothane	Hepatotoxicity	Acyl halide
Diclofenac	Hepatotoxicity	Quinone imine/acyl glucuronide
Tacrine	Hepatotoxicity	Quinone methide
Indomethacin	Hepatotoxicity	Quinone imine/chloro-indole
Valproic acid	Hepatotoxicity	α, β-Unsaturated carbonyl
Vesnarinone	Hepatotoxicity	Iminium ion
Phenacetin	Hepatotoxicity	Quinone imine
Phenytoin	Teratogenicity/hepatotoxicity	Free radical
Clozapine	Agranulocytosis	Nitrenium ion
Aminopyrene	Agranulocytosis	iminium
Ticlopidine	Agranulocytosis	*S*-oxide
Sulfamethoxazole	Toxic epidermal necrolysis	Hydroxylamine/nitroso
Lamotrigine	Toxic epidermal necrolysis	epoxide
Carbamazepine	Hypersensitivity	Quinone imine/epoxide
Tienilic acid	Hypersensitivity	S-oxide
Felbamate	Aplastic anemia	Atropaldehyde
Remoxipride	Aplastic anemia	Hydroquinone

does not automatically result in bioactivation and toxicity [1]. A combinatory effect of a number of other factors will ultimately determine whether a RM will be either detoxified and eliminated or cause toxicity. Dose and duration of therapy of a drug will control the levels of exposure to the drug a patient receives; genetic factors may also affect rates of RM formation, cellular defense mechanisms, and susceptibility to immune-mediated toxicities; and environmental factors and disease state of a patient can influence their ability to detoxify and/or eliminate RMs.

This chapter is focused on the role of drug metabolism and bioactivation in oxidative stress and adverse drug reactions (ADRs). These main themes are discussed in a general manner and then further explored, as case studies, to demonstrate these principles.

6.2 DRUG METABOLISM AND BIOACTIVATION

Drug metabolism and bioactivation are catalyzed by a range of enzymes that occur naturally in the body and have endogenous substrates. Metabolism is divided into two phases. Phase I involves the functionalization of the drug; functional groups are added or revealed, thereby making the metabolite more water soluble and available for conjugation (phase II metabolism). Phase II metabolism involves conjugation of the phase I metabolite, at the functional groups, rendering them less reactive and more easily eliminated.

6.2.1 Involvement of Cytochrome P450 Enzymes in Bioactivation

The cytochrome P450 (CYP) superfamily of enzymes is responsible for the majority of phase I metabolism and is, therefore, responsible for the majority of RM formation

[2,3]. Within this enzyme superfamily exist a number of isoforms that differ in substrate specificity. Expression of different CYP isoforms varies between species, sex, and individual and can be altered through environmental changes, diet, and disease state [4]. Each organ, in a single individual, will have a different complement of CYP isoforms, making them differentially susceptible to drug-induced toxicity [2]. The CYP enzymes are found predominantly in the liver and most drug biotransformation occurs here, making the liver a frequent target of drug-induced toxicity [5].

Quantitatively, the CYP isoforms in the endoplasmic reticulum are the most important group of enzymes involved in this process. However, products of phase II metabolism can also lead to toxicity [6]. Additionally, non-CYP oxidative enzymes, such as myeloperoxidase and prostaglandin H synthetase, have been implicated in the bioactivation of drugs and other chemicals [7], the metabolites of which are thought to be responsible for observed toxicity, for example, clozapine and agranulocytosis, benzene, and aplastic anemia [8–11].

6.3 REACTIVE METABOLITES OF DRUG MOLECULES AND INITIATION OF TOXICITY

The concept that small organic molecules can undergo bioactivation to electrophiles and free radicals, and elicit toxicity by covalent modification of cellular macromolecules, has its basis in chemical carcinogenicity and the pioneering work of the Millers [12,13], who studied the hepatotoxic effects of *p*-dimethylaminoazobenzene in the rat and found that aminoazo dyes become tightly bound to the protein constituents of liver tissue. The application of such concepts to human drug-induced hepatotoxicity was established through the studies of Brodie, Gillette, and Mitchell on the covalent binding to hepatic proteins of toxic doses (samples were obtained from overdose patients) of the widely used analgesic acetaminophen (APAP) [14,15].

6.3.1 Chemical Nature of Reactive Drug Metabolites

RMs may be broadly classified as either electrophiles or free radicals [16]. In the vast majority of cases, the ultimate reactive species is electrophilic in nature [1], for example, epoxides and quinones. Electrophiles are reactive because they are electron deficient and have either a high positive charge density (hard electrophiles) or a low positive charge density (soft electrophiles) at the electrophilic center [17]. RMs that possess unpaired electrons can be described as free radicals. Free radicals usually abstract a hydrogen atom from other molecules rather than becoming covalently bound; however, they can also add to double bonds [6]. Free radical reactions can be self-propagating: abstraction of a hydrogen atom from a lipid can initiate a chain reaction leading to lipid peroxidation, oxidative stress, or modification of other types of biological molecules by free radicals [7].

6.3.2 Mechanisms Underlying Drug-Induced Toxicity

RMs have the ability to interact with cellular proteins, lipids, and nucleic acids, leading to protein dysfunction, lipid peroxidation, DNA damage, and oxidative stress (Fig. 6.1)

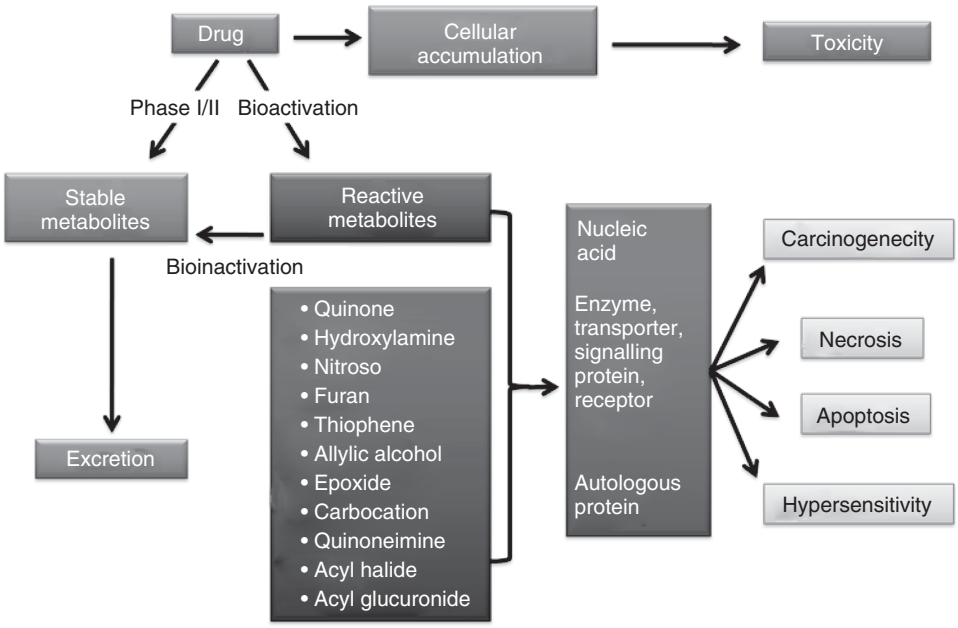

Figure 6.1 Relationship between drug metabolism and toxicity. Toxicity may accrue through accumulation of parent drug or, via metabolic activation, through the formation of a chemically reactive metabolite, which, if not detoxified, can effect covalent modification of biological macromolecules. The identity of the target macromolecule and the functional consequence of its modification will dictate the resulting toxicological response [18]. (See color insert.)

[18]. Additionally, the metabolites may induce disruption of ionic gradients and intracellular calcium stores, resulting in mitochondrial dysfunction and loss of energy production. This impairment of cellular function can result in cell death and possible liver failure [18].

6.4 ADVERSE DRUG REACTIONS

ADRs are defined as a response to a drug that is noxious, unintended, or undesired occurring at doses normally used for the prevention, diagnosis, or treatment of disease [19]. Despite intensive investigation in the fields of chemical toxicology and molecular biology, ADRs remain a major complication of drug therapy [20,21]. ADRs account for a significant number of hospital admissions each year and contribute to patient morbidity and mortality [21]. A pilot study investigating hospital in-patient ADR development found that 19% of patients suffered an ADR, the majority of which were avoidable [22]. In addition, ADRs represent a major issue for pharmaceutical industries, accounting for 30% of compound attrition during the drug development process [23]. Drug toxicity can mimic natural disease and almost any body system can be adversely affected by drugs [24].

6.4.1 Classification of Adverse Drug Reactions

ADRs can be classified as "on-target" or "off-target/idiosyncratic" reactions [25–27]. On-target reactions can be predicted from the known primary or secondary pharmacology of the drug and often represent an exaggeration of the pharmacological effect of the drug. They show simple and clear dose–response relationships and, therefore, can usually be avoided by dose reduction and are only rarely life threatening [26]. In contrast, idiosyncratic adverse reactions cannot be predicted from knowledge of the basic pharmacology of the drug. They are extremely host-dependent and uncommon reactions [28].

Drug–drug interactions can influence both pharmacodynamic (PD) and pharmacokinetic (PK) properties of coadministered drugs. An understanding of the role of DDIs in on-target toxicity requires an understanding of PKPD properties of all coadministered agents. Pharmacodynamic interactions can produce synergistic and additive effects, leading to toxicology. For example, consumption of alcohol while taking antihistamines causing drowsiness can lead to impaired psychomotor skills [29]. PK interactions influence concentration of drug by alteration in absorption, distribution, metabolism, and elimination of one agent by another. Coadministration of a drug with an agent that inhibits its clearance mechanisms leads to an increase in plasma levels, potentially hitting a toxic threshold. The anticoagulant warfarin is metabolized by CYP enzymes in the liver, specifically CYP2C9 and CYP3A4. Therefore, agents that inhibit either of these two enzymes, such as fluconazole [30], will lead to an increase in prothrombin time and possible bleeding.

6.4.2 Idiosyncratic Adverse Drug Reactions

Idiosyncratic drug reactions represent a major problem for the pharmaceutical industry because they add significant uncertainty to the process of drug development and can ultimately lead to drug withdrawal or warnings in drug labeling [31,32]. They are particularly difficult to deal with because they are likely to be discovered late in development or after the drug has been approved; this has important implications as the latter a drug fails, the more expensive is the failure [33,34]. Drug attrition rates are at their highest; the overall success rate for approval of a new chemical entity (NCE) is 11% and with 62% of attrition occurring at phase II clinical trials, the financial cost is huge [32]. During 1975–2000, over 10% of newly approved drugs in the United States were withdrawn or given black-box warnings [31]. In 1991, adverse PK and bioavailability results contributed to a total of 40% of attrition. In 2000, this had been reduced to 10%, but lack of efficacy and safety each contribute to 30% of drug attrition [32].

The exact mechanisms of idiosyncratic drug reactions are still unclear; however, it is believed that some idiosyncratic reactions are initiated by RM [28,35], which bind covalently to macromolecules and either cause direct cell damage or trigger an immune response leading to cell death [34,36,37]. Therefore, the propensity of a NCE to undergo bioactivation needs to be determined at an early stage of the drug development process. Considerable efforts have been made to develop screening systems for RM based on subcellular liver fractions [38,39] or isolated hepatocytes [39]. However, a noninvasive, generic, and quantitative method for assessing bioactivation of drugs in experimental animals, human volunteers, and patients is not currently available.

6.5 ADVERSE DRUG REACTIONS, BIOACTIVATION, AND OXIDATIVE STRESS

It is a widely accepted hypothesis that some idiosyncratic reactions are initiated by RM [28,35], which bind covalently to macromolecules and either cause direct cell damage or trigger an immune response leading to cell death [34,36,37]. Metabolic activation of drug molecules to electrophilic RM can contribute to stress in the cell and interfere with normal cellular homeostasis. Drug-induced oxidative stress can be defined as an imbalance between prooxidants and antioxidants in a cell caused by either parent drug or a reactive intermediate of metabolism [40,41]. Oxidative stress is most commonly mediated by reactive oxygen species (ROS); these include the oxygen free radicals, superoxide anion ($O_2^{-\cdot}$), hydroxyl radical ($HO^{\cdot}$), and nonradical hydrogen peroxide (H_2O_2) [42]. Other species and processes that can mediate oxidative stress include reactive nitrogen species (RNS), RMs of xenobiotics, reduced transition metals, γ-radiation in the presence of oxygen, activated neutrophils, UV light, and by-products of lipid peroxidation [43]. Oxidative stress can lead to cell damage through a number of mechanisms including lipid peroxidation, protein oxidation, DNA oxidation, and mitochondrial damage [44]. Products of oxidative damage can also have prooxidative effects, thereby propagating oxidative damage in the cell [45].

6.5.1 Generation of Reactive Oxygen Species

ROS are generated, in small amounts, in the cell by normal physiological processes [46]. The mitochondria serves as a source of ROS through the electron transport chain, which has redox centers that can leak electrons to molecular oxygen generating $O_2^{-\cdot}$ [47–49]. Peroxisomes contain high amounts of H_2O_2 that can leak out into the cytosol if degradation via catalase is inefficient [50–52]. A number of enzymatic processes can increase the amount of oxidants in the cell, including the CYP monooxygenase system [53]. This enzyme system oxidizes xenobiotics in phase I metabolism; however, incomplete oxidation of a substrate can lead to reduction of O_2 [54]. Electrons can also escape from flavins in the NADPH/P450 reductase enzyme [55]. The cell has an extensive antioxidant defense system that is put into play when an imbalance in pro- and antioxidant species is detected [41,56,57]. These systems can degrade ROS, provide ROS scavengers, prevent/repair oxidation of lipids, and maintain the thiol status of the cell [44].

6.5.2 Cellular Defense against Drug-Induced Oxidative Stress

Once cellular stress has been detected, the cell can activate an immediate adaptive defense response via the upregulation of gene expression that is mediated by transcription factors such as activator protein-1 (AP-1) [58], nuclear factor κ B (NFκB) [59], and nuclear factor erythroid-2 p45-related factor 2 (Nrf2) [60,61]. These are regulatory proteins that often reside in the cytoplasm and translocate to the nucleus on activation where they bind to specific DNA sequences or response elements within gene promoter regions. They can then recruit RNA polymerase and other binding proteins to initiate gene transcription [62]. Activation and translocation of a transcription factor is often oxidant and thiol status sensitive. GSH synthesis is highly inducible on conditions of oxidative stress and GSH depletion. Transcription factor controlling the expression of

enzymes involved in GSH synthesis can be directly activated by electrophilic species that conjugate to GSH depletion as a positive feedback mechanism of the adaptive response [63,64].

Although oxidative stress alone is not enough to kill a cell, all, the ability of RM to bind to cellular macromolecules, initiate oxidative stress, and promote GSH depletion, contribute to damage to key intracellular organelles and can subsequently lead to cell death.

6.5.3 The Role of Glutathione in Cellular Defense

GSH is a tripeptide, derived from the amino acids cysteine, glutamate, and glycine. It is a major endogenous antioxidant within hepatocytes owing to its free thiol group and its relatively high abundance ($\sim$10 mM in hepatocytes). It is present in two forms: reduced (GSH) and oxidized (GSSG). The change in the ratio between the two forms is directly reflective of intracellular redox alterations [65]. GSH homeostasis within hepatocytes is maintained through GSH synthesis and utilization. Synthesis occurs in the cytosol through a two-step ATP consuming reaction. The first step comprises the synthesis of γ-glutamylcysteine. This occurs through the condensation of cysteine and glutamate, catalyzed by γ-glutamylcysteinyl ligase. GSH synthetase then further catalyzes the formation of GSH from γ-glutamylcysteine and glycine [66,67]. It is then used either as an enzyme cosubstrate or as a scavenger molecule in the presence of an oxidized substrate, resulting in the formation of GSSG. Under normal physiological conditions, GSSG can then be recycled back to GSH by the enzyme GSH reductase (Fig. 6.2) [67].

GSH is critical for cellular function and cell survival, playing a major determinant of redox potential. The antioxidant role of GSH is to quench endogenous oxidative species and to overcome exogenous oxidative stress. Increased levels of ROS occur as a result of infection, inflammation, xenobiotics, and intracellular stress. This is potentially hazardous for the cell as ROS can oxidize proteins, lipids, and DNA, causing oxidative damage. GSH also acts as a hydrogen donor for GSH peroxidase. This enzyme is responsible for the subsequent conversion of H_2O_2 to water in parallel with catalase. The level of GSH consequently has a direct impact on the function of GSH peroxidase. GSH peroxidase also plays a role in the repair of lipid peroxidation. Additionally, GSH acts as a scavenger of RNS such as peroxynitrite ($ONOO^-$). An imbalance of endogenous oxidants and antioxidants could result in oxidative stress, emphasizing the crucial role GSH plays in cellular defense. Furthermore, GSH can act as a cofactor substrate in conjugation reactions of xenobiotics under the catalysis of GSH transferases. This acts as a detoxifying mechanism enabling the exportation of drugs and xenobiotics through the multidrug-resistance-associated transporter family [67]. As GSH plays a fundamental role in cellular defense, perturbation of cellular levels can to lead to oxidative stress and/or potential irreversible binding of RMs to cellular macromolecules, resulting in direct cell toxicity or an immunological reaction.

6.5.4 Reactive Metabolites and Mechanisms and Consequences of Oxidative Stress

The formation of RM through bioactivation of drug molecules can push the balance toward a more oxidative environment in the cell by stimulating overproduction of ROS,

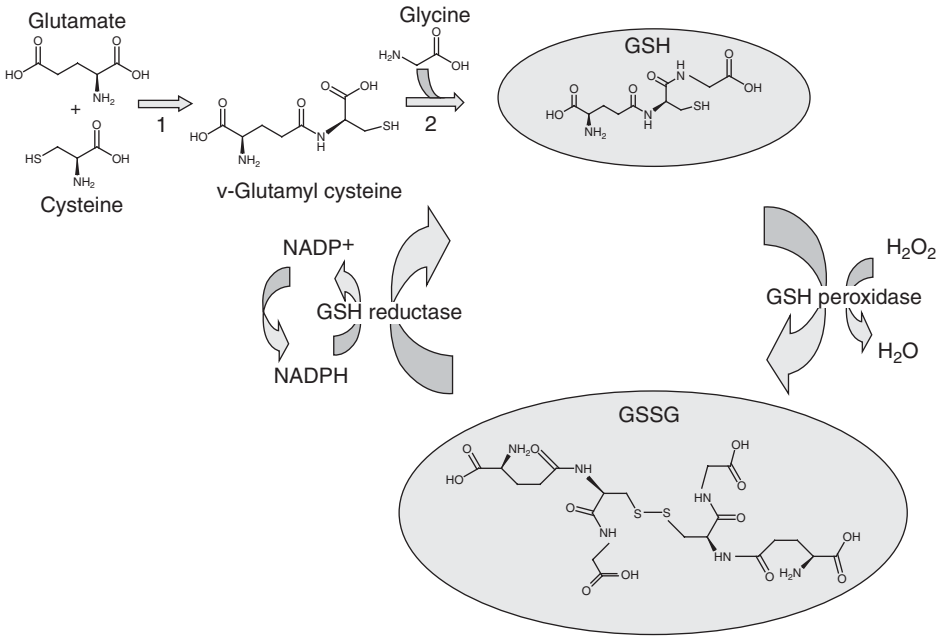

Figure 6.2 Enzyme regulation of GSH synthesis and recycling pathway. GSH is synthesized by a two-step ATP-dependent pathway. γ-Glutamylcysteine is formed from L-glutamate and cysteine, which is catalyzed by Glutamylcysteine ligase γ-GCL (1). Glycine is then added at the C-terminal of γ-glutamylcysteine through the catalysis of GSH synthetase (2). Redox cycling between GSH and GSSG is mediated by GSH reductase and GSH peroxidase [68]. *Source*: Adapted from DeLeve *et al*., 1991.

introduction of electrophilic species, and disruption of thiol status. Some mechanisms by which ROS and other prooxidant species can be generated and how they cause oxidative damage to cellular organelles and macromolecules are outlined below; often, these mechanisms propagate and amplify the oxidative insult.

6.5.4.1 Redox Cycling of Drug Metabolites and Their Role in ROS Generation. RM of xenobiotics can increase ROS by redox cycling, as in the classic case of paraquat [69,70]. Oxidative stress arises when the xenobiotic is reduced by reductases to a semiquinone radical that reduces molecular oxygen to superoxide radicals and reforms the drug or metabolite; repeated reduction and oxidation of a xenobiotic can generate cytotoxic levels of hydrogen peroxide and GSSG [44,71]. Structures capable of redox cycling include catechols and other quinone compounds, iron chelates, and aromatic nitro compounds [72]. Iron chelators, administered in cases of iron overload following blood transfusion, are associated with redox-cycling-mediated toxicity [73]. Drug-induced redox cycling is often associated with quinone moieties in the drug molecules. Cytotoxicity of quinones has been attributed to free radical generation and to arylation of cellular nucleophiles. For redox-cycling quinones, cell injury is associated with mitochondrial permeability transition, whereas arylating quinones directly depolarize the mitochondrial membrane and deplete ATP [74]. The quinone containing anticancer agent doxorubicin (Adriamycin) induced a dose-limiting cardiotoxicity by

inducing reactive species in the heart via redox cycling of the drug at complex I of the electron transport chain [75]. Diclofenac (DCF) quinone-imine-related redox cycling has been implicated in DCF-induced hepatotoxicity [76]. Redox cycling can instigate lipid peroxidation through the generation of ROS.

6.5.4.2 Role of Lipid Peroxidation in Generation and Propagation of ROS and Involvement in Cellular Damage. RM can initiate lipid peroxidation either directly if the RM is electrophilic or indirectly by increasing the generation of free radicals [16,77]. Lipid peroxidation can damage biological membranes [78], leading to further damage to the cell through increased permeability of the plasma membrane or the membranes of organelles [79]. Hepatotoxic mechanisms of carbon tetrachloride are thought to be mediated through lipid peroxidation and disruption of cellular homeostasis, which can lead to disruption of the cytoskeleton, cell signaling, and gene expression pathways [80,81]. Incubation of paraquat with liver microsomes or a system containing NADPH-cytochrome *c* reductase, NADPH, and microsomal lipid greatly increases the formation of malonaldehyde; the amount of malonaldehyde formed in these studies was dependent on the concentration of paraquat in the incubation mixture [82,83]. Oxidative stress has been implicated in the hepatotoxicity associated with the aromatic antiepileptic drugs, carbamazepine, phenytoin, and phenobarbital, as their metabolites increased malondialdehyde levels, oxidation of cardiolipin, oxidation of sulfhydryl proteins, and alteration of the cellular redox status [84].

6.5.4.3 Mitochondrial Dysfunction as a Consequence of Drug-induced Oxidative Stress. Products of lipid peroxidation, ROS, and electrophilic RMs can disrupt mitochondrial function through membrane damage, uncoupling oxidative phosphorylation [85,86] and critical protein oxidation [87], leading to depletion of cellular ATP levels that could ultimately lead to apoptosis and necrosis [49]. The anti-HIV drug class of nucleoside reverse transcriptase inhibitors (NRTIs), used to inhibit viral replication in host cells, induces a variety of serious ADRs including lactic acidosis and cardio and skeletal myopathies [88]. These prodrugs are phosphorylated into the active state and are believed to induce ADR though mitochondrial dysfunction and altered mitochondrial DNA [88]. Nefazodone is an antidepressant that was withdrawn from the market in 2004 owing to severe cases of hepatotoxicity resulting in 1 case of liver failure resulting in death or transplant per 250,000–300,000 patient years. The effect of nefazodone on the mitochondria and resulting hepatotoxicity has been investigated in isolated oxidative phosphorylation (OXPHOS) complexes, HepG2 cells, and sandwich human hepatocytes [89], in which it has been demonstrated that in isolated OXPHOS complexes nefazodone inhibits complex I.

6.5.4.4 Glutathione and Thiol Status Disruption in Drug-Induced Oxidative Stress. As discussed previously, drug metabolites are generally electrophilic species and can form GSH conjugates either enzymatically or nonenzymatically [90]. During alular GSH depletion, such as that seen during high RM exposure, other thiol groups, especially those on critical proteins, can become adducted or vulnerable to oxidation, cross-linking, and formation of mixed disulfides. APAP provides a good example of how thiol status is critical for the successful handling of electrophilic drug intermediates (Section 6.7.1).

6.5.4.5 Irreversible Binding of Reactive Metabolites to Cellular Macromolecules. RMs usually have a low electron density (electrophilic) and as a result are capable of reacting with nucleophiles, molecular centers of high electron density. Nucleophilic targets are dependent on whether the electrophile is chemically defined as hard or soft. Hard electrophiles tend to react with nucleophilic N, O, and C in biological molecules. Hard electrophiles are likely to be genotoxic as they have the potential to react with DNA, forming DNA adducts [91]. This acts as the initiatory stage of carcinogenesis when the mechanism is genotoxic. Aflatoxin B_1 is a genotoxic carcinogen, which is bioactivated to 8,9 epoxide metabolite, which is then able to bind to DNA, forming B_1-N_7-guanine adducts [92,93]. This emphasizes both the importance of drug bioactivation and covalent binding in aflatoxin B_1 causation of drug-induced liver injury (DILI).

Soft electrophiles react more readily with sulfur and target cellular protein. Target proteins for adduction usually contain strong nucleophilic sites such as cysteine thiols, lysine amines, histidine imidazoles, and protein N-terminal amines [94–96]. As discussed previously, GSH plays a crucial role in protecting the thiol status of the cell by quenching endogenous oxidative species such as some drug RMs. However, in some cases, RM can overwhelm the GSH store and cellular GSH levels can become depleted, as in the case of APAP. The depletion of hepatic GSH has been shown to be an obligatory step to enable *N*-acetyl-*p*-benzoquinone imine (NAPQI) to covalently modify and potentially inhibit the function of a number of critical proteins within cells, leading to cell death and has been discussed previously [97]. A number of therapeutic drugs have been reported to undergo irreversible binding to various target proteins despite having different chemical structures and pharmacological actions. Metabolic bioactivation is usually a prerequisite, resulting in the formation of RMs, which are able to bind to proteins by nucleophilic substitution and/or Schiff base formation [98–100]. Irreversible binding with cellular protein has been well documented in retrospective association with drug toxicity, with a number of drugs incriminated, including APAP, amodiaquine, and clozapine [101–103]. However, protein adduct formation does not always lead to toxicity. This concept is best illustrated by APAP and its structural isomer 3′-hydroxyacetanilide (*N*-acetyl-*m*-aminophenol, 3-acetamidophenol; AMAP), where AMAP at the same molar dose as APAP was found to covalently bind to cellular protein and yet did not result in toxicity. The RM of APAP and AMAP, a quinone imine (NAPQI) and quinone, respectively, are chemically similar species, yet there is an intrinsic difference between them that accounts for the difference in ability to cause hepatotoxicity [104]. This introduced the concept of the critical protein hypothesis, delineating certain proteins as critical targets in hepatotoxicity [18,105]. Supporting this, the RM of APAP, NAPQI binds to a number of proteins. In mice, one of the major targets of NAPQI is urate oxidase, an enzyme lost during primate evolution. As a result, it is thought unlikely that irreversible binding to urate oxidase represents a role in APAP-induced hepatotoxicity in mice, as both humans and mice develop centrilobular necrosis with similar per kilogram doses [106]. This highlights the need to identify the critical protein adducts involved in the mechanism of DILI.

Drug-protein adducts can act as neoantigens and induce immune-mediated toxicity. Halothane is the best-studied drug with respect to immunoallergic hepatitis. A significant proportion of patients exposed to this inhalation anesthetic develop asymptomatic rises in transaminases. Fulminant irreversible hepatitis is a rare but life-threatening phenomenon. Most of the patients recorded in the literature with immunoallergic hepatitis had more than one exposure [107]. Antibodies have been detected in such patients

that recognize autoantigens and neoantigens created by trifluoroacetylation of hepatic proteins. Preincubation of halothane pretreated, but not control, rabbit hepatocytes with sera from patients with halothane-induced fulminant hepatic failure rendered the hepatocytes susceptible to the cytotoxic effects of normal lymphocytes *in vitro* [108]. This phenomenon was not observed in control hepatocytes. It is, thus, likely that drug-specific T cells may play a role in the pathogenesis of hepatocyte injury, but direct evidence for this lacks.

Halothane is metabolized by CYP2E1, such as APAP, but forms a chemically reactive acyl halide and bioactivation to the acyl halide is substantial as it is the only metabolic route available. It is hypothesized that the formation of the acyl halide triggers an immune response. Evidence for this concept of an immune trigger is both direct and indirect, with drug-specific antibodies showing that the drug has initiated an immune response in affected patients and newer metabolically inert inhaled anesthetics, such as enflurane and isoflurane, being rarely associated with hepatotoxicity (Fig. 6.3). Target proteins modified by the acyl halide have been identified with the principal chemical modification trifluoroacetylation of lysine residue [109–112]. Chemical modification of protein(s) is associated with the immune response observed with halothane hepatitis; however, there is no conclusive evidence that the antibodies mediate liver injury. Antidrug antibodies have also been observed with idiosyncratic drug

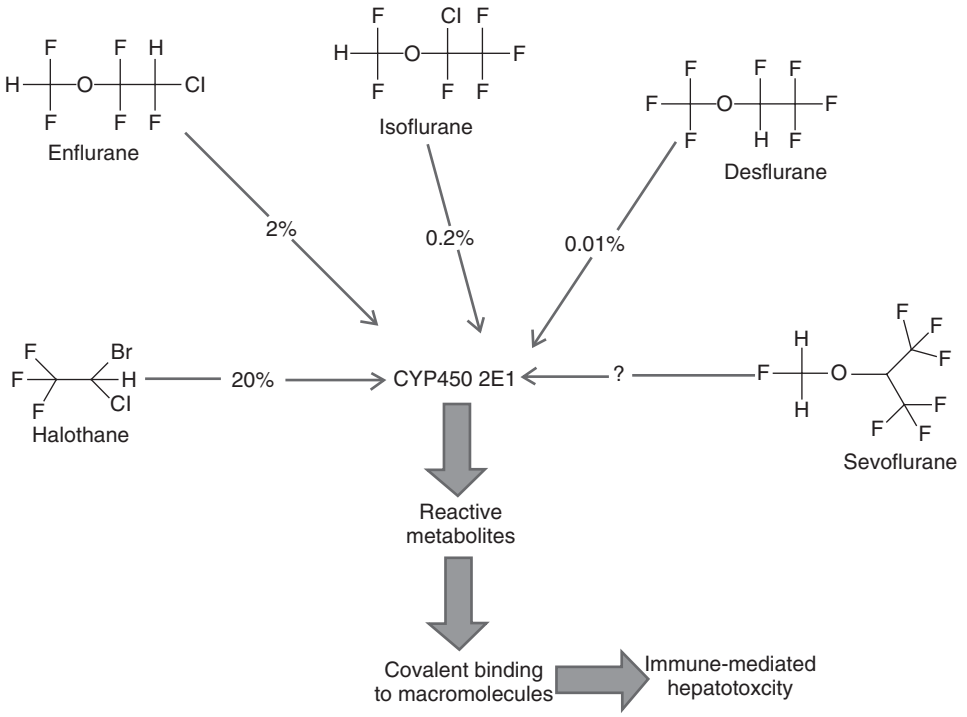

Figure 6.3 Metabolic bioactivation of inhaled anesthetics potential of new inhaled anesthetics to cause immune-mediated hepatotoxicity in man correlates with extent of metabolic bioactivation and liver protein adduct formation, which can be quantified experimentally (*in vivo*/*in vitro*).

TABLE 6.2 Idiosyncratic Drug Reactions with Evidence of Immune Involvement

Drug Class	Drug Name	Is There Evidence to Support Immune Involvement?
Anesthetic	Halothane	Yes
Analgesic	Diclofenac	Yes/no
	Sulindac	Yes
Antibiotic	Amoxicillin	Yes
	Erythromycins	Yes
	Minocycline	Yes
	Nitrofluantoin	Yes
	Rifampicin	Yes/no
Anticonvulsant	Phenytoin	Yes
	Phenothiazines	Yes
Antiepileptic	Carbamazepine	Yes/no
Antihypertensive	Dihydralazine	Yes
	Methyldopa	Yes
	Tienilic acid	Yes
Antithyroid	Propylthiouracil	Yes
Depression	Tricyclic antidepressants	Yes
Gout	Allopurinol	Yes
Hypertension	ACE inhibitors	Yes

Source: Amended from Ref. 115.

reactions to tienilic acid [113] and dihydralazine [114], but there is equivocal evidence that DILI such as that caused by halothane is immune mediated. Most of the information available is compatible with the hapten hypothesis where the drug undergoes bioactivation in hepatocytes leading to drug–protein conjugate formation in the liver. The current theory suggests that the occurrence of idiosyncratic drug hypersensitivity in man represents interplay between a number of factors, which include the chemistry of the drug, drug metabolism, and the immune system of the patient (Table 6.2). Whether cytokines may be useful biomarkers in such idiosyncratic cases is currently unknown, as is the mechanistic details. However, if the hapten hypothesis is correct it is clear that the inflammatory response induced by chemical insult such as APAP overdose compared to that of idiosyncratic DILI may be very different.

6.6 BIOMARKERS OF DRUG BIOACTIVATION

Clearly, drug metabolism can play an important initiating step in the development of ADRs, and RMs have been correlated with the toxicity of several drugs. Therefore, a major interest in biological and analytical techniques has been to minimize metabolic activation of drug candidates during the drug discovery process [1,116]. A variety of experimental approaches have been developed and applied to early optimization phases through to development stages of lead candidates. These studies are largely based on the discovery and quantitation of drug conjugates with endogenous molecules and *in vitro* trapping agents that serve as indicators of drug safety [117]. By allowing for

improved decision making, these indicators, referred to as *biomarkers*, can significantly improve the efficiency of drug discovery and development. However, these studies are time consuming and, in some cases, may delay the process of drug discovery without sufficient improvement in the safety of NCE.

Generally, chemically RMs are detected and estimated indirectly through the irreversible binding of uncharacterized radiolabeled material to hepatic protein [118] and/or the formation of stable metabolites such as GSH conjugates [119]. Until recently, most assays of RM formation *in vivo* and *in vitro* depended on measuring irreversible binding of radioactivity, using relatively laborious methods of exhaustive solvent extraction [118–120].

6.6.1 Trapping Agents and *In Vitro* Biomarkers of Bioactivation

Most RMs are electrophilic in nature because of the inherent reactivity and instability and can readily be trapped with a suitable nucleophile to form stable adducts; the strategy of trapping these intermediates *in vitro* is widely used [121,122]. The *in vitro* assay most widely used to screen for RMs is liver microsomes supplemented with a NADPH/NADPH regenerating system, as cofactor for CYP450-catalyzed reactions and a trapping agent [116,118]. Table 6.3 lists chemical traps that have been used in microsomal incubations for several reactive intermediates.

Methods of trapping RMs commonly include the use of GSH, GSH-ethyl ester, and *N*-acetyl cysteine (NAC), because of their nucleophilic nature they are able to conjugate spontaneously with electrophilic compounds. The most widely used trapping agent, GSH, contains a free sulfydryl group, a soft nucleophile, that can react directly with soft electrophiles, including quinones, epoxides, nitrenium ions, alkyl halides, Michael acceptors, and so on to form stable GSH adducts [117,118]. GSH is present in a concentration of $\sim$10 mM in the liver and can conjugate readily with metabolites with an electrophilic center, generated through the bioactivation by CYP450 enzymes. Hence, GSH serves as a natural trapping agent for chemically RMs and has been routinely used *in vitro* to screen and evaluate RM formation [119].

β-Mercaptoethanol has been successfully used to trap several RMs generated by microsomes and isolated enzymes. In most cases, where the structure of the intermediate can be deduced, the precursor of the β-mercaptoethanol adduct has been an α, β-unsaturated carbonyl [123], a quinone [124], or a quinone imine [124].

TABLE 6.3 Examples of Reactive Intermediates and Trapping Agents in Microsomal Incubation

Trapping Agents	Reactive Intermediate
GSH, GSH-ethyl ester, mercaptoethanol, cysteine, *N*-acetyl cysteine	Quinones, enones
GSH (ester), mercaptoethanol, cysteine	Thiophenes
Cyanide	Iminium ions
Semicarbazide, methoxylamine	Aldehydes, furans
Lysine	Imides, aryl halides
lysine + cysteine	Furan, epoxide
TEMPO	Free radical trap

GSH-ethyl ester has been used as a more lipophilic thiol [124] and was shown to increase the mass spectrometry (MS) sensitivity by ~10-fold for the detection of RMs [124]. Trapping of free radicals employs spin-trapped reagents, for example, electron paramagnetic resonance spectroscopy in the presence and absence of the spin trap, 5,5-dimethyl-1-pyrroline-*N*-oxide [125]. Iminium ions can be trapped with cyanide as well as detection of the trapped intermediate, and commonly the incorporation of radiolabeled cyanide is used as confirmation of iminium formation [122,126]. Hard electrophiles such as aldehydes are trapped with hard nucleophiles such as semicarbazide [127,128] and methoxylamine [129,130]. Semicarbazide has also been used to identify furan ring opening and thiophene bioactivation.

While the detection of adducts (GSH/NAC/cyano, etc.) early in the drug discovery process provides the information about RM formation, it is absolutely essential that the data need to be placed in proper context before discarding drug candidates associated with this liability. There are numerous examples of drugs that form GSH conjugates in these assays and also exhibit covalent protein binding, but which are not associated with a significant incidence of toxicity. Moreover, toxic drugs that form RMs through non-CYP-mediated bioactivation process will not be detected by the routinely used microsomal assays [131].

6.6.2 Mercapturate (NAC) Conjugate as *In Vivo* Indicators of Drug Bioactivation

A common mechanism for the detoxification of toxic electrophilic compounds occurs via GSH conjugation [16]. The GSH conjugates can be further processed into breakdown products such as mercapturate conjugates (Fig. 6.4) and are excreted in urine [132]. Therefore, mercapturate conjugates are potential biomarkers of bioactivation that are not dependent on cell death to be released into an accessible compartment (e.g., urine) [133]. GSH conjugates are unsuitable biomarkers of metabolic activation *in vivo* because they are only very rarely eliminated in urine and seemingly only at very low concentrations [134]. They have been found as *N*-acetylated derivatives [135].

Human urinary mercapturates have been recognized for many years as particularly useful biomarkers in assessments of human exposure to chemically diverse environmental and biogenic electrophiles [132,136,137]. Recently, through the employment of advanced MS techniques [138], there has been a revival of interest in the bioanalytical possibilities of mercapturates [139,140]. Mercapturate biosynthesis (Fig. 6.4) has been modeled as an interorgan pathway, with the liver serving as the major site of GSH conjugation and the kidney as the primary site for the conversion of GSH conjugates to cysteine conjugates [132,141]; however, in some cases, it can be an entirely intrahepatic process even in rats [142]. GSH adducts of bendamustine are metabolized completely in human liver, only the mercapturates and further metabolites appearing in bile [115]. The cysteine adduct is the sole thioether metabolite of APAP eliminated in human bile, with the APAP cysteine adduct and mercapturate being excreted in urine [143]. Any drug mercapturate produced extrahepatically might also be eliminated in bile: rat liver possesses an efficient mechanism for uptake and biliary excretion of circulating mercapturates [144]. Although the metabolism of GSH adducts can yield numerous S-linked products via modifications of the tripeptide moiety [115,135], as in the case of carbamazepine [145,146] mercapturates are not invariably found among

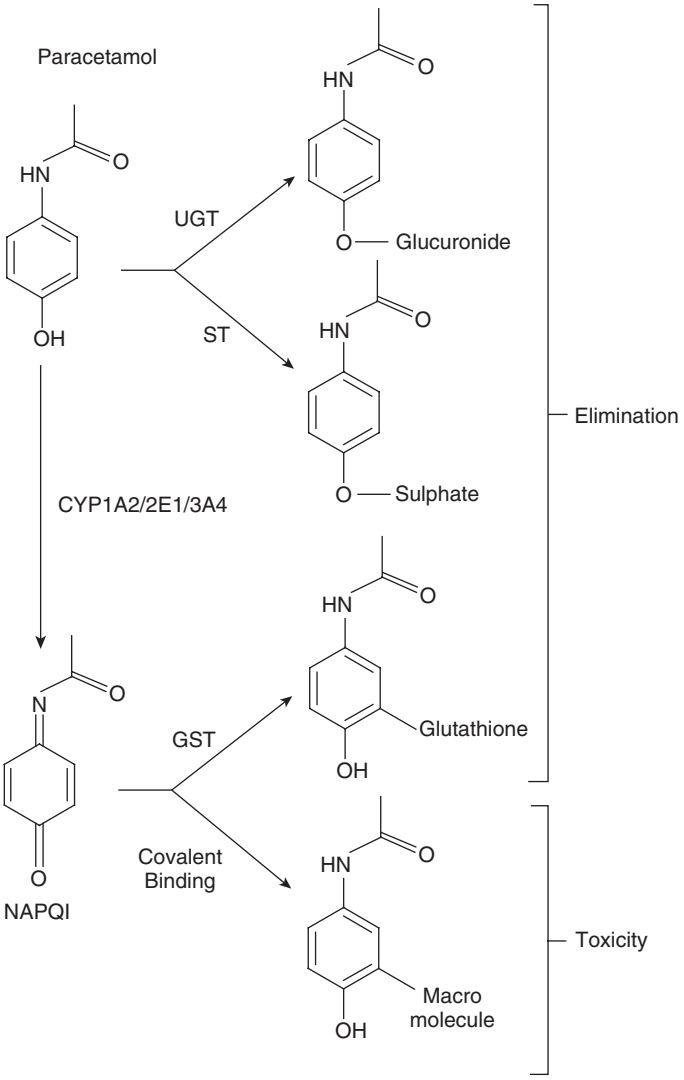

Figure 6.4 Metabolic activation of APAP. APAP is primarily detoxified by glucuronidation and sulfation in the liver. APAP can also undergo conversion to the chemically reactive species *N*-acetyl-*p*-benzoquinone imine (NAPQI) by cytochrome P450. NAPQI can undergo bioinactivation via GSH conjugation, but when GSH stores are depleted, free NAPQI can oxidize and covalently modify proteins resulting in hepatotoxicity. The toxicological and pharmacological properties of the molecule are a function of the redox potential of the molecule [151].

the excreted metabolites, and there is evidence for a species-selective absence of carbamazepine mercapturates from human urine [145,147]. When considered alongside the frequent observation that a compound can be metabolized to several GSH adducts [145,146,148–150], these findings indicate that a careful analysis of a drug's metabolites is necessary to identify a thioether derivative suitable for use as a biomarker of metabolic activation.

S-Phenyl mercapturic acid and *N*-methylcarbamoyl mercapturic acids have been proposed by American Conference of Governmental Industrial Hygienists (ACGIH) [152] as biomarkers for purposes of evaluating exposure to benzene and *N*, *N*-dimethylformamide, respectively. Among the human pharmaceuticals the mercapturate conjugates (Fig. 6.5) of APAP [143], phenacetin [153], felbamate [154], and valproic acid [155] have been used for the quantitative assessment of *in vivo* bioactivation.

6.7 CASE STUDIES

6.7.1 Acetaminophen

6.7.1.1 The Role of Metabolism in APAP Hepatotoxicity. APAP is a commonly used analgesic and antipyretic, which is safe at therapeutic doses (4 g/day). The pharmacological activity is thought to be attributed to the inhibition of cyclooxygenase activity and a reduction in prostaglandin synthesis [156–158]. However, in cases of overdose, acute liver failure develops that is owing to centrilobular hepatic necrosis. To date, APAP hepatotoxicity still remains a major clinical problem and is the single biggest cause of acute liver failure in the United Kingdom and United States [159]. APAP provides an important tool with clinical relevance to study toxicological consequences of drug metabolism across *in vitro*, *in vivo*, and clinical systems.

At therapeutic doses, about 55% and 30% of renally excreted metabolites are as the glucuronide and sulfate conjugates, respectively [160]. A small proportion of the

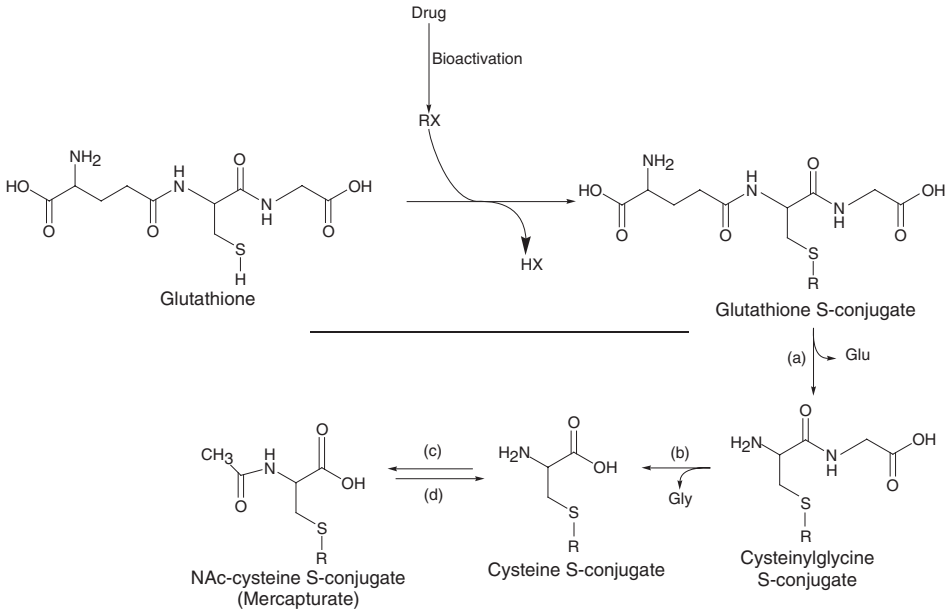

Figure 6.5 Mercapturate biosynthesis pathway. Steps are catalyzed by (a) γ-glutamyltranspeptidase; (b) dipeptidase: cysteinylglycine dipeptidase, and aminopeptidase; (c) cysteine conjugate *N*-acetyltransferase; and (d) *N*-deacetylase [132]. RX, reactive species; Glu, glutamate; and Gly, glycine.

therapeutic dose (5%) is bioactivated primarily by CYP2E1 and also CYP3A4 and CYP1A2 oxidations to the electrophilic intermediate NAPQI [161]. NAPQI is readily detoxified by GSH conjugation and is excreted in the urine as a cysteine or mercapturate product [162].

In cases of overdose, low-capacity sulfation pathways become saturated and a greater fraction of the dose undergoes glucuronidation and oxidation. Under these conditions, NAPQI accumulates and cellular stores of GSH become depleted owing to the shift in NAPQI formation and GSH synthesis (Fig. 6.6) [162–166]. The standard treatment for APAP intoxication is NAC, which replaces hepatic GSH after depletion or conjugates to NAPQI and prevents toxicity, although this is most beneficial if given within 16 h of the overdose.

6.7.1.2 Cellular Consequences of APAP Bioactivation and Bioinactivation. The depletion of hepatic GSH has been shown to be an obligatory step to enable NAPQI to

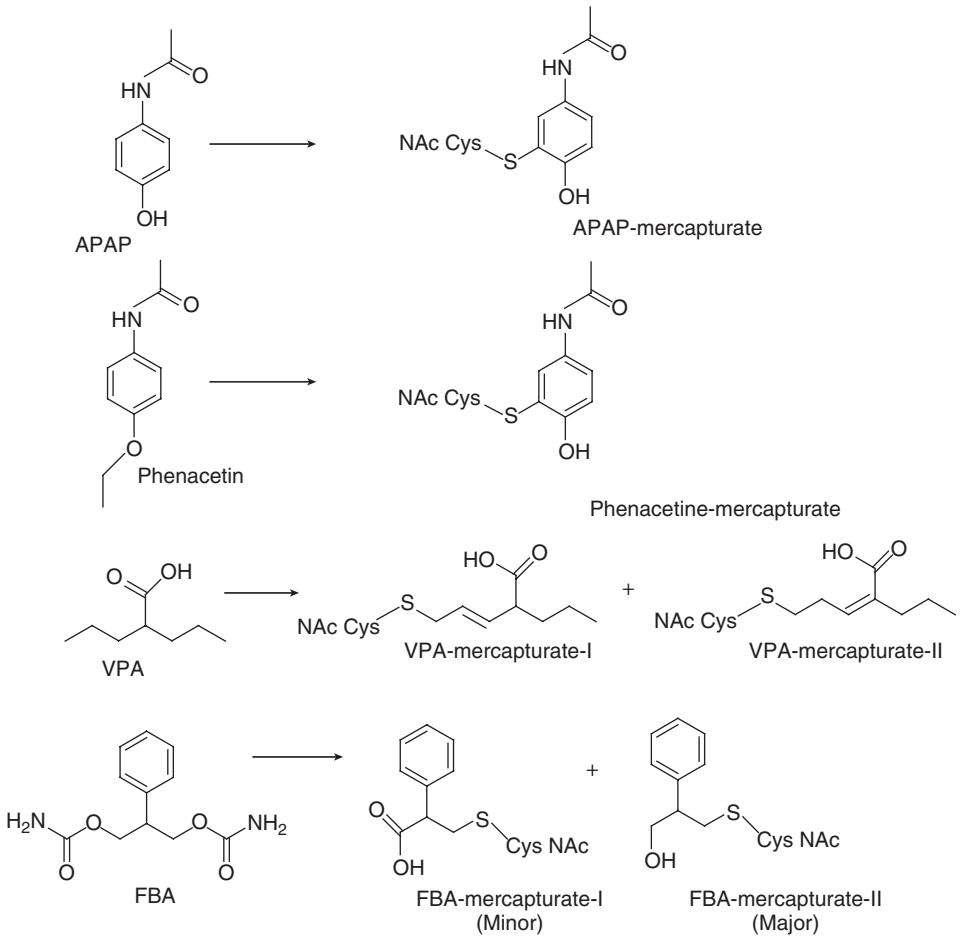

Figure 6.6 Structures of APAP, phenacetin, valproic acid, felbamate, and their mercapturate conjugates. APAP, acetaminophen; VPA, valproic acid; and FBA, felbamate.

covalently modify and potentially inhibit the function of a number of critical proteins within cells, leading to cell death and has been discussed previously [97]. Examples of critical proteins whose function is inhibited by NAPQI modification include γ-glutamylcysteine ligase catalytic subunit [167], glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [168], aldehyde dehydrogenase [169], and Ca^{2+}/Mg^{2+} ATPase [170]. As outlined earlier in the chapter, covalent binding has been hypothesized to contribute to mitochondrial dysfunction, and the resulting disruption in Ca^{2+} and ATP homeostasis is thought to be a critical step in the development of cell death associated with APAP overdose. Moreover, the importance of mitochondrial protein binding in the mechanism of APAP toxicity is highlighted by observations with the nontoxic regioisomer of APAP, AMAP. AMAP undergoes an overall similar level of covalent binding but significantly less covalent binding to mitochondrial protein than APAP [105]. Therefore, the extent of covalent binding and which proteins are modified, rather than covalent binding per se, represent a major factor underlying APAP hepatotoxicity.

The massive chemical stress mediated by an APAP overdose leads to an immediate adaptive defense response in the hepatocyte. This involves various mechanisms, including the nuclear translocation of redox-sensitive transcription factors such as Nrf2, which "sense" chemical danger and orchestrate cell defense. Thus, with respect to APAP, Nrf2-dependent genes of immediate significance are those involved in GSH synthesis such as γ-glutamate cysteine ligase, GSH transferases, glucuronyltransferases, and heme oxygenase. Importantly, it has been observed that nuclear translocation occurs at nontoxic doses of APAP and at time points before overt toxicity [171]. However, with increasing doses of APAP, there is progressive dislocation of nuclear translocation, transcription, translation, and protein activity [167] as the rate of drug bioactivation overwhelms cell defense through the oxidative modification of critical proteins. The cytoplasmic protein Kelch-like ECH-associated protein 1 (Keap1) directly binds to Nrf2 and represses transcription by promoting proteasome-dependent degradation of the protein. It has also been demonstrated that Keap1 is an adaptor molecule for the ubiquitin ligase complex and directs the rapid degradation of Nrf2. When cells are exposed to electrophiles, Nrf2 is liberated from the Keap1-mediated degradation process and accumulates in the nucleus to activate antioxidant response element (ARE)-mediated gene transcription [172]. Somatic disruption of the *Keap1* gene does not interfere with the development of the morphological and physiological integrity of the liver. However, specific knockout of the *Keap1* gene in mouse hepatocytes confers a strong resistance to drug-induced toxicity [172]. This would indicate that the constitutive activation of Nrf2 and concomitantly its target genes is advantageous for mice to overcome xenobiotic toxicity.

6.7.1.3 Inflammation in an Acetaminophen Model of Drug-Induced Liver Injury. A widely studied model of DILI has been acetaminophen-induced liver injury (AILI) in mice (Table 6.4). Evidence suggests that the initial hepatocellular damage caused by NAPQI may lead to the release of cellular contents known as *damage-associated molecular patterns*. These include High mobility group box protein 1 (HMGB1) and the heat shock proteins that act as signals that can activate cells of the innate immune system, which reside in the liver. This, in turn, leads to hepatic infiltration of inflammatory cells that may contribute to the progression of liver injury by producing proinflammatory mediators such as cytokines and chemokines as well as reactive oxygen and nitrogen

TABLE 6.4 Summary of Inflammatory Models Used in APAP-Induced Hepatotoxicity

References	Inflammatory Model(s)	Reported Effect
100,101	KC depletion	Protection against liver injury at early time points, more susceptible at later time points
114	Neutrophil depletion	Significantly protected against liver injury
102	NK/NKT cell depletion	Significantly protected against liver injury
107	Anti-TNF-α/IL-1-α/IL-1ra	Increased susceptibility to liver injury
106	Anti-TNF-α, TNF-p55 receptor $1^{-/-}$ mice	Reduced mortality and liver injury
122	TNFR1$^{-/-}$ mice	Increased susceptibility to liver injury
109	IFN-$\gamma^{-/-}$ mice	Significantly protected against liver injury
119	IL-6$^{-/-}$ mice	Increased susceptibility to liver injury
110	IL-10/4/6$^{-/-}$ mice	Significantly protected against liver injury
	IL-10/4$^{-/-}$ mice	Increased susceptibility to liver injury
120	IL-13$^{-/-}$ mice	Increased susceptibility to liver injury
112	Anti-TLR9, TLR9$^{-/-}$, Nalp3$^{-/-}$ mice	Reduced mortality and liver injury
113	Soluble RAGE	Reduced mortality and liver injury
117	LPS	Increased susceptibility to liver injury

species [173]. Neutrophils accumulating in necrotic areas of the liver after APAP overdose were first recognized by Mitchell *et al*., but the relevance of this accumulation to the pathophysiology is still unclear [174]. A study in C3Heb/FeJ mice revealed that neutrophils accumulate in the liver shortly after hepatic injury, but there was no evidence of neutrophil activation. Additionally, antibodies targeted at these cells did not protect against AILI [175]. In a rat model of AILI, a significant reduction in neutrophil accumulation showed no protection of liver injury [176]. These findings are in contrast to other models in which neutrophils are involved in the course of injury where pretreatment with an antineutrophil antiserum attenuated AILI in rats [177].

Other studies have demonstrated that mice treated with gadolinium chloride to inactivate Kupffer cells (KCs) [178,179] and liposome/clodronate to deplete KCs [179,180] are protected against APAP toxicity. Nevertheless, reports suggest that KC depletion confers protection at early times after APAP treatment [180] but can lead to more severe injury at later time points [179]. The use of an anti-NK1.1 monoclonal antibody to deplete natural killer (NK) cells and natural killer T (NKT) cells significantly protected mice from APAP toxicity probably owing to the reduction in the levels of the proinflammatory cytokine IFN-γ [181]. However, as has been observed with KC depletion, there are also conflicting reports suggesting that NK/NKT cells play little

role in the overall outcome of liver injury in the mouse model of APAP hepatotoxicity [182,183]. This has been supported by a recent study that suggested NK and NKT cells only contributed to liver injury when dimethyl sulfoxide was used to solubilize APAP [183]. Given the contradictory data available on neutrophils and KCs, it is clear that more studies are required to determine their precise role in DILI.

The involvement of various proinflammatory mediators such as TNF-α [184–186], IL-1ra [187], and IFN-γ [181,188], in promoting tissue damage have been demonstrated, whereas other cytokines such as IL-10 and IL-4 [189] have been found to be hepatoprotective in models of APAP hepatotoxicity. Receptors involved in promoting an inflammatory response such as IL-1 receptor [186], p55 TNF-α receptor-1 [185], Toll-like receptors TLR4 and -9 [190,191], and receptor for advanced glycation end products (RAGEs) [192] have been found to be important in promoting an inflammatory response and subsequent pathogenesis.

6.7.1.4 Comparison of the Inflammatory Response in Animal Models of Acetaminophen-Induced Liver Injury. Several different models of DILI in which inflammation has been modulated are being investigated in an attempt to dissect the processes of inflammation from those on DILI. NK and NKT cells were found to play a critical role in the severity and progression of AILI (500 mg/kg, i.p.) by secretion of cytokines and recruitment of inflammatory cells (mainly neutrophils) into the liver [181,193]. An increase of hepatic neutrophil accumulation as early as 4–6 h with a significant threefold increase by 24 h was observed in APAP-treated mice with an associated increase in mRNA levels for cytokines TNF-α and IFN-γ [181]. Depletion of NK and NKT cells (using an anti-NK/NKT cell antibody or an antineutrophil antibody) inhibited liver injury with markedly reduced accumulation of neutrophils in the liver. Subsequent depletion of neutrophils also significantly protected mice against AILI. However, in a recently published fed CD-1 mouse model of AILI, no evidence of hepatic inflammatory cell infiltration was observed over a 24 h time course [27].

Nontoxic doses of APAP (100–400 mg/kg, i.p.) administered 2 h after a dose of Lipopolysaccharide LPS (44×10^6 EU/kg) to fasted C57BL6 mice have been shown to sensitize animals to AILI [194]. Application of LPS led to significantly greater TNF-α production than APAP alone. With APAP treatment alone there was a modest, sustained accumulation of neutrophils from 3 to 24 h after 300 mg/kg APAP. However, neutrophils were not apparent in livers of mice treated with a nontoxic dose of APAP (175 mg/kg), but significant accumulation occurred when this dose was coadministered with a noninjurious dose of LPS. Two hours after treatment with LPS (0 h after APAP), serum TNF-α levels were large and rapidly decreased thereafter, confirming that the inflammatory response observed in this model was due to LPS [194]. These observations support the hypothesis that inflammation induced by LPS and associated elevated levels of TNF-α increase sensitivity to AILI.

Fasted transgenic C57BL6 mice lacking anti-inflammatory mediators such as IL-10, IL-4, IL-6, migration inhibitory factor (MIF), and IL-13 dosed with APAP (200–300 mg/kg, i.p.) displayed increased susceptibility to AILI [189,,195–197]. Neutrophils were found to accumulate to a greater extent in IL-13 knockout (KO) mice than wild type (WT) treated with APAP with the level of TNF-α, IL-6, and IFN-γ increased at 4 h in WT and IL-13 KO APAP-treated animals compared to saline-treated controls [197]. These data suggest a hepatoprotective role for anti-inflammatory mediators in these models and that the balance between the Th1 and Th2 response

is an important determinant for AILI. To test this hypothesis, susceptibility to AILI was compared between two mouse strains, C57BL6 (more susceptible to AILI and that develop predominantly a Th1 response) and BALB/c (less susceptible to AILI and that develop a Th2 response). About 24 h after exposure to APAP C57BL6 mice produce high levels of TNF-α (proinflammatory), whereas levels in BALB/c mice show no difference. On the other hand, IL-6 (anti-inflammatory) levels were higher in BALB/c than C57BL6 24 h after administration. These data support the hypothesis that AILI is associated with a Th1-dominant response in Th1/Th2 cytokine balance and TNF-α may play a role in toxicity [196].

However, the role of TNF-α remains controversial with contradictory reports, suggesting that TNF-α is involved in pathogenesis of DILI [185,186]; it is not involved in toxicity [27,184,185,187,189–198]; or it has a role in defense against toxicity and repair [199,200]. Data suggest that as most cytokines the effects of TNF-α in liver toxicity are pleiotropic with other cytokine networks being involved and it is the interplay between these different cytokine cascades that determine the nature of the inflammatory response associated with toxicity. Furthermore, the understanding of the regulatory mechanisms in the context of DILI that control the potential for damage-associated molecular pattern molecules or cytokines to activate immune cells and initiate an inflammatory response require further investigation. For example, recent evidence suggests that the caspase-dependent oxidation of HMGB1 is critical for inhibiting its immune stimulatory potential following apoptosis [27].

As well as APAP, other model hepatotoxins have been used to investigate the role inflammation plays during DILI (Table 6.5). Given the complexity of interplay between chemically mediated toxicity and inflammation and the differences observed between strains of mice, species, and *in vivo* model pretreatment, further investigation is required to define the precise role a cytokine has in response to the chemical insult or as part of the inflammatory response. Once this has been defined, then the value of the cytokine as a biomarker can be assessed.

6.7.1.5 APAP Toxicity and Circadian Rhythm. Susceptibility to AILI has been reported to follow a circadian rhythm [201,202]. Rodents administered with APAP at the start of the circadian light phase (early subjective day) were more protected than those dosed at the start of the dark phase (early subjective night). This temporal difference in hepatotoxicity can be explained by concomitant changes in drug disposition. Hepatic GSH content fluctuates over a 24-h cycle linked closely with feeding patterns. In rodents, GSH abundance peaks at early morning and reaches a nadir at early night. This reduction in GSH lowers the potential for the liver to bioinactivate NAPQI and allows the chemically RM to cause extensive damage as defined by serum biomarkers

TABLE 6.5 Inflammation in Other Models of DILI

DILI Model	Inflammatory Factors Investigated	References
LPS	Neutrophils, TNF-α, Kupffer cells	124–126
EtOH	Kupffer cells, TNF-α	127–130
Halothane	Macrophage inhibitory factor	118
Cocaine + LPS	Kupffer cells, nitric oxide	131,132
Galactosamine + LPS	TNF-α, TNF-p55 receptor	133,134

of DILI. Moreover, it has also been shown that the bioactivation pathway, Cyp2e1-mediated biotransformation of APAP, does not remain constant over the course of the day. Cyp2e1 has been shown to exhibit a mild oscillation with an increased expression at early night. The molecular mechanism underlying Cyp2e1 oscillation has been suggested to be linked with hepatocyte nuclear factor 1 α (HNF1α) and the biological clock [203], but other mechanisms have been suggested, for example, diurnal rhythms in liver energy status. This parallel of increased bioactivation and reduced bioinactivation over the light phase in the rodent model increases the exposure of the liver to the toxic metabolite and suggests the temporal variation in APAP-induced hepatotoxicity.

6.7.2 Nefazodone

Nefazodone is an antidepressant that was withdrawn from the US market in 2004, following withdrawal in Canada and Europe, owing to incidences of hepatotoxicity. A black-box warning for hepatotoxicity stated that there was one case of liver failure resulting in death or transplant per 250,000–300,000 patient years and more than 20 deaths were reported to the FDA [204]. Patients who presented with nefazodone induced hepatotoxicity did so 1–8 months after commencement of treatment and had been taking a dose of 200–400 mg daily [204–206]. In those patients with acute liver failure, histology demonstrated bile duct proliferation, cholestasis, and centrilobular necrosis [205]. It is still unclear how nefazodone caused hepatotoxicity in man and the three mechanisms currently presented in the literature are discussed below.

6.7.2.1 Reactive Metabolite Formation and Covalent Binding. Nefazodone is predominantly metabolized by CYP3A4 [207]. During metabolism in human and rat liver microsomes, it is bioactivated to several quinone imine and iminium ions that can be trapped with GSH and cyanide, respectively (Fig. 6.7) [39,208]. The major quinone imine is formed from hydroxylation para to the piperazinyl nitrogen that can then undergo two electron addition to form the corresponding quinone imine [208]. The bioactivation of nefazodone *in vitro* has led to the investigation of binding to liver protein as a mechanism of toxicity. It has been shown to covalently bind to human liver protein in microsomes, S9 fraction, and hepatocytes [39,208,209]. In human liver microsomes (HLMs), it has been demonstrated to bind at 1364 pmol/mg protein [209], a level of binding over 20 times that of the 50 pmol/mg threshold, which Evans *et al*. suggested as an upper level of "safe" binding. This binding was significantly reduced to 306 pmol/mg with a mixture of Uridine 5′-diphospho-glucuronic acid (UDPGA) and GSH and 254 pmol/mg in the more complete metabolizing system of hepatocytes. Studies that took into the account the intrinsic clearance also showed that there was a high rate of binding in liver microsomes [210]. Although it has been established that nefazodone covalent binds to liver protein, there is currently no literature that indicates which proteins are the binding targets of nefazodone and whether this binding may contribute to cell death.

6.7.2.2 Role of Bile Salt Export Pump (BSEP) Inhibition in Nefazodone Toxicity. Nefazodone is predominantly excreted in the bile. Approximately 60% of radiolabeled nefazodone that was intravenously administered to dogs was excreted in the feces [211] and in humans 49% of nefazodone was cleared in the urine [212], suggesting that the other route of clearance was through bile. Bile salt export pump (BSEP) is a

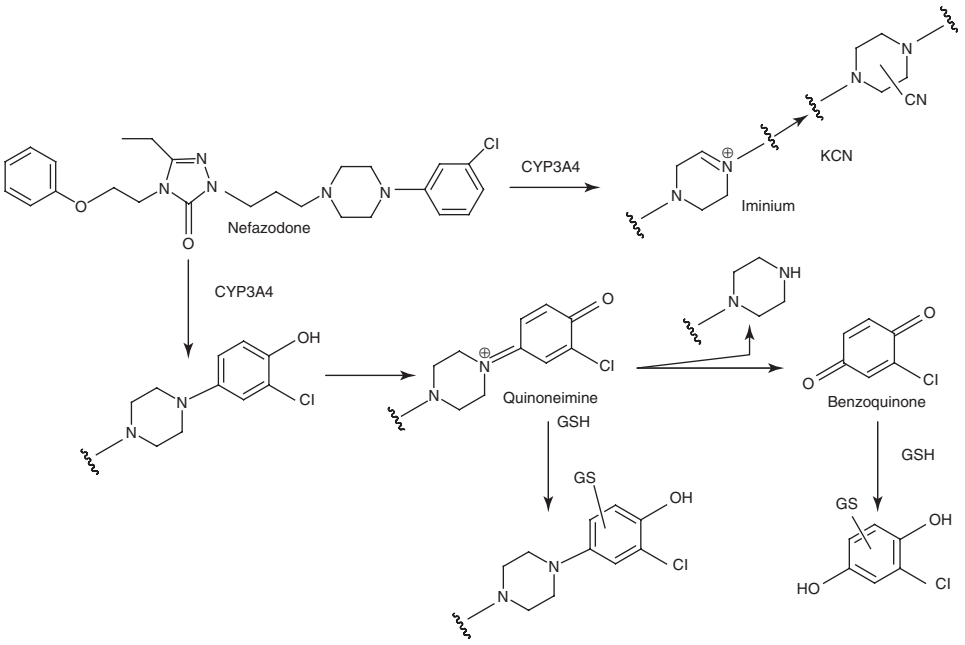

Figure 6.7 Characterized bioactivation pathway of nefazodone in human liver microsomes [122].

member of the ATP-binding cassette transporters and mainly responsible for the transport of bile salts and any other cholephilic compounds across hepatocytes from the blood plasma and into the bile canaliculi [213]. There have been investigations into the inhibition of BSEP as a cause of nefazodone hepatotoxicity [214]. Sandwich human hepatocytes, expressed BSEP in membrane vesicles and rats, were used to investigate the potential for nefazodone to be causing toxicity through inhibition of hepatobiliary transport [214]. Nefazodone caused a concentration-dependent inhibition of taurocholic acid in canaliculi with an $IC_{50} = 14\,\mu M$ [214]. As the hepatocytes were fully capable of metabolism, it was not clear whether metabolites or parent compound was causing the inhibition. This led to the investigation of BSEP-mediated transport inhibition activity in membrane vesicles, which again resulted in concentration-dependent inhibition of taurocholic acid transport with $IC_{50} = 9\,\mu M$ [214]. This suggested that the parent compound of nefazodone was causing the inhibition and not metabolites. The authors also explored the toxicity of nefazodone in hepatocytes using protein synthesis as an indicator of toxicity. Nefazodone produced a concentration-dependent decrease in protein synthesis. The effect of metabolism on toxicity was also considered, the authors used 1-aminobenzotriazole (ABT), a nonspecific inhibitor of CYP, to inhibit the metabolism of nefazodone in hepatocytes [214]. Incubation with nefazodone and ABT for 24 h resulted in a 45% decrease in total protein synthesis. Nefazodone alone resulted in almost full recovery after 24 h. This indicates that the bioactivation and covalent binding of nefazodone may not be the cause for hepatotoxicity.

6.7.2.3 Role of Mitochondrial Dysfunction in Nefazodone Toxicity. Increasingly, mitochondrial toxicity is being implicated in drug-induced organ toxicity. Patients with

underlying mitochondrial diseases may not have any symptoms until additional stress is placed on the mitochondria, such as drug-induced oxidative stress, that results in organ toxicity. As described earlier, the effect that nefazodone has on the mitochondria and the resulting hepatotoxicity has been investigated in isolated OXPHOS complexes, HepG2 cells, and sandwich human hepatocytes [89]. It has been demonstrated that in isolated OXPHOS complexes nefazodone inhibits complex I ($IC_{50} = 14\,\mu M$) [89]. Using culture medium in which the primary energy source, glucose, has been replaced with galactose, HepG2 cells, which are more reliant on oxidative phosphorylation, exhibits 100% depletion of ATP when treated with $200\,\mu M$ nefazodone [89]. A concentration curve in galactose grown HepG2 cells results in an $IC_{50} = 9\,\mu M$ [89]. The HepG2 cells did not express CYP3A4, again suggesting that metabolism is not required for toxicity. Investigations of mitochondrial injury were also carried out in sandwich human hepatocytes. This resulted in significant cell loss, increased ROS, elimination of mitochondrial membrane potential, and depletion of intracellular GSH [89]. Maximum plasma concentrations of $2–4\,\mu M$ has been reported in patients taking 200–400 mg/day, although there is no available data on plasma:liver ratio of nefazodone [215]. However, if nefazodone does inhibit its own excretion as discussed above, then accumulation in the liver could reach levels similar to that seen *in vitro*.

6.7.3 Acyl Glucuronides

Human populations are exposed to numerous chemical entities possessing a carboxylic acid moiety, including nonsteroidal anti-inflammatory drugs (NSAIDs), hypolipidemic drugs (clofibrate), anticonvulsants (valproic acid), and diuretics (furosemide). Several carboxylic acid drugs have been withdrawn from the market over the years as a consequence of rare but occasionally severe adverse effects. From a toxicological perspective, attention has been focused on the bioactivation of this functional group to form RMs, namely, coenzyme A thioesters and acyl glucuronides (AGs) [63], implicating drug bioactivation as an early step in the pathogenesis of ensuing adverse effects. Acyl glucuronidation represents one of the metabolic pathways in the elimination of many carboxylic-acid-containing drugs and metabolites [216], and therefore as a physiological clearance process is potentially important to the pharmacological effects of those compounds. This reaction is catalyzed by multiple human UDP-glucuronosyltransferases (UGTs) [217,218]. Generally, glucuronides are not only less biologically active than the parent aglycone [219] but also subject to more rapid biliary excretion because of their enhanced affinity for transporter proteins [220–225]. However, AGs represent an exception to this accepted principle, possessing intrinsic electrophilic reactivity. This reactivity is manifested through three main pathways: hydrolysis; acyl migration, and irreversible binding with cellular macromolecules. As a number of carboxylated drugs known to form AG metabolites are associated with ADRs in patients, AGs have come to be almost generically implicated in the possible causation of these toxicities. Although bioactivation liabilities connected with oxidative metabolism appear to have caused concern much more frequently than acyl glucuronidation [116] and initiate increasingly sophisticated structural optimization programs to minimize bioactivation [226], medicinal chemists have still derived reassurance from any evidence that an AG metabolite of a new drug is relatively stable [117,227,228]. As a further indication of an established perception of toxicological risk, attempts to connect drug metabolites with the toxicity that has forced the withdrawal

of a compound from development, as in the recent case of torcetrapib [229], still consider the possible involvement of AGs. Torcetrapib, a cholesteryl ester transfer protein inhibitor, was terminated in phase 3 trials because of an unexpected increase in cardiovascular events and death [230]. Administration of torcetrapib was shown to acutely increase blood pressure in both rodent and nonrodent species [230]. Glucuronide conjugates of two major carboxylic acid metabolites can be detected in different species [229]. What may be characterized as a lingering but ill-defined anxiety seems to surround drugs that are metabolized extensively to AGs in humans. Nevertheless, with the notable exception of the acute enteral toxicity of DCF in rats [231], the toxicological implications of AG formation have not been delineated clearly *in vivo*. Certainly, the use of probenecid [232] and the recent developments of bicifadine [233] and deferasirox [234] have continued, despite these drugs being metabolized by humans to unstable AGs.

6.7.3.1 Acyl Glucuronide Reactivity. The reactivity of an AG derives ultimately from the electrophilicity of its ester carbonyl group resulting in hydrolysis to the parent aglycone, intramolecular rearrangement, and intermolecular transacylation reactions. A structure effect on the degree of reactivity has been demonstrated with the rate order: acetic acid > propionic acid > benzoic acid derivatives. It was proposed that this order could be attributed to the inherent electronic and steric properties of each specific aglycone [235]. Consequently, as a number of drugs are associated with AG formation but not necessarily toxicity, the reactivity of the corresponding AGs has been under much investigation with the aim to delineate a relationship between AG reactivity and ensuing toxicity.

1-β-*O*-Acyl glucuronides may undergo intramolecular rearrangement through the migration of the acyl group to positions C-2, C-3, and C-4 of the carbohydrate moiety [236] (Fig. 6.8). The mechanism is thought to involve nucleophilic attack by the hydroxyl group on the adjacent carbon to generate an *o*-acid ester intermediate [237,238]. The intramolecular rearrangement of AGs is of particular importance because it leads to re-exposure of the hemiacetal function of the glucuronic acid moiety, thus allowing two chemical reactions: anomerization and nonenzymatic glycation [239]. Stability and, therefore, reactivity of the AG could potentially have significant implications on the likelihood of a drug causing toxicity.

In addition, 1-β-*O*-acyl glucuronides are particularly susceptible to hydrolysis, resulting in the release of the parent aglycone. A number of catalysts, including β-glucuronidases, nonspecific esterases, serum albumin, and hydroxide ion, can be involved in this reaction. The rate of hydrolysis is dependent on a range of parameters including pH, temperature, and aglycone structure [241]. It has been determined that the rate of hydrolysis, in parallel with acyl migration, can be a relevant parameter to investigate in connection to AG reactivity with cellular macromolecules [242]. An almost linear correlation ($r^2 = 0.995$) between the overall degradation rate (hydrolysis and intramolecular rearrangement) of AGs and the amount of drug bound to Human serum albumin (HSA) *in vitro* was reported for 9AGs. Subsequent investigations of AG reactivity also resulted in similar results with an improved correlation obtained when the fraction of bound drug (as a percentage of initial glucuronide present) was plotted against the degradation rate [235,243]. Exact degradation half-lives are variable across the AGs and is most likely dependent on the structure of the aglycone [244].

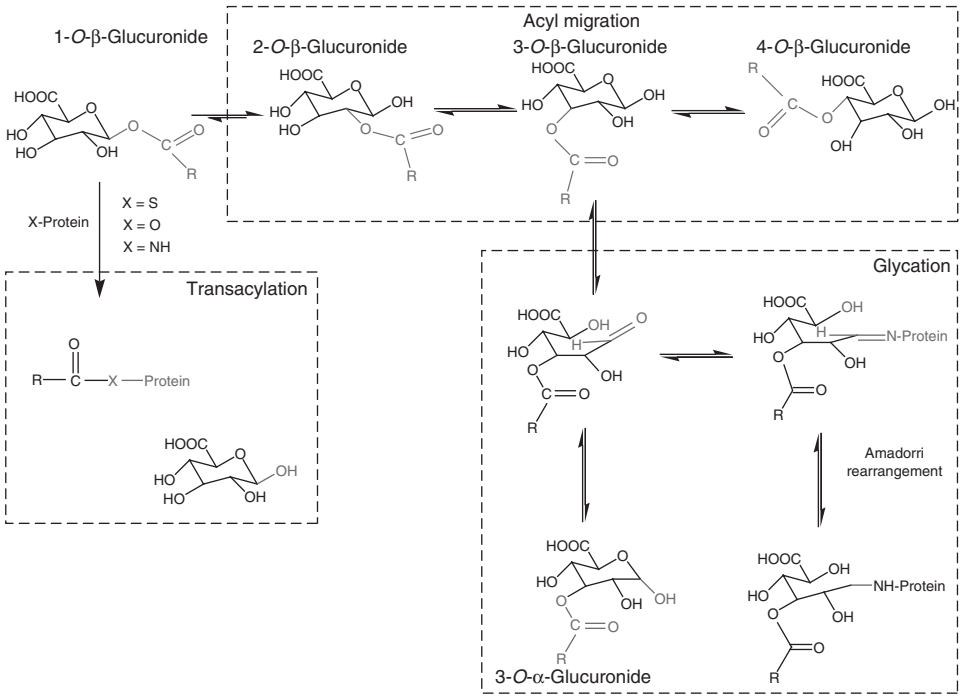

Figure 6.8 Mechanisms of AG rearrangement and covalent binding to protein. Rearrangement of the AG is shown, as the aglycone moves around the ring to the 2-, 3-, and 4-glucuronide positions. An example of rearrangement between the β and α positions is shown by the 3-isomer, with its corresponding covalent binding to protein via the glycation mechanism. This also occurs for the 2- and 4-isomers. Covalent modification of protein by the transacylation pathway is represented by the 1β isomer. $RCOO^-$ represents the aglycone. X represents nucleophilic groups on proteins susceptible to covalent adduct formation from the AG through transacylation. *Source:* Adapted from Bailey *et al*. [240]. (See color insert.)

The electrophilic properties of AGs also enable them to covalently bind to specific nucleophilic sites on biological macromolecules. While this reaction is considered to be a quantitatively a minor pathway of AGs, it has been implicated in the underlying mechanism of toxicity of certain carboxylic-acid-containing drugs such as hypersensitivity reactions [237,,245–247]. Specifically, these immune-mediated reactions are governed by the generation of neoantigens, formed as a result of RMs binding to hepatic protein. These immunogenic adducts are then recognized by the immune system and elicit either antibody [107,173] or cytotoxic T-cell responses [173,245].

Several studies have been carried out to elucidate the mechanism for the formation of protein covalent adducts, with two pathways identified. The first is a transacylation mechanism involving nucleophilic displacement of the glucuronic acid from the ester group by macromolecular nucleophilic sites such as $-NH_2$ of lysine residues [248], −SH of cysteine residues [249], or −OH groups from tyrosine residues [250]. An alternative mechanism reported is the glycation pathway, where the aglycone moiety forms a protein adduct via the glucuronic acid moiety. Specifically, this requires acyl migration before binding, enabling the ring opening of the carbohydrate moiety and subsequent

anomerization. This results in the formation of open-chain aldehyde intermediates and the further condensation of the aldehyde group with the amine groups on lysines or DNA nucleosides to form imines (Schiff bases), which can further undergo rearrangement to a more stable 1-amino-keto-product (Amadori rearrangement) [251–254].

The formation of protein adducts is not only limited to plasma protein alone but can also involve other organs including the liver, kidney, skeletal muscle, and intestine, indicated by a number of animal studies [255,256]. Therefore, the formation of AGs could have implications in specific organ-directed toxicities and also systemic hypersensitivity reactions through both the binding to specific organs and plasma protein. However, the toxicological significance of covalent binding between drug and macromolecule remain speculative and still require definitive proof as protein adduct formation does not always lead to toxicity. This concept is best illustrated by APAP and its structural isomer AMAP, where AMAP at the same molar dose as APAP was found to covalently bind to cellular protein and yet did not result in toxicity. This introduced the concept of the critical protein hypothesis, delineating certain proteins as critical targets in hepatotoxicity [18,105]. Supporting this, the RM of APAP, NAPQI binds to a number of proteins. In mice, one of the major targets of NAPQI is urate oxidase, an enzyme lost during primate evolution. As a result, it is thought unlikely that irreversible binding to urate oxidase represents a role in APAP-induced hepatotoxicity in mice, as both humans and mice develop centrilobular necrosis with similar per kilogram doses [257]. This highlights the need to identify the critical protein adducts involved in the mechanism of toxicity.

Glucuronides of propionic acids have been reported to be more stable and less chemically reactive than those formed from acetic acid NSAIDs, generally possessing a much longer half-life *in vitro* at physiological pH. It is thought that this can most likely be attributed to the steric hindrance and electron donating effects of the α-methyl group [247]. Despite this, Benoxaprofen BNX-AG has been reported to react with HSA *in vitro*, forming protein adducts through both transacylation and glycation pathways [257]. The use of tandem mass spectrometry further enabled the identification of specific binding sites, which included Lys-159 and Lys-199 to a lesser extent. Ser-312, Ser-480, and Arg-222 were the other three major binding sites, which reacted only via nucleophilic displacement.

It is, therefore, well established that AGs are intrinsically reactive electrophiles, with the potential to bind to cellular macromolecules. However, the contribution of AG formation to underlying toxicological mechanisms remains unclear and is consequently under much investigation.

6.7.3.2 Toxicological Implications of Acyl Glucuronides. A number of carboxylate drugs have been found to undergo extensive metabolism to an AG, but with varying degrees of toxicity associated with administration. Specifically, the use of DCF has been associated with a number of adverse effects, the most prominent being induction of hepatic injury [242,,258–261]. It has been reported that 15% develop abnormalities in aminotransferase plasma levels [258,262]. Cases of severe hepatic reactions are much rarer in frequency with estimates ranging from 1–2 cases per million prescriptions [263] to 6–18 cases per 100,000 person years [264]. Published cases of severe DCF hepatotoxicity equate to ~250 reports with a case fatality rate of 10% [265]. The high case numbers can be attributed to the large number of patients who

are prescribed DCF worldwide [76]. The injury associated with DCF-induced hepatotoxicity is consistently hepatocellular, resembling hepatitis, characterized by jaundice, and to varying degrees, fatigue anorexia, nausea, and vomiting [262]. The underlying pathogenesis associated with DCF hepatotoxicity is unknown, but drug bioactivation has been implicated. Consequently, investigations have been undertaken to delineate the importance of AG reactivity and to a lesser degree AG exposure.

The majority of investigations concerning AG adduct formation have focused on binding to HSA, which would explain systemic adverse effects associated with carboxylate drugs. However, certain drugs are associated with specific organ-related toxicity, such as DCF, which represents a paradigm for idiosyncratic hepatotoxicity. Consequently, the focus of investigations has centered on assessment of binding directly to liver macromolecules. In rodents, the majority of adducts were located on the canalicular plasma domain of the hepatocytes [266,267]. In addition, DCF adducts were also identified on the apical domain of enterocytes; however, it has not be conclusively proven that these are as a result of AG formation [268]. DCF adduct formation with enterocytes through acyl glucuronidation has been suggested based on a study by Seitz *et al*. [267]whereby administration of bile containing DCF-AG resulted in intestinal ulcer formation in Multidrug resistance-associated protein 2 MRP2-deficient rats, which did not occur when DCF alone was administered. The ability of DCF-AG to bind to serum albumin has yet to be determined in either *in vitro* or *in vivo*.

6.7.4 Nevirapine

Nevirapine (NVP) is a highly effective nonnucleoside reverse transcriptase inhibitor (nnRTI) used in the treatment of HIV-1 infection. NVP is used widely in developing countries as it is inexpensive and does not require refrigeration; however, it is associated with clinically restrictive side effects, skin reaction and hepatotoxicity that can occur either simultaneously or separately. Toxicity is thought to be mediated through RM formation either directly or through the induction of the immune system. Although NVP is highly effective, the incidence of severe or life-threatening hepatotoxicity has lead to the FDA giving a black-box warning. The nature of NVP-induced liver injury is not clear; cases of hepatotoxicity vary in terms of severity, time to onset, and can occur with or without a hypersensitivity reactions [269,270]. It is possible that the late-occurring reactions may be nonimmune in nature, while those occurring in the first three months may have an immune pathogenesis [271]. The time of onset and the nature of the NVP-induced skin rash suggest that the reaction is immune mediated and because of the low incidence it is likely that the reaction may be idiosyncratic [271,272].

6.7.4.1 Possible Pathways of Nevirapine Bioactivation. There appear to be several pathways for NVP bioactivation (Fig. 6.9). The cyclopropylamine group has the potential, via N-dealkylation, to become bioactivated to an aminium cation radical [120]. 12-OH NVP, a major metabolite in HLMs, is a substrate for sulfotransferase in rats [272], and it has been proposed that the sulfate ester dissociates to form a reactive quinone methide intermediate [272]. The quinone methide could also be generated, additionally or alternatively, by enzymic oxidation [117]. Hydroxyheteroaryl metabolites [273] are potential precursors of reactive quinone imines [117]. Finally, NVP might form one or more heteroarene epoxide intermediates in either of the pyridine

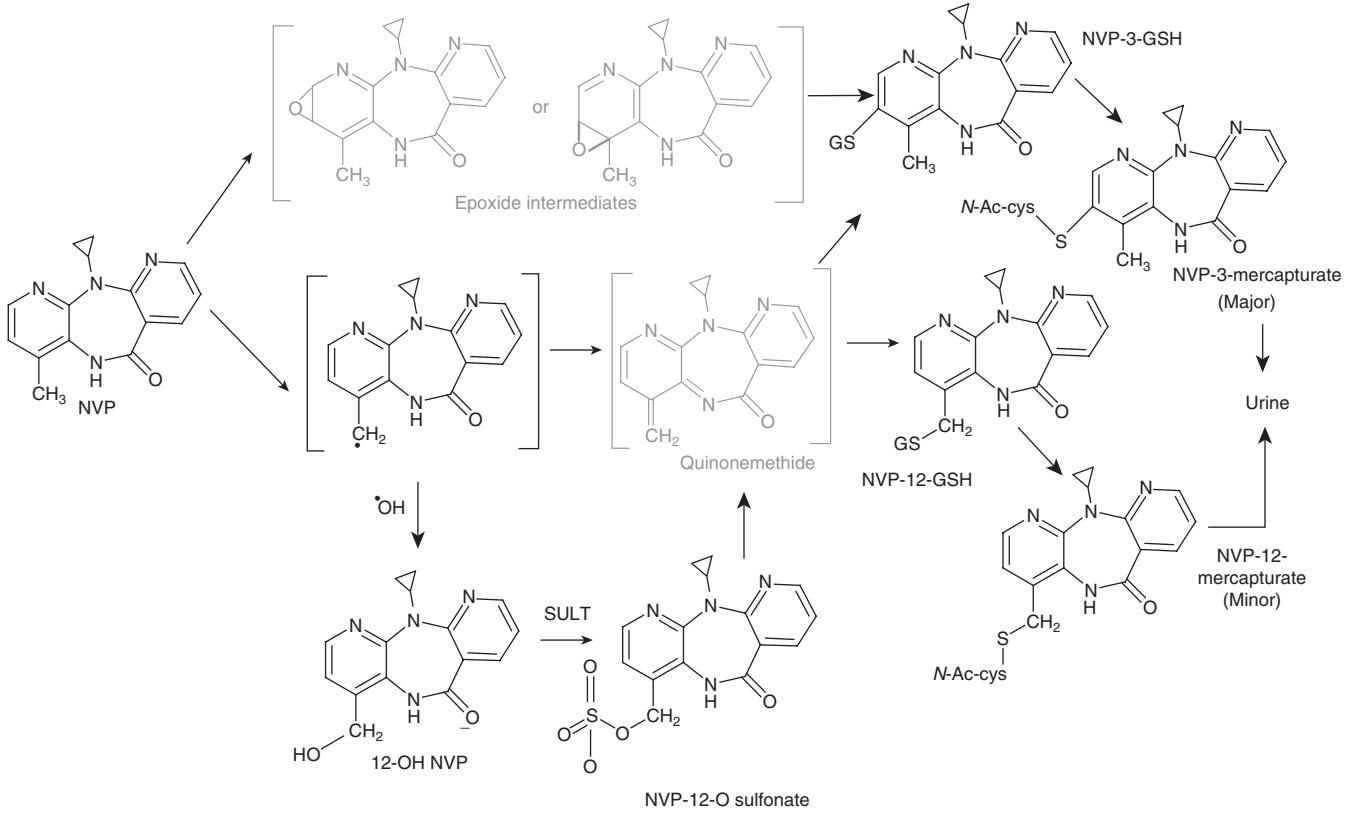

Figure 6.9 Proposed scheme for the bioactivation of NVP and possible reactive metabolites.

rings. Novel NVP GSH conjugates and mercapturates, potentially formed from multiple RMs, have been characterized [274]. Mercapturates were isolated from rat bile and characterized definitively by NMR as thioethers substituted at the C-3 and exocyclic C-12 positions of the methylpyridoring of NVP. It is proposed that NVP undergoes bioactivation to arene oxide and quinone methide intermediates [274]. Further evidence for metabolic activation of NVP includes NADPH-dependent irreversible binding of radiolabeled drug to rat liver microsomes (RLMs) [120], mechanism-based inhibition of microsomal CYP3A4 [117], and the metabolism of NVP by HLM and CYP3A4 to a single GSH adduct [117].

6.7.4.2 Animal Model of Nevirapine-Induced Skin Rash. Uetrecht and colleagues have characterized extensively a dose-dependent, NVP-induced skin rash in female Brown Norway (BN) rats that resembles the idiosyncratic cutaneous reaction seen in humans [275,276] and appears to be immune mediated [277]. The NVP-induced skin rash in BN rats is similar to that in human in terms of: the time of onset, sex dimorphism, the fact that incidence increases with dose, that an escalating dose of NVP can reduce incidence, and that rechallenge in rats and humans results in more severe rashes than on initial exposure [275]. Chen *et al*. [272] have proposed that the skin rash induced by NVP and 12-OH NVP in BN rats may be due to NVP quinone methide formed through dissociation of 12-OH NVP sulfonate in the skin. A mild hepatotoxicity, assessed by histopathological and plasma enzyme indicators, is induced in rats pretreated with NVP or dexamethasone [278]. This implies one or more NVP metabolites are causal agents of hepatotoxicity but no mechanistic explanation of the liver injury has emerged. Chen *et al*. [272] have suggested that the hepatotoxicity of NVP in humans is due to quinone methide formed *in situ* by CYP enzymes.

Evidence suggests that the 12-OH NVP pathway is responsible for NVP-induced skin rash, as administration of 12-OH NVP induces skin rash at 50 mg/kg/day as opposed to 150 mg/kg/day with NVP [272]. Cotreatment with ABT decreased urinary excretion of 2-OH NVP, 3-OH NVP, and 4-COOH NVP while increasing the urinary excretion of 12-OH NVP. The result was the formation of skin rash at 50 mg/kg/day in female BN rats and at 150 mg/kg/day in male BN rats, which normally unaffected [272]. However, on rechallenge, 12-OH NVP failed to elicit a skin rash, even if the initial rash was induced by 12-OH NVP [272]. It may be that NVP is needed to induce the CYP isoforms required for bioactivation to RM.

6.7.4.3 Potential Markers of Nevirapine Bioactivation. Novel NVP GSH conjugates and mercapturates, potentially formed from multiple RMs, have been characterized [274]. The formation of drug-GSH conjugates is often used as an indicator of bioactivation. GSH conjugates are generally excreted in the urine as mercapturates. Drug mercapturates have been used as biomarkers of exposure to an RM [154,155]. The identification of two novel NVP mercapturates in humans and rats and the development of a mass spectrometric assay to quantify them pose the opportunity to develop the mercapturates as biomarkers of NVP-induced skin rash [274]. To justify the use of a NVP mercapturate as a biomarker of exposure to an RM, a direct link between mercapturate formation and adverse event must be confirmed; the female BN rat model of NVP-induced skin rash can be utilized to do this. A direct link between mercapturate formation and skin rash incidence could be proved by using specific CYP450 inhibitors to modulate NVP metabolism.

6.8 SUMMARY POINTS

- There is overwhelming evidence to suggest bioactivation of drugs to chemically RMs plays a major role in toxicity and ADRs.
- Chemically RMs can induce oxidative stress in the cell by introducing electrophilic species, stimulating production of ROS and disrupting thiol status through depletion of GSH.
- The cell can activate an immediate adaptive defense response via the upregulation of gene expression when the cell undergoes oxidative stress.
- If the oxidative insult overwhelms the cell's protective mechanisms cell death can ensue through a number of mechanisms, including mitochondrial dysfunction, DNA damage, protein binding, and induction of the immune response and inflammation.
- As a result of drug attrition occurring late in drug development—one of the main reasons for which is related to safety—we need to be able to detect the ability of a NCE to be bioactivated and whether the reactive intermediate is likely to cause hazards.
- Many pharmaceutical companies have used covalent binding to microsomal proteins as an indicator of a safety liability. However, the degree of covalent binding in microsomes is an indicator of bioactivation, but is not always indicative of toxicity and, hence, is poorly translated into safety outcomes in the clinic covalent binding. Work is currently ongoing to develop relevant human hepatic cell lines as an alternative to microsomes with the hope of generating a more physiologically relevant system and improving clinical translation of data.
- The development of clinically relevant biomarkers, which can successfully assess the propensity of a chemical to cause toxicity and will bridge the "disconnect" between preclinical *in vitro* and *in vivo* studies and clinical studies, in terms of successful prediction of safety liability, is crucial.

REFERENCES

1. Kalgutkar AS, Gardner I, Obach RS, *et al*. A comprehensive listing of bioactivation pathways of organic functional groups Curr Drug Metab 2005; 6(3):161–225.
2. Van der Weide J, Steijns LSW. Cytochrome P450 enzyme system: Genetic polymorphisms and impact on clinical pharmacology. Ann Clin Biochem 1999;36(6):722–729.
3. Rooney PH, Telfer C, McFadyen MCE, *et al*. The role of cytochrome P450 in cytotoxic bioactivation: future therapeutic directions. Curr Cancer Drug Targets 2004;4(3):257–265.
4. Pelkonen O, Raunio H. Metabolic activation of toxins: tissue-specific expression and metabolism in target organs. Environ Health Perspect 1997;105,Suppl 4:767–774.
5. Uetrecht JP. The role of leukocyte-generated reactive metabolities in the pathogenesis of idiosyncratic drug reactions. Drug Metab Rev 1992;24(3):299–366.
6. Rose R, Levi P, Hodgson E. Reactive metabolites: a textbook of modern toxicology. Hoboken (NJ): John Wiley & Sons, Inc.; 2004. pp. 149–160.
7. Uetrecht JP. Myeloperoxidase as a generator of drug free radicals. Biochem Soc Symp 1995;61:163–170.
8. Mason RP, Fischer V. Possible role of free radical formation in drug-induced agranulocytosis. Drug Saf 1992;7,Suppl 1:45–50.

9. Fischer V, Haar JA, Greiner L, *et al*. Possible role of free radical formation in clozapine (clozaril)-induced agranulocytosis. Mol Pharmacol 1991;40(5):846–853.
10. Ross D, Siegel D, Schattenberg DG, *et al*. Cell-specific activation and detoxification of benzene metabolites in mouse and human bone marrow: identification of target cells and a potential role for modulation of apoptosis in benzene toxicity. Environ Health Perspect 1996;104,Suppl 6:1177–1182.
11. Smith MT, Yager JW, Steinmetz KL, *et al*. Peroxidase-dependent metabolism of benzene's phenolic metabolites and its potential role in benzene toxicity and carcinogenicity. Environ Health Perspect 1989;82:23–29.
12. Miller E, Miller J. *in vivo* combinations between carcinogens and tissue constituents and their possible role in carcinogenesis. Cancer Res 1952;12(8):547–556. 0008–5472 (Print); 0008–5472 (Linking).
13. Gelboin HV, Miller JA, Miller EC. The formation *in vitro* of protein-bound derivatives of aminoazo dyes by rat liver and its enhancement by benzypyrene pretreatment. Biochim Biophys Acta 1958;27(C):655–656.
14. Gillette JR, Mitchell JR, Brodie BB. Biochemical mechanisms of drug toxicity. Annu Rev Pharmacol 1974;14(1):271–288.
15. Brodie BB, Reid WD, Cho AK, *et al*. Possible mechanism of liver necrosis caused by aromatic organic compounds. Proc Natl Acad Sci U S A 1971;68(1):160–164.
16. Williams DP, Kitteringham NR, Naisbitt DJ, *et al*. Are chemically reactive metabolites responsible for adverse reactions to drugs. Curr Drug Metab 2002;3(4):351–366.
17. Pearson RG, Songstad J. Application of the principle of hard and soft acids and bases to organic chemistry. J Am Chem Soc 1967;89(8):1827–1836.
18. Park BK, Kitteringham NR, Maggs JL, *et al*. The role of metabolic activation in drug-induced hepatotoxicity. Annu Rev Pharmacol Toxicol 2005;45:177–202.
19. Edwards IR, Aronson JK. Adverse drug reactions: definitions, diagnosis, and management. Lancet 2000;356(9237):1255–1259.
20. Lazarou J, Pomeranz BH, Corey PN. Incidence of adverse drug reactions in hospitalized patients: a meta- analysis of prospective studies. J Am Med Assoc 1998; 279(15):1200–1205.
21. Pirmohamed M, James S, Meakin S, *et al*. Adverse drug reactions as cause of admission to hospital: prospective analysis of 18 820 patients. Br Med J 2004;329(7456):15–19.
22. Davies EC, Green CF, Mottram DR, *et al*. Adverse drug reactions in hospital in-patients: a pilot study. J Clin Pharm Ther 2006;31(4):335–341.
23. Greene N, Naven R. Early toxicity screening strategies. Curr Opin Drug Discov Devel 2009;12(1):90–97.
24. Park BK, Kitteringham NR, Powell H, *et al*. Advances in molecular toxicology - towards understanding idiosyncratic drug toxicity. Toxicology 2000;153(1–3):39–60.
25. Park K. Molecular aspects of adverse drug reactions – from molecule to man. Toxicol Lett 2009;189, Suppl 1:S14–S14.
26. Testa B, Krämer SD. The biochemistry of drug metabolism - an introduction part 5. metabolism and bioactivity. Chem Biodivers 2009;6(5):591–684.
27. Antoine DJ, Mercer AE, Williams DP, *et al*. Mechanism-based bioanalysis and biomarkers for hepatic chemical stress. Xenobiotica 2009;39(8):565–577.
28. Uetrecht J. Idiosyncratic drug reactions: past, present, and future. Chem Res Toxicol 2008;21(1):84–92.
29. Hindmarch I, Bhatti JZ. Psychomotor effects of astemizole and chlorpheniramine, alone and in combination with alcohol. Int Clin Psychopharmacol 1987;2(2):117–119.
30. Kunze KL, Wienkers LC, Thummel KE, *et al*. Warfarin-fluconazole I - inhibition of the human cytochrome P450-dependent metabolism of warfarin by fluconazole: *in vitro* studies. Drug Metab Dispos 1996;24(4):414–421.

31. Lasser KE, Allen PD, Woolhandler SJ, *et al*. Timing of new black box warnings and withdrawals for prescription medications. J Am Med Assoc 2002;287(17):2215–2220.
32. Kola I, Landis J. Can the pharmaceutical industry reduce attrition rates. Nat Rev Drug Discov 2004;3(8):711–715.
33. Uetrecht J. Screening for the potential of a drug candidate to cause idiosyncratic drug reactions. Drug Discov Today 2003;8(18):832–837.
34. Uetrecht J. Evaluation of which reactive metabolite, if any, is responsible for a specific idiosyncratic reaction. Drug Metab Rev 2006;38(4):745–753.
35. Smith DA, Obach RS. Metabolites and safety: what are the concerns, and how should we address them. Chem Res Toxicol 2006;19(12):1570–1579.
36. Williams DP, Park BK. Idiosyncratic toxicity: the role of toxicophores and bioactivation. Drug Discov Today 2003;8(22):1044–1050.
37. Guengerich FP. Cytochrome P450s and other enzymes in drug metabolism and toxicity. AAPS J 2006;8(1):E105–E111.
38. Gan J, Ruan Q, He B, *et al*. *In vitro* screening of 50 highly prescribed drugs for thiol adduct formation - comparison of potential for drug-induced toxicity and extent of adduct formation. Chem Res Toxicol 2009;22(4):690–698.
39. Bauman JN, Kelly JM, Tripathy S, *et al*. Can *in vitro* metabolism-dependent covalent binding data distinguish hepatotoxic from nonhepatotoxic drugs? An analysis using human hepatocytes and liver S-9 fraction. Chem Res Toxicol 2009;22(2):332–340.
40. Kannan K, Jain SK. Oxidative stress and apoptosis. Pathophysiology 2000;7(3):153–163.
41. Sies H. Oxidative stress: from basic research to clinical application. Am J Med 1991;91(3Suppl 3):S31–S38.
42. Halliwell B. Reactive oxygen species and the central nervous system. J Neurochem 1992;59(5):1609–1623.
43. Niki E. Lipid peroxidation products as oxidative stress biomarkers. Biofactors 2008;34(2):171–180.
44. Boelsterli UA. Xenobiotic-induced oxidative stress. Mechanistic toxicology. London: Taylor & Francis; 2003. pp. 93–97, 100–103, 112–119.
45. Cejas P, Casado E, Belda-Iniesta C, *et al*. Implications of oxidative stress and cell membrane lipid peroxidation in human cancer (Spain). Canc Causes Contr 2004; 15(7):707–719.
46. Valko M, Leibfritz D, Moncol J, *et al*. Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol 2007;39(1):44–84.
47. Turrens JF. Mitochondrial formation of reactive oxygen species. J Physiol (Lond) 2003;552(2):335–344.
48. Turrens JF. Superoxide production by the mitochondrial respiratory chain. Biosci Rep 1997;17(1):3–8.
49. Ott M, Gogvadze V, Orrenius S, *et al*. Mitochondria, oxidative stress and cell death. Apoptosis 2007;12(5):913–922.
50. Schrader M, Fahimi HD. Peroxisomes and oxidative stress. Biochim Biophys Acta Mol Cell Res 2006;1763(12):1755–1766.
51. Singh I. Mammalian peroxisomes: metabolism of oxygen and reactive oxygen species. Ann N Y Acad Sci 1996;804:612–627.
52. Boveris A, Oshino N, Chance B. The cellular production of hydrogen peroxide. Biochem J 1972;128(3):617–630.
53. Sies H. Oxidative stress: oxidants and antioxidants. Exp Physiol 1997;82(2):291–295.
54. Gonzalez FJ. Role of cytochromes P450 in chemical toxicity and oxidative stress: studies with CYP2E1. Mutat Res Fund Mol Mech Mutagen 2005;569(1,2):101–110.
55. Schlezinger JJ, White RD, Stegeman JJ. Oxidative inactivation of cytochrome P-450 1A (CYP1A) stimulated by 3,3′,4,4′-tetrachlorobiphenyl: production of reactive oxygen by vertebrate CYP1As. Mol Pharmacol 1999;56(3):588–597.

56. Davies KJA. Oxidative stress, antioxidant defenses, and damage removal, repair, and replacement systems. IUBMB Life 2000;50(4,5):279–289.
57. Eşrefoğlu M. Cell injury and death: oxidative stress and antioxidant defense system: review. Turkiye Klinikleri J Med Sci 2009;29(6):1660–1676.
58. Matsui M, Tokuhara M, Konuma Y, *et al*. Isolation of human fos-related genes and their expression during monocyte-macrophage differentiation. Oncogene 1990;5(3):249–255.
59. Baldwin AS Jr. The NF-κB and IκB proteins: new discoveries and insights. Annu Rev Immunol 1996;14:649–681.
60. Venugopal R, Jaiswal AK. Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD(P)H:quinone oxidoreductase1 gene. Proc Natl Acad Sci U S A 1996;93(25):14960–14965.
61. Venugopal R, Jaiswal AK. Nrf2 and Nrf1 in association with Jun proteins regulate antioxidant response element-mediated expression and coordinated induction of genes encoding detoxifying enzymes. Oncogene 1998;17(24):3145–3156.
62. Sun Y, Oberley LW. Redox regulation of transcriptional activators. Free Radic Biol Med 1996;21(3):335–348.
63. Boelsterli UA. Xenobiotic acyl glucuronides and acyl CoA thioesters as protein-reactive metabolites with the potential to cause idiosyncratic drug reactions. Curr Drug Metab 2002;3(4):439–450.
64. Copple IM, Goldring CE, Jenkins RE, *et al*. The hepatotoxic metabolite of acetaminophen directly activates the keap1-Nrf2 cell defense system. Hepatology 2008;48(4):1292–1301.
65. Meister A, Anderson ME. Glutathione. Annu Rev Biochem 1983;52:711–760.
66. Meister A. Transport and metabolism of glutathione and γ-glutamyl amino acids. Biochem Soc Trans 1983;11(6):793–794.
67. Yuan L, Kaplowitz N. Glutathione in liver diseases and hepatotoxicity. Mol Aspects Med 2009;30(1,2):29–41.
68. DeLeve LD, Kaplowitz N. Glutathione metabolism and its role in hepatotoxicity. Pharmacology and Therapeutics 1991;52:287–305.
69. Adam A, Smith LL, Cohen GM. An assessment of the role of redox cycling in mediating the toxicity of paraquat and nitrofurantoin. Environ Health Perspect 1990;85:113–117.
70. Bonneh-Barkay D, Reaney SH, Langston WJ, *et al*. Redox cycling of the herbicide paraquat in microglial cultures. Mol Brain Res 2005;134(1):52–56.
71. O'Brien PJ. Molecular mechanisms of quinone cytotoxicity. Chem Biol Interact 1991; 80(1):1–41.
72. Kappus H, Sies H. Toxic drug effects associated with oxygen metabolism: redox cycling and lipid peroxidation. Experientia 1981;37(12):1233–1241.
73. Merkofer M, Kissner R, Hider RC, *et al*. Redox properties of the iron complexes of orally active iron chelators CP20, CP502, CP509, and ICL670. Helv Chim Acta 2004;87(12):3021–3034.
74. Tan AS, Berridge MV. Differential effects of redox-cycling and arylating quinones on trans-plasma membrane electron transport. Biofactors 2009;34(3):183–190.
75. Berthiaume JM, Wallace KB. Adriamycin-induced oxidative mitochondrial cardiotoxicity. Cell Biol Toxicol 2007;23(1):15–25.
76. Boelsterli UA. Diclofenac-induced liver injury: a paradigm of idiosyncratic drug toxicity. Toxicol Appl Pharmacol 2003;192(3):307–322.
77. Kehrer JP. Free radicals as mediators of tissue injury and disease. Crit Rev Toxicol 1993;23(1):21–48.
78. Kamat JP, Devasagayam TPA, Priyadarsini KI, *et al*. Reactive oxygen species mediated membrane damage induced by fullerene derivatives and its possible biological implications. Toxicology 2000;155(1–3):55–61.
79. Maridonneau-Parini I, Harpey C. Effect of trimetazidine on membrane damage induced by oxygen free radicals in human red cells. Br J Clin Pharmacol 1985;20(2):148–151.

80. Clawson GA. Mechanisms of carbon tetrachloride hepatotoxicity. Pathol Immunopathol Res 1989;8(2):104–112.
81. Recknagel RO, Glende EA, Dolak JA, *et al*. Mechanisms of carbon tetrachloride toxicity. Pharmacol Ther 1989;43(1):139–154.
82. Bus JS, Aust SD, Gibson JE. Superoxide and singlet oxygen catalyzed lipid peroxidation as a possible mechanism for paraquat (methyl viologen) toxicity. Biochem Biophys Res Commun 1974;58(3):749–755.
83. Plaa GL, Witschi H. Chemicals, drugs, and lipid peroxidation. Annu Rev Pharmacol Toxicol 1976;16:125–141.
84. Santos NAG, Medina WSG, Martins NM, *et al*. Involvement of oxidative stress in the hepatotoxicity induced by aromatic antiepileptic drugs. Toxicol *In vitro* 2008;22(8):1820–1824.
85. Brody TM. The uncoupling of oxidative phosphorylation as a mechanism of drug action. Pharmacol Rev 1955;7(3):335–363.
86. Moreno-Sánchez R, Bravo C, Vásquez C, *et al*. Inhibition and uncoupling of oxidative phosphorylation by nonsteroidal anti-inflammatory drugs: study in mitochondria, submitochondrial particles, cells, and whole heart. Biochem Pharmacol 1999;57(7):743–752.
87. Park KB, Dalton-Brown E, Hirst C, *et al*. Selection of new chemical entities with decreased potential for adverse drug reactions. Eur J Pharmacol 2006;549(1–3):1–8.
88. Day BJ, Lewis W. Oxidative stress in NRTI-induced toxicity: evidence from clinical experience and experiments *in vitro* and *in vivo*. Cardiovasc Toxicol 2004;4(3):207–216.
89. Dykens JA, Jamieson JD, Marroquin LD, *et al*. *In vitro* assessment of mitochondrial dysfunction and cytotoxicity of nefazodone, trazodone, and buspirone. Toxicol Sci 2008;103(2):335–345.
90. Esaki H, Kumagai S. Glutathione-S-transferase activity toward aflatoxin epoxide in livers of mastomys and other rodents. Toxicon 2002;40(7):941–945.
91. LoPachin RM, DeCaprio AP. Protein adduct formation as a molecular mechanism in neurotoxicity. Toxicol Sci 2005;86(2):214–225.
92. Essigmann JM, Croy RG, Nadzan AM. Structural identification of the major DNA adduct formed by aflatoxin B1 *in vitro*. Proc Natl Acad Sci U S A 1977;74(5):1870–1874.
93. Gurtoo HL, Dave CV. *In vitro* metabolic conversion of aflatoxins and benzo(a)pyrene to nucleic acid binding metabolites. Cancer Res 1975;35(2):382–389.
94. Casini A, Scozzafava A, Supuran CT. Cysteine-modifying agents: a possible approach for effective anticancer and antiviral drugs. Environ Health Perspect 2002;110,Suppl 5:801–806.
95. Guengerich FP, Cai H, Johnson WW, *et al*. Reactive intermediates in biological systems: what have we learned and where are we going. Adv Exp Med Biol 2001;500:639–650.
96. Guengerich FP, Liebler DC. Enzymatic activation of chemicals to toxic metabolites. Crit Rev Toxicol 1985;14(3):259–307.
97. Davis DC, Potter WZ, Jollow DJ, *et al*. Species differences in hepatic glutathione depletion, covalent binding and hepatic necrosis after acetaminophen. Life Sci 1974; 14(11):2099–2109.
98. Pumford NR, Halmes NC, Hinson JA. Covalent binding of xenobiotics to specific proteins in the liver. Drug Metab Rev 1997;29(1,2):39–57.
99. Reichardt P, Schreiber A, Wichmann G, *et al*. Identification and quantification of *in vitro* adduct formation between protein reactive xenobiotics and a lysine-containing model peptide. Environ Toxicol 2003;18(1):29–36.
100. Skipper PL. Influence of tertiary structure on nucleophilic substitution reactions of proteins. Chem Res Toxicol 1996;9(6):918–923.
101. Harrison AC, Kitteringham NR, Clarke JB, *et al*. The mechanism of bioactivation and antigen formation of amodiaquine in the rat. Biochem Pharmacol 1992;43(7):1421–1430.

102. Jollow DJ, Thorgeirsson SS, Potter WZ. Effects of drug pretreatments on acetaminophen induced hepatic necrosis, glutathione depletion, covalent binding and pattern of acetaminophen metabolites in hamsters. Fed Proc 1973; 32(3):1.
103. Pirmohamed M, Williams D, Madden S, *et al*. Metabolism and bioactivation of clozapine by human liver *in vitro*. J Pharmacol Exp Ther 1995;272(3):984–990.
104. Rashed MS, Nelson SD. Characterization of glutathione conjugates of reactive metabolites of 3′-hydroxyacetanilide, a nonhepatotoxic positional isomer of acetaminophen. Chem Res Toxicol 1989;2(1):41–45.
105. Tirmenstein MA, Nelson SD. Subcellular binding and effects on calcium homeostasis produced by acetaminophen and a nonhepatotoxic regioisomer, 3′-hydroxyacetanilide, in mouse liver. J Biol Chem 1989;264(17):9814–9819.
106. Qiu Y, Benet LZ, Burlingame AL. Identification of the hepatic protein targets of reactive metabolites of acetaminophen *in vivo* in mice using two-dimensional gel electrophoresis and mass spectrometry. J Biol Chem 1998;273(28):17940–17953.
107. Kenna JG. Immunoallergic drug-induced hepatitis: lessons from halothane. J Hepatol Suppl 1997;26(1):5–12.
108. Vergani D, Mieli-Vergani G, Alberti A. Antibodies to the surface of halothane-altered rabbit hepatocytes in patients with severe halothane-associated hepatitis. N Engl J Med 1980;303(2):66–71.
109. Bourdi M, Chen W, Peter RM, *et al*. Human cytochrome P450 2E1 is a major autoantigen associated with halothane hepatitis. Chem Res Toxicol 1996;9(7):1159–1166.
110. Martin DC, Dennison RL, Introna RPS, *et al*. Influence of halothane on the interactions of serotonin(1A) and adenosine A1 receptors with G proteins in rat brain membranes. Biochem Pharmacol 1991;42(6):1313–1316.
111. Martin JL, Pumford NR, LaRosa AC, *et al*. A metabolite of halothane covalently binds to an endoplasmic reticulum protein that is highly homologous to phosphatidylinositol-specific phospholipase C-α but has no activity. Biochem Biophys Res Commun 1991; 178(2):679–685.
112. Pohl LR, Thomassen D, Pumford NR, *et al*. Hapten carrier conjugates associated with halothane hepatitis. Adv Exp Med Biol 1991;283:111–120.
113. Lecoeur S, André C, Beaune PH. Tienilic acid-induced autoimmune hepatitis: anti-liver and -kidney microsomal type 2 autoantibodies recognize a three-site conformational epitope on cytochrome P4502C9. Mol Pharmacol 1996;50(2):326–333.
114. Bourdi M, Tinel M, Beaune PH, *et al*. Interactions of dihydralazine with cytochromes P4501A: a possible explanation for the appearance of anti-cytochrome P4501A2 autoantibodies. Mol Pharmacol 1994;45(6):1287–1295.
115. Teichert J, Sohr R, Hennig L, *et al*. Identification and quantitation of the N-acetyl-L-cysteine S-conjugates of bendamustine and its sulfoxides in human bile after administration of bendamustine hydrochloride. Drug Metab Dispos 2009;37(2):292–301.
116. Kalgutkar AS, Soglia JR. Minimising the potential for metabolic activation in drug discovery. Expert Opin Drug Metab Toxicol 2005;1(1):91–142.
117. Wen B, Chen Y, Fitch WL. Metabolic activation of nevirapine in human liver microsomes: dehydrogenation and inactivation of cytochrome P450 3A4. Drug Metab Dispos 2009;37(7):1557–1562.
118. Evans DC, Watt AP, Nicoll-Griffith DA, *et al*. Drug-protein adducts: an industry perspective on minimizing the potential for drug bioactivation in drug discovery and development. Chem Res Toxicol 2004;17(1):3–16.
119. Masubuchi N, Makino C, Murayama N. Prediction of *in vivo* potential for metabolic activation of drugs into chemically reactive intermediate: correlation of *in vitro* and *in vivo* generation of reactive intermediates and *in vitro* glutathione conjugate formation in rats and humans. Chem Res Toxicol 2007;20(3):455–464.

120. Takakusa H, Masumoto H, Yukinaga H, *et al*. Covalent binding and tissue distribution/retention assessment of drugs associated with idiosyncratic drug toxicity. Drug Metab Dispos 2008;36(9):1770–1779.
121. Ma L, Wen B, Ruan Q, *et al*. Rapid screening of glutathione-trapped reactive metabolites by linear ion trap mass spectrometry with isotope pattern-dependent scanning and postacquisition data mining. Chem Res Toxicol 2008;21(7):1477–1483.
122. Argoti D, Liang L, Conteh A, *et al*. Cyanide trapping of iminium ion reactive intermediates followed by detection and structure identification using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Chem Res Toxicol 2005;18(10):1537–1544.
123. Madden S, Spaldin V, Hayes RN, *et al*. Species variation in the bioactivation of tacrine by hepatic microsomes. Xenobiotica 1995;25(1):103–116.
124. Soglia JR, Harriman SP, Zhao S, *et al*. The development of a higher throughput reactive intermediate screening assay incorporating micro-bore liquid chromatography-microelectrospray ionization-tandem mass spectrometry and glutathione ethyl ester as an *in vitro* conjugating agent. J Pharm Biomed Anal 2004;36(1):105–116.
125. Sanders SP, Bassett DJP, Harrison SJ, *et al*. Measurements of free radicals in isolated, ischemic lungs and lung mitochondria. Lung 2000;178(2):105–118.
126. Gorrod JW, Whittlesea CMC, Lam SP. Trapping of reactive intermediates by incorporation of 14C-sodium cyanide during microsomal oxidation. Adv Exp Med Biol 1991;283:657–664.
127. O'Donnell JP, Dalvie DK, Kalgutkar AS, *et al*. Mechanism-based inactivation of human recombinant P450 2C9 by the nonsteroidal anti-inflammatory drug suprofen. Drug Metab Dispos 2003;31(11):1369–1377.
128. Dalvie DK, Kalgutkar AS, Khojasteh-Bakht SC, *et al*. Biotransformation reactions of five-membered aromatic heterocyclic rings. Chem Res Toxicol 2002;15(3):269–299.
129. Chauret N, Nicoll-Griffith D, Friesen R, *et al*. Microsomal metabolism of the 5-lipoxygenase inhibitors L-746,530 and L- 739,010 to reactive intermediates that covalently bind to protein: the role of the 6,8-dioxabicyclo[3.2.1]octanyl moiety. Drug Metab Dispos 1995;23(12):1325–1334.
130. Zhang KE, Naue JA, Arison B, *et al*. Microsomal metabolism of the 5-lipoxygenase inhibitor L-739,010: evidence for furan bioactivation. Chem Res Toxicol 1996; 9(2):547–554.
131. Kalgutkar AS, Fate G, Didiuk MT, *et al*. Toxicophores, reactive metabolites and drug safety: when is it a cause for concern. Expert Rev Clin Pharmacol 2008;1(4):515–531.
132. De Rooij BM, Commandeur JNM, Vermeulen NPE. Mercapturic acids as biomarkers of exposure to electrophilic chemicals: applications to environmental and industrial chemicals. Biomarkers 1998;3(4,5):239–303.
133. Kuiper HC, Miranda CL, Sowell JD, *et al*. Mercapturic acid conjugates of 4-hydroxy-2-nonenal and 4-oxo-2-nonenal metabolites are *in vivo* markers of oxidative stress. J Biol Chem 2008;283(25):17131–17138.
134. Johnson KA, Plumb R. Investigating the human metabolism of acetaminophen using UPLC and exact mass oa-TOF MS. J Pharm Biomed Anal 2005;39(3,4):805–810.
135. Tsuruda LS, Lame MW, Jones AD. Metabolism of [14C]naphthalene in the B6C3F1 murine isolated perfused liver. Drug Metab Dispos 1995;23(1):129–136.
136. Seutter Berlage F, Van Dorp HL, Kosse HGJ, *et al*. Urinary mercapturic acid excretion as a biological parameter of exposure to alkylating agents. Int Arch Occup Environ Health 1977;39(1):45–51.
137. Henderson RF, Bechtold WE, Bond JA, *et al*. The use of biological markers in toxicology. Crit Rev Toxicol 1989;20(2):65–79.
138. Scholz K, Dekant W, Völkel W, *et al*. Rapid detection and identification of N-acetyl-L-cysteine thioethers using constant neutral loss and theoretical multiple reaction monitoring

combined with enhanced product-ion scans on a linear ion trap mass spectrometer. J Am Soc Mass Spectrom 2005;16(12):1976–1984.
139. Norrgran J, Bravo R, Bishop AM, *et al*. Quantification of six herbicide metabolites in human urine. J Chromatogr B Analyt Technol Biomed Life Sci 2006;830(2):185–195.
140. Wagner S, Scholz K, Sieber M, *et al*. Tools in metabonomics: an integrated validation approach for LC-MS metabolic profiling of mercapturic acids in human urine. Anal Chem 2007;79(7):2918–2926.
141. Inoue M, Okajima K, Morino Y. Metabolic coordination of liver and kidney in mercapturic acid biosynthesis *in vivo*. Hepatology 1982;2(3):311–316.
142. Hinchman CA, Ballatori N. Glutathione conjugation and conversion to mercapturic acids can occur as an intrahepatic process. J Toxicol Environ Health 1994;41(4):387–409.
143. Siegers CP, Loeser W, Gieselmann J, *et al*. Biliary and renal excretion of paracetamol in man. Pharmacology 1984;29(5):301–303.
144. Hinchman CA, Rebbeor JF, Ballatori N. Efficient hepatic uptake and concentrative biliary excretion of a mercapturic acid. Am J Physiol Gastrointest Liver Physiol 1998;275(438-4): G612–G619.
145. Amore BM, Kalhorn TF, Skiles GL, *et al*. Characterization of carbamazepine metabolism in a mouse model of carbamazepine teratogenicity. Drug Metab Dispos 1997; 25(8):953–962.
146. Madden S, Maggs JL, Park BK. Bioactivation of carbamazepine in the rat *in vivo* : evidence for the formation of reactive arene oxide(s). Drug Metab Dispos 1996;24(4): 469–479.
147. Maggs JL, Pirmohamed M, Kitteringham NR, *et al*. Characterization of the metabolites of carbamazepine in patient urine by liquid chromatography/mass spectrometry. Drug Metab Dispos 1997;25(3):275–280.
148. Bu HZ, Zhao P, Dalvie DK, *et al*. Identification of primary and sequential bioactivation pathways of carbamazepine in human liver microsomes using liquid chromatography/tandem mass spectrometry. Rapid Commun Mass Spectrom 2007;21(20):3317–3322.
149. Rousu T, Pelkonen O, Tolonen A. Rapid detection and characterization of reactive drug metabolites *in vitro* using several isotope-labeled trapping agents and ultra-performance liquid chromatography/time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 2009;23(6):843–855.
150. Wang Y, Zhong D, Chen X, *et al*. Identification of quinone methide metabolites of dauricine in human liver microsomes and in rat bile. Chem Res Toxicol 2009; 22(5):824–834.
151. Zhou S, Chan E, Duan W, Huang M, and Chen Y. Drug bioactivation, covalent binding to target proteins and toxicity relevance. Drug Metab Rev 2005;37:41–213.
152. ACGIH. Benzene BEI. Documentation of the Biological Exposure Indices. 7th edn. Cincinnati, OH: American Conference of Governmental Industrial Hygienists. p. 114. 2001.
153. Veronese ME, McLean S, D'Souza CA, *et al*. Formation of reactive metabolites of phenacetin in humans and rats. Xenobiotica 1985;15(11):929–940.
154. Dieckhaus CM, Thompson CD, Roller SG, *et al*. Mechanisms of idiosyncratic drug reactions: the case of felbamate. Chem Biol Interact 2002;142(1,2):99–117.
155. Gopaul S, Farrell K, Abbott F. Effects of age and polytherapy, risk factors of valproic acid (VPA) hepatotoxicity, on the excretion of thiol conjugates of (E)-2,4-diene VPA in people with epilepsy taking VPA. Epilepsia 2003;44(3):322–328.
156. Botting R. Paracetamol-inhibitable COX-2. Journal of Physiology and Pharmacology 2000;51:609–618.
157. Boutaud O, Aronoff DM, Richardson JH, *et al*. Determinants of the cellular specificity of acetaminophen as an inhibitor of prostaglandin H2 synthases. Proc Natl Acad Sci U S A 2002;99:7130–7135.

158. Greco A, Ajmone-Cat MA, Nicolini A, *et al*. Paracetamol effectively reduces prostaglandin E2 synthesis in brain macrophages by inhibiting enzymatic activity of cyclooxygenase but not phospholipase and prostaglandin E synthase. J Neurosci Res 2003;71:844–852.
159. Ostapowicz G, Fontana RJ, Schioødt FV, *et al*. Results of a prospective study of acute liver failure at 17 tertiary care centers in the United States. Ann Intern Med 2002; 137(12):947–954.
160. Howie D, Adriaenssens PI, Prescott LF. Paracetamol metabolism following overdosage: application of high performance liquid chromatography. J Pharm Pharmacol 1977;29(4):235–237.
161. Dahlin DC, Miwa GT, Lu AY, *et al*. N-acetyl-p-benzoquinone imine: a cytochrome P-450-mediated oxidation product of acetaminophen. Proc Natl Acad Sci U S A 1984;81(5):1327–1331.
162. Mitchell JR, Jollow DJ, Potter WZ. Acetaminophen induced hepatic necrosis. IV. Protective role of glutathione. J Pharmacol Exp Ther 1973;187(1):211–217.
163. Massey TE, Racz WJ. Protection against acetaminophen toxicity in isolated mouse hepatocytes by N-acetylcysteine. Pharmacologist 1981;23(3):456.
164. Mitchell JR, Jollow DJ, Potter WZ. Acetaminophen induced hepatic necrosis. I. Role of drug metabolism. J Pharmacol Exp Ther 1973;187(1):185–194.
165. Potter WZ, Davis DC, Mitchell JR. Acetaminophen induced hepatic necrosis. III. Cytochrome P450 mediated covalent binding *in vitro*. J Pharmacol Exp Ther 1973;187(1):203–210.
166. Reid AB, Kurten RC, McCullough SS, *et al*. Mechanisms of acetaminophen-induced hepatotoxicity: role of oxidative stress and mitochondrial permeability transition in freshly isolated mouse hepatocytes. J Pharmacol Exp Ther 2005;312(2):509–516.
167. Kitteringham NR, Powell H, Clement YN, *et al*. Hepatocellular response to chemical stress in CD-1 mice: Induction of early genes and γ-glutamylcysteine synthetase. Hepatology 2000;32(2):321–333.
168. Dietze EC, Schäfer A, Omichinski JG, *et al*. Inactivation of glyceraldehyde-3-phosphate dehydrogenase by a reactive metabolite of acetaminophen and mass spectral characterization of an arylated active site peptide. Chem Res Toxicol 1997;10(10):1097–1103.
169. Landin JS, Cohen SD, Khairallah EA. Identification of a 54-kDa mitochondrial acetaminophen-binding protein as aldehyde dehydrogenase. Toxicol Appl Pharmacol 1996;141(1):299–307.
170. Tsokos-Kuhn JO, Hughes H, Smith CV, *et al*. Alkylating toxins and the liver plasma membrane calcium pump/calcium atpase. Adv Exp Med Biol 1988;232:151–158.
171. Goldring CEP, Kitteringham NR, Elsby R, *et al*. Activation of hepatic Nrf2 *in vivo* by acetaminophen in CD-1 Mice. Hepatology 2004;39(5):1267–1276.
172. Okawa H, Motohashi H, Kobayashi A, *et al*. Hepatocyte-specific deletion of the keap1 gene activates Nrf2 and confers potent resistance against acute drug toxicity. Biochem Biophys Res Commun 2006;339(1):79–88.
173. Holt MP, Ju C. Mechanisms of drug-induced liver injury. AAPS J 2006;8(1):E48–E54.
174. Cover C, Liu J, Farhood A, *et al*. Pathophysiological role of the acute inflammatory response during acetaminophen hepatotoxicity. Toxicol Appl Pharmacol 2006; 216(1):98–107.
175. Lawson JA, Farhood A, Hopper RD, *et al*. The hepatic inflammatory response after acetaminophen overdose: role of neutrophils. Toxicol Sci 2000;54(2):509–516.
176. Bauer I, Vollmar B, Jaeschke H, *et al*. Transcriptional activation of heme oxygenase-1 and its functional significance in acetaminophen-induced hepatitis and hepatocellular injury in the rat. J Hepatol 2000;33(3):395–406.
177. Smith GS, Nadig DE, Kokoska ER, *et al*. Role of neutrophils in hepatotoxicity induced by oral acetaminophen administration in rats. J Surg Res 1998;80(2):252–258.

178. Gardner CR, Laskin JD, Dambach DM, *et al*. Reduced hepatotoxicity of acetaminophen in mice lacking inducible nitric oxide synthase: potential role of tumor necrosis factor-α and interleukin-10. Toxicol Appl Pharmacol 2002;184(1):27–36.
179. Ju C, Reilly TP, Bourdi M, *et al*. Protective role of kupffer cells in acetaminophen-induced hepatic injury in mice. Chem Res Toxicol 2002;15(12):1504–1513.
180. Goldin RD, Ratnayaka ID, Breach CS, *et al*. Role of macrophages in acetaminophen (paracetamol)-induced hepatotoxicity. J Pathol 1996;179(4):432–435.
181. Liu ZX, Govindarajan S, Kaplowitz N. Innate immune system plays a critical role in determining the progression and severity of acetaminophen hepatotoxicity. Gastroenterology 2004;127(6):1760–1774.
182. Jaeschke H. How relevant are neutrophils for acetaminophen hepatotoxicity. Hepatology 2006;43(6):1191–1194.
183. Masson MJ, Carpenter LD, Graf ML, *et al*. Pathogenic role of natural killer T and natural killer cells in acetaminophen-induced liver injury in mice is dependent on the presence of dimethyl sulfoxide. Hepatology 2008;48(3):889–897.
184. Boess F, Bopst M, Althaus R, *et al*. Acetaminophen hepatotoxicity in tumor necrosis factor/lymphotoxin-α gene knockout mice. Hepatology 1998;27(4):1021–1029.
185. Ishida Y, Kondo T, Tsuneyama K, *et al*. The pathogenic roles of tumor necrosis factor receptor p55 in acetaminophen-induced liver injury in mice. J Leukoc Biol 2004; 75(1):59–67.
186. Blazka ME, Wilmer JL, Holladay SD, *et al*. Role of proinflammatory cytokines in acetaminophen hepatotoxicity. Toxicol Appl Pharmacol 1995;133(1):43–52.
187. Ishibe T, Kimura A, Ishida Y, *et al*. Reduced acetaminophen-induced liver injury in mice by genetic disruption of IL-1 receptor antagonist. Lab Invest 2009;89(1):68–79.
188. Ishida Y, Kondo T, Ohshima T, *et al*. A pivotal involvement of IFN-γ in the pathogenesis of acetaminophen-induced acute liver injury. FASEB J 2002;16(10):1227–1236.
189. Bourdi M, Eiras DP, Holt MP, *et al*. Role of IL-6 in an IL-10 and IL-4 double knockout mouse model uniquely susceptible to acetaminophen-induced liver injury. Chem Res Toxicol 2007;20(2):208–216.
190. Yohe HC, O'Hara KA, Hunt JA, *et al*. Involvement of toll-like receptor 4 in acetaminophen hepatotoxicity. Am J Physiol Gastrointest Liver Physiol 2006;290(6):G1269–G1279.
191. Imaeda AB, Watanabe A, Sohail MA, *et al*. Acetaminophen-induced hepatotoxicity in mice is dependent on Tlr9 and the Nalp3 inflammasome. J Clin Invest 2009; 119(2):305–314.
192. Ekong U, Zeng S, Dun H, *et al*. Blockade of the receptor for advanced glycation end products attenuates acetaminophen-induced hepatotoxicity in mice. J Gastroenterol Hepatol 2006;21(4):682–688.
193. Liu ZX, Han D, Gunawan B, *et al*. Neutrophil depletion protects against murine acetaminophen hepatotoxicity. Hepatology 2006;43(6):1220–1230.
194. Maddox JF, Amuzie CJ, Li M, *et al*. Bacterial- and viral-induced inflammation increases sensitivity to acetaminophen hepatotoxicity. J Toxicol Environ Health A 2010; 73(1):58–73.
195. Bourdi M, Reilly TP, Elkahloun AG, *et al*. Macrophage migration inhibitory factor in drug-induced liver injury: a role in susceptibility and stress responsiveness. Biochem Biophys Res Commun 2002;294(2):225–230.
196. Masubuchi Y, Bourdi M, Reilly TP, *et al*. Role of interleukin-6 in hepatic heat shock protein expression and protection against acetaminophen-induced liver disease. Biochem Biophys Res Commun 2003;304(1):207–212.
197. Yee SB, Bourdi M, Masson MJ, *et al*. Hepatoprotective role of endogenous interleukin-13 in a murine model of acetaminophen-induced liver disease. Chem Res Toxicol 2007;20(5):734–744.

198. Simpson KJ, Lukacs NW, McGregor AH, *et al*. Inhibition of tumour necrosis factor alpha does not prevent experimental paracetamol-induced hepatic necrosis. J Pathol 2000;190(4):489–494.
199. Chiu H, Gardner CR, Dambach DM, *et al*. Role of tumor necrosis factor receptor 1 (p55) in hepatocyte proliferation during acetaminophen-induced toxicity in mice. Toxicol Appl Pharmacol 2003;193(2):218–227.
200. Gardner CR, Laskin JD, Dambach DM, *et al*. Exaggerated hepatotoxicity of acetaminophen in mice lacking tumor necrosis factor receptor-1: potential role of inflammatory mediators. Toxicol Appl Pharmacol 2003;192(2):119–130.
201. Kim YC, Lee SJ. Temporal variation in hepatotoxicity and metabolism of acetaminophen in mice. Toxicology 1998;128(1):53–61.
202. Matsunaga N, Nakamura N, Yoneda N, *et al*. Influence of feeding schedule on 24-h rhythm of hepatotoxicity induced by acetaminophen in mice. J Pharmacol Exp Ther 2004;311(2):594–600.
203. Matsunaga N, Ikeda M, Takiguchi T, *et al*. The molecular mechanism regulating 24-hour rhythm of CYP2E1 expression in the mouse liver. Hepatology 2008;48(1):240–251.
204. U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER).
205. Aranda-Michel J, Koehler A, Bejarano PA, *et al*. Nefazodone-induced liver failure: Report of three cases. Ann Intern Med 1999;130(4 I):285–288.
206. Eloubeidi MA, Gaede JT, Swaim MW. Reversible nefazodone-induced liver failure. Dig Dis Sci 2000;45(5):1036–1038.
207. Rotzinger S, Baker GB. Human CYP3A4 and the metabolism of nefazodone and hydroxynefazodone by human liver microsomes and heterologously expressed enzymes. Eur Neuropsychopharmacol 2002;12(2):91–100.
208. Kalgutkar AS, Vaz ADN, Lame ME, *et al*. Bioactivation of the nontricyclic antidepressant nefazodone to a reactive quinone-imine species in human liver microsomes and recombinant cytochrome P450 3A4. Drug Metab Dispos 2005;33(2):243–253.
209. Usui T, Mise M, Hashizume T, *et al*. Evaluation of the potential for drug-induced liver injury based on *in vitro* covalent binding to human liver proteins. Drug Metab Dispos 2009;37(12):2383–2392.
210. Obach RS, Kalgutkar AS, Soglia JR, *et al*. Can *in vitro* metabolism-dependent covalent binding data in liver microsomes distinguish hepatotoxic from nonhepatotoxic drugs? An analysis of 18 drugs with consideration of intrinsic clearance and daily dose. Chem Res Toxicol 2008;21(9):1814–1822.
211. Shukla UA, Marathe PH, Pittman KA, *et al*. Pharmacokinetics, absolute bioavailability, and disposition of [14C] nefazodone in the dog. Drug Metab Dispos 1993;21(3): 502–507.
212. Barbhaiya RH, Dandekar KA, Greene DS. Pharmacokinetics, absolute bioavailability, and disposition of [14C]nefazodone in humans. Drug Metab Dispos 1996;24(1):91–95.
213. Stieger B, Meier Y, Meier PJ. The bile salt export pump. Pflugers Arch 2007;453(5):611–620.
214. Kostrubsky SE, Strom SC, Kalgutkar AS, *et al*. Inhibition of hepatobiliary transport as a predictive method for clinical hepatotoxicity of nefazodone. Toxicol Sci 2006;90(2):451–459.
215. Barbhaiya RH, Marathe PH, Greene DS, *et al*. Safety, tolerance, and preliminary pharmacokinetics of nefazodone after administration of single and multiple oral doses to healthy adult male volunteers: a double-blind, phase I study. J Clin Pharmacol 1995;35(10):974–984.
216. Miners JO, Mackenzie PI. Drug glucuronidation in humans. Pharmacol Ther 1991; 51(3):347–369.

217. Argikar UA, Remmel RP. Effect of aging on glucuronidation of valproic acid in human liver microsomes and the role of UDP-glucuronosyltransferase UGT1A4, UGT1A8, and UGT1A10. Drug Metab Dispos 2009;37(1):229–236.
218. Sakaguchi K, Green M, Stock N, *et al*. Glucuronidation of carboxylic acid containing compounds by UDP-glucuronosyltransferase isoforms. Arch Biochem Biophys 2004;424(2):219–225.
219. Zheng Y, Sun D, Sharma AK, *et al*. Elimination of antiestrogenic effects of active tamoxifen metabolites by glucuronidation. Drug Metab Dispos 2007;35(10):1942–1948.
220. Bruin GJ, Faller T, Wiegand H, *et al*. Pharmacokinetics, distribution, metabolism, and excretion of deferasirox and its iron complex in rats. Drug Metab Dispos 2008; 36(12):2523–2538.
221. Kim MS, Liu DQ, Strauss JR, *et al*. Metabolism and disposition of gemfibrozil in Wistar and multidrug resistance-associated protein 2-deficient TR- rats. Xenobiotica 2003; 33(10):1027–1042.
222. Nishino A, Kato Y, Igarashi T, *et al*. Both cMOAT/MRP2 and another unknown transporter(s) are responsible for the biliary excretion of glucuronide conjugate of the nonpeptide angiotensin II antagonist, telmisaltan. Drug Metab Dispos 2000;28(10):1146–1148.
223. Sabordo L, Sallustio BC, Evans AM, *et al*. Hepatic disposition of the acyl glucuronide 1-O-gemfibrozil-beta-D-glucuronide: effects of clofibric acid, acetaminophen, and acetaminophen glucuronide. J Pharmacol Exp Ther 2000;295(1):44–50.
224. Sallustio BC, Fairchild BA, Shanahan K, *et al*. Disposition of gemfibrozil and gemfibrozil acyl glucuronide in the rat isolated perfused liver. Drug Metab Dispos 1996; 24(9):984–989.
225. Sasabe H, Tsuji A, Sugiyama Y. Carrier-mediated mechanism for the biliary excretion of the quinolone antibiotic grepafloxacin and its glucuronide in rats. J Pharmacol Exp Ther 1998;284(3):1033–1039.
226. Doss GA, Baillie TA. Addressing metabolic activation as an integral component of drug design. Drug Metab Rev 2006;38(4):641–649.
227. Baillie TA. Metabolism and toxicity of drugs. Two decades of progress in industrial drug metabolism. Chem Res Toxicol 2008;21(1):129–137.
228. Ebner T, Heinzel G, Prox A, *et al*. Disposition and chemical stability of telmisartan 1-O-acylglucuronide. Drug Metab Dispos 1999;27(10):1143–1149.
229. Srinivasan K, Nouri P, Kavetskaia O. Challenges in the indirect quantitation of acylglucuronide metabolites of a cardiovascular drug from complex biological mixtures in the absence of reference standards. Biomed Chromatogr 2009;24:759–767.
230. Forrest MJ, Bloomfield D, Briscoe RJ, *et al*. Torcetrapib-induced blood pressure elevation is independent of CETP inhibition and is accompanied by increased circulating levels of aldosterone. Br J Pharmacol 2008;154(7):1465–1473.
231. Treinen-Moslen M, Kanz MF. Intestinal tract injury by drugs: importance of metabolite delivery by yellow bile road. Pharmacol Ther 2006;112(3):649–667.
232. Akira K, Uchijima T, Hashimoto T. Rapid internal acyl migration and protein binding of synthetic probenecid glucuronides. Chem Res Toxicol 2002;15(6):765–772.
233. Krieter PA, Gohdes M, Musick TJ, *et al*. Pharmacokinetics, disposition, and metabolism of bicifadine in humans. Drug Metab Dispos 2008;36(2):252–259.
234. Waldmeier FJ, Bruin GJ, Glaenzel U, *et al*. Pharmacokinetics, metabolism and disposition of deferasirox in beta-thalassemia patients with transfusion-dependent iron overload who are at pharmacokinetic steady state. Drug Metab Dispos 2010;38:808–816.
235. Wang J, Davis M, Li F, *et al*. A novel approach for predicting acyl glucuronide reactivity via Schiff base formation: development of rapidly formed peptide adducts for LC/MS/MS measurements. Chem Res Toxicol 2004;17(9):1206–1216.
236. Hasegawa J, Smith PC, Benet LZ. Apparent intramolecular acyl migration of zomepirac glucuronide. Drug Metab Dispos 1982;10(5):469–473.

237. Faed EM. Properties of acyl glucuronides: implications for studies of the pharmacokinetics and metabolism of acidic drugs. Drug Metab Rev 1984;15(5,6):1213–1249.
238. Ritter JK. Roles of glucuronidation and UDP-glucuronosyltransferases in xenobiotic bioactivation reactions. Chem Biol Interact 2000;129(1,2):171–193.
239. Shipkova M, Armstrong VW, Oellerich M, *et al*. Acyl glucuronide drug metabolites: toxicological and analytical implications. Ther Drug Monit 2003;25(1):1–16.
240. Bailey MJ, Dickinson RG. Acyl glucuronide reactivity in perspective: biological consequences. Chem Biol Interact 2003;145:117–137, 10.1016/S0009-2797(03)00020-6
241. Sallustio BC, Sabordo L, Evans AM, *et al*. Hepatic disposition of electrophilic acyl glucuronide conjugates. Curr Drug Metab 2000;1(2):163–180.
242. Skonberg C, Olsen J, Madsen KG, *et al*. Metabolic activation of carboxylic acids. Expert Opin Drug Metab Toxicol 2008;4(4):425–438.
243. Bolze S, Bromet N, Gay-Feutry C, *et al*. Development of an *in vitro* screening model for the biosynthesis of acyl glucuronide metabolites and the assessment of their reactivity toward human serum albumin. Drug Metab Dispos 2002;30(4):404–413.
244. Stachulski AV, Harding JR, Lindon JC, *et al*. Acyl glucuronides: biological activity, chemical reactivity, and chemical synthesis. J Med Chem 2006;49(24):6931–6945.
245. Naisbitt DJ, Sanderson LS, Meng X, *et al*. Investigation of the immunogenicity of diclofenac and diclofenac metabolites. Toxicol Lett 2007;168(1):45–50.
246. Smith PC, McDonagh AF, Benet LZ. Irreversible binding of zomepirac to plasma protein *in vitro* and *in vivo*. J Clin Invest 1986;77(3):934–939.
247. Spahn-Langguth H, Benet LZ. Acyl glucuronides revisited: is the glucuronidation process a toxification as well as a detoxification mechanism? Drug Metab Rev 1992;24(1):5–47.
248. McDonagh AF, Palma LA, Lauff JJ, *et al*. Origin of mammalian biliprotein and rearrangement of bilirubin glucuronides *in vivo* in the rat. J Clin Invest 1984;74(3):763–770.
249. van Breemen RB, Fenselau C. Acylation of albumin by 1-O-acyl glucuronides. Drug Metab Dispos 1985;13(3):318–320.
250. Ruelius HW, Kirkman SK, Young EM, *et al*. Reactions of oxaprozin-1-O-acyl glucuronide in solutions of human plasma and albumin. Adv Exp Med Biol 1986;197:431–441.
251. Ding A, Ojingwa JC, McDonagh AF, *et al*. Evidence for covalent binding of acyl glucuronides to serum albumin via an imine mechanism as revealed by tandem mass spectrometry. Proc Natl Acad Sci U S A 1993;90(9):3797–3801.
252. Grubb N, Weil A, Caldwell J. Studies on the *in vitro* reactivity of clofibryl and fenofibryl glucuronides, evidence for protein binding via a Schiff's base mechanism. Biochem Pharmacol 1993;46(3):357–364.
253. Hayball PJ. Formation and reactivity of acyl glucuronides: the influence of chirality. Chirality 1995;7(1):1–9.
254. Smith PC, Benet LZ, McDonagh AF. Covalent binding of zomepirac glucuronide to proteins: evidence for a Schiff base mechanism. Drug Metab Dispos 1990;18(5):639–644.
255. Dickinson RG, King AR. Studies on the reactivity of acyl glucuronides– V. Glucuronide-derived covalent binding of diflunisal to bladder tissue of rats and its modulation by urinary pH and beta-glucuronidase. Biochem Pharmacol 1993;46(7):1175–1182.
256. Dong JQ, Liu J, Smith PC. Role of benoxaprofen and flunoxaprofen acyl glucuronides in covalent binding to rat plasma and liver proteins *in vivo*. Biochem Pharmacol 2005;70(6):937–948.
257. Qiu Y, Burlingame AL, Benet LZ. Mechanisms for covalent binding of benoxaprofen glucuronide to human serum albumin. Studies By tandem mass spectrometry. Drug Metab Dispos 1998;26(3):246–256.
258. Banks AT, Zimmerman HJ, Ishak KG, *et al*. Diclofenac-associated hepatotoxicity: analysis of 180 cases reported to the Food and Drug Administration as adverse reactions. Hepatology 1995;22(3):820–827.

259. Breen EG, McNicholl J, Cosgrove E, *et al*. Fatal hepatitis associated with diclofenac. Gut 1986;27(11):1390–1393.
260. Deshayes P, Leloet X, Bercoff E, *et al*. Diclofenac hepatitis: a case. Presse Med 1984;13(30):1847.
261. Scully LJ, Clarke D, Barr RJ. Diclofenac induced hepatitis. 3 cases with features of autoimmune chronic active hepatitis. Dig Dis Sci 1993;38(4):744–751.
262. Boelsterli UA, Zimmerman HJ, Kretz-Rommel A. Idiosyncratic liver toxicity of nonsteroidal antiinflammatory drugs: molecular mechanisms and pathology. Crit Rev Toxicol 1995;25(3):207–235.
263. Purcell P, Henry D, Melville G. Diclofenac hepatitis. Gut 1991;32(11):1381–1385.
264. Walker AM. Quantitative studies of the risk of serious hepatic injury in persons using nonsteroidal antiinflammatory drugs. Arthritis Rheum 1997;40(2):201–208.
265. Lewis SC, Langman MJ, Laporte JR, *et al*. Dose-response relationships between individual nonaspirin nonsteroidal anti-inflammatory drugs (NANSAIDs) and serious upper gastrointestinal bleeding: a meta-analysis based on individual patient data. Br J Clin Pharmacol 2002;54(3):320–326.
266. Hargus SJ, Martin BM, George JW, *et al*. Covalent modification of rat liver dipeptidyl peptidase IV (CD26) by the nonsteroidal anti-inflammatory drug diclofenac. Chem Res Toxicol 1995;8(8):993–996.
267. Seitz S, Kretz-Rommel A, Oude Elferink RP, *et al*. Selective protein adduct formation of diclofenac glucuronide is critically dependent on the rat canalicular conjugate export pump (Mrp2). Chem Res Toxicol 1998;11(5):513–519.
268. Atchison CR, West AB, Balakumaran A, *et al*. Drug enterocyte adducts: possible causal factor for diclofenac enteropathy in rats. Gastroenterology 2000;119(6):1537–1547.
269. Buyse S, Vibert E, Sebagh M, *et al*. Liver transplantation for fulminant hepatitis related to nevirapine therapy. Liver Transpl 2006;12(12):1880–1882.
270. De Maat MMR, Ter Heine R, Van Gorp ECM, *et al*. Case series of acute hepatitis in a non-selected group of HIV-infected patients on nevirapine-containing antiretroviral treatment. AIDS 2003;17(15):2209–2214.
271. Pirmohamed M, Drummond NS, Naisbitt DJ, *et al*. Drug hypersensitivity reactions in patients with HIV disease. Expert Rev Clin Immunol 2007;3(3):395–410.
272. Chen J, Mannargudi BM, Xu L, *et al*. Demonstration of the metabolic pathway responsible for nevirapine-induced skin rash. Chem Res Toxicol 2008;21(9):1862–1870.
273. Riska P, Lamson M, Macgregor T, *et al*. Disposition and biotransformation of the antiretroviral drug nevirapine in humans. Drug Metab Dispos 1999;27(8):895–901.
274. Srivastava A, Lian L-, Maggs JL, *et al*. Quantifying the metabolic activation of nevirapine in patients by integrated applications of NMR and mass spectrometries. Drug Metab Dispos 2010;38(1):122–132.
275. Shenton JM, Teranishi M, Abu-Asab MS, *et al*. Characterization of a potential animal model of an idiosyncratic drug reaction: nevirapine-induced skin rash in the rat. Chem Res Toxicol 2003;16(9):1078–1089.
276. Shenton JM, Chen J, Uetrecht JP. Animal models of idiosyncratic drug reactions. Chem Biol Interact 2004;150(1):53–70.
277. Shenton JM, Popovic M, Chen J, *et al*. Evidence of an immune-mediated mechanism for an idiosyncratic nevirapine-induced reaction in the female brown Norway rat. Chem Res Toxicol 2005;18(12):1799–1813.
278. Walubo A, Barr S, Abraham AM. Rat CYP3A and CYP2B1/2 were not associated with nevirapine-induced hepatotoxicity. Methods Find Exp Clin Pharmacol 2006;28(7): 423–431