Deleterious effects of reactive metabolites

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Key words: metabolism, reactive metabolites, adverse drug reactions, drug design

A number of drugs have been withdrawn from the market or severely restricted in their use because of unexpected toxicities that become apparent only after the launch of new drug entities. Circumstantial evidence suggests that, in most cases, reactive metabolites are responsible for these unexpected toxicities. In this review, a general overview of the types of reactive metabolites and the consequences of their formation are presented. The current approaches to evaluate bioactivation potential of new compounds with particular emphasis on the advantages and limitation of these procedures will be discussed. Reasonable reasons for the excellent safety record of certain drugs susceptible to bioactivation will also be explored and should provide valuable guidance in the use of reactive-metabolite assessments when nominating drug candidates for development. This will, in turn, help us to design and bring safer drugs to the market.

Introduction

The World Health Organization defines adverse drug reactions as “a response to a drug that is noxious, unintended and occurs at doses normally used in man for the prophylaxis, diagnosis, or therapy of disease or for modification of the physiological function.” Adverse drug reactions can be generally classified into type-A and type B reactions. Type-A reactions are associated with the primary pharmacology of the drug (e.g., risk of hypotension with antihypertensives) and are responsible for 80% of all side effects. Type-A adverse drug reactions can be detected in animal models of pharmacology and/or toxicology; they exhibit simple dose-response relationships and are usually avoided in the clinic via dose adjustments. In contrast, type-B (bizarre or idiosyncratic) adverse drug reactions are unrelated to known drug pharmacology, and although they are dose dependent in susceptible individuals, they do not occur at any dose in most patients. Because the frequency of occurrence of idiosyncratic adverse drug reactions is very low these reactions are often not detected, until the drug has gained broad exposure in a large patient population. Importantly, standard regulatory animal toxicity studies have traditionally shown a poor concordance with occurrence of idiosyncratic adverse drug reactions in humans.

Type B adverse drug reactions can affect any organ, but the most common organs involved are liver, skin, and bone marrow. The life-threatening type B adverse drug reactions noted for drugs include hepatotoxicity, severe cutaneous reactions, aplastic anemia and blood dyscrasias. Amongst these, hepatotoxicity is the most frequent reason for drug withdrawal and is also the major cause of attrition in drug discovery/development. Manifestations of liver injury can range from mild, asymptomatic changes in serum transaminases, which occur at high frequency with a number of drugs, to fulminant liver failure, which, although rare, can be potentially life-threatening and may necessitate a liver transplant. Considering that the liver is exposed to high concentrations of drug/metabolite(s) after oral administration, it is not altogether surprising that the organ is particularly vulnerable to damage by xenobiotics including drugs. The role of drug metabolism and reactive metabolites that cause these serious reactions have been investigated over the past 25 years and will be the focus of this review. Clinical management is still empirical, but recognition of a drug induced disease is important for future management of the patient.

Reactive Metabolites

The basic principle of drug metabolism is to convert a lipophilic drug or xenobiotic to hydrophilic metabolites that can be more readily excreted from the body. Sometimes during this process of biotransformation some of the drug or xenobiotic may be activated to chemically reactive species, i.e. reactive metabolites. This biotransformation of relatively inert chemicals to highly reactive intermediary metabolites is commonly referred to as metabolic activation or bioactivation, and it is known to be the initial event in many chemically induced toxicities. Some toxicants are direct acting and require no activation, whereas other chemicals may be activated nonenzymatically. The focus of this review, however, is on xenobiotics requiring metabolic activation and to those processes involved in activation.

In the 1940s and 1950s the pioneering studies of James and Elizabeth Miller provided early evidence for in vivo conversion of chemical carcinogens to reactive metabolites. They found that reactive metabolites of the aminoazo dye N,N-dimethyl-4-aminoazobenzene (DAB), a hepatocarcinogen in rats, would bind covalently to proteins and nucleic acids. The term, metabolic activation, was coined by the Millers to describe this process. Moreover they demonstrated that covalent binding of these chemicals was an essential part of the carcinogenic process. The overall scheme of metabolism for potentially toxic xenobiotics is outlined in Figure 1. As illustrated by this diagram, xenobiotic
metabolism can produce not only nontoxic metabolites, which are more polar and readily excreted (detoxification), but also highly reactive metabolites, which can interact with vital intracellular macromolecules, resulting in toxicity. In addition reactive metabolites can be detoxified for example, by interaction with glutathione (GSH).

Within the tissue a variety of reactions may occur depending on the nature of the reactive species and the physiology of the organism. Reactive metabolites are usually electron deficient molecules and are referred to as electrophiles (molecules containing positive centers). If not detoxified properly, these electrophiles can react with electron rich species, i.e. nucleophiles (molecules containing negative centers), through covalent bond formation. The nucleophiles usually contain atoms such as S, N, or O that have a lone pair of electrons, which can form a new bond to the electrophile. Such nucleophiles are present on macromolecules such as proteins, nucleic acids and lipids. Chemically reactive metabolites can directly react with proteins causing changes in protein structure or protein folding.11,12 These modified proteins are processed by antigen presenting cells and can look “foreign” to the immune system leading to an immune response. Chemically reactive electrophiles can also covalently react with nucleic acids on the DNA thereby causing changes in DNA structure or gene expression. Changes in DNA can lead to mutagenicity, teratogenicity or carcinogenicity.13-16

### Types of reactive metabolites

Reactive metabolites include such diverse groups as epoxides, quinones, free radicals, reactive oxygen species, and unstable conjugates. As a result of their high reactivity, reactive metabolites are often considered to be short-lived. This is not always true, however, because reactive intermediates can be transported from one tissue to another, where they may exert their deleterious effects.9 For example, carcinogenic aromatic amines are metabolized in the liver to the N-hydroxylated derivatives that, following sulfation and/or acetylation conjugation, are transported to the bladder, where the N-hydroxy derivative is released under the acidic conditions of urine.16

As mentioned above, reactive metabolites are usually electrophiles or free radicals. Electrophiles can be further subdivided into hard or soft depending on how concentrated or diffuse their electron deficient site is. Soft electrophiles are generally uncharged and are less electrophilic. Example of soft electrophiles are Michael acceptor, quinine methide and iminoquinone. Hard electrophiles are generally small and charged such as alkyl carbocation, carbonyl carbocation and nitrenium ion.17 Soft electrophiles react with soft nucleophiles through orbital interactions, while hard electrophiles react with hard nucleophiles to a large degree through electrostatic interactions. Examples of soft nucleophiles are RSH, GSH, RS-, F-, RSe-, alkenes and R,P (soft because the nucleophilic site is a sulfur atom, which is relatively large and hence its electron cloud is more polarizable). In contrast, R-NH2, R-OH, RO-, SO2- and Cl- are hard nucleophiles because nitrogen is small and much less polarizable relative to sulfur. Several factors play a role in the formation and reactivity of these reactive metabolites. These factors include the presence of a good leaving group (such as sulfate, sulfonate, chloride, and acetate), ring strain, polarization of a double bond by a carbonyl group (Michael acceptor), and the presence of electron withdrawing groups.11

Free radicals are another kind of reactive metabolite that can be formed by xenobiotics.39 Free radical refers to compounds having an unpaired electron. Since electrons “like” to be paired to form a chemical bond, a free radical cannot react covalently with nucleophiles. Rather they react with another free radical to form a covalent bond, abstract a hydrogen atom from a neutral molecule to generate a new radical, or abstract an electron to form an anion and generate a radical cation. An example of a free radical mechanism is the ring opening of cyclopropyl ring of tertiary amine as in trovofloxacin (Fig. 2). It has been reported that trovofloxacin-induced hepatotoxicity may be mediated through the oxidation of the cyclopropylamine substructure to reactive intermediates that may form covalent adducts to hepatic proteins, resulting in damage to liver tissue.19

Free radicals can occur, for example, in lipids, amino acids, nucleotides, and oxygen compounds. Oxygen radicals are of particular importance as they can trigger the formation of all other radicals. Other oxygen containing species that are chemically not radicals also have high reactivity with biological substances. Jointly, these substances are known as reactive oxygen species (ROS) (Fig. 3). In addition to ROS, there are radicals that contain an additional nitrogen atom for example, nitric oxide (NO). NO can be generated enzymatically by the so-called NO synthases (NOS) or non-enzymatically by nitrite (NO2). NO has important signalling and protective functions; in 1998, a Nobel Prize was awarded for their discovery. NO/NO2- and ROS, in turn, can react with each other. This generates peroxyxinitrite (ONOO-), the most reactive compound of all ROS, which can oxidize and nitrate proteins, lipids, and nucleic acids.20

It is worthwhile to note that free radicals are a two-edged sword. On the one hand, they have important physiological functions. In addition to NO, which is an important protective factor in the vasculature and a neurotransmitter in the nervous system,21 oxygen radicals are, for example, essential in the immune defence, as well as in the regulation of cellular growth and gene expression.22 But too much of a good thing can literally be harmful,
because radicals are also highly dangerous by-products of the cellular metabolism. An oxidative imbalance, i.e., a disturbance in the balance between the production of ROS (especially free radicals) and antioxidant defenses, has been described as an oxidative stress status. This can result in several kinds of cell damage, leading to a loss of function and integrity. Undesirable effects include inactivation of NO as a result of a direct chemical reaction with ROS and oxidative damage of cell components such as DNA and proteins. These effects are potentially involved in the development of a large number of pathological conditions, including cardiovascular diseases, neurological disorders, cancer and aging process.

**Reactivity and toxicity of reactive metabolites.** The relationship between drug metabolism and adverse drug reactions was first demonstrated with the analgesic agent paracetamol. Paracetamol is a major cause of drug-related morbidity and mortality in humans, capable of producing hepatic necrosis after a single toxic overdose. At normal therapeutic doses, paracetamol is safe, but can be hepatotoxic at high doses. The major portion of paracetamol is conjugated with either sulfate or glucuronic acid to form water-soluble, readily excreted metabolites and only small amounts of the reactive intermediate, believed to be N-acetyl-p-benzoquinonimine (NAPQI), are formed by the cytochrome P450 enzymes (Figure 4). When therapeutic doses of paracetamol are ingested, the small amount of reactive intermediate forms is efficiently deactivated by conjugation with GSH. When large doses are ingested, however, the sulfate and glucuronide cofactors (PAPS and UDPGA) become depleted, resulting in more of the paracetamol being metabolized to the reactive intermediate.

As long as GSH is available, most of the reactive intermediate can be detoxified. When the concentration of GSH in the liver also becomes depleted, however, covalent binding to sulphydryl (-SH) groups of various cellular proteins increases, resulting in...
replicated in animals.35-37 In contrast, drugs like the antidiabetic consider as idiosyncratic as they are dose dependent and can be 
the hepatotoxic effects of paracetamol in humans can hardly be 
dose-dependent and dose-independent adverse drug reactions. 
important to make a distinction here between drugs that exhibit 
demonstrated.38 These examples provide a circumstantial link 
between reactive-metabolite formation and toxicity. It is very 
paracetamol, which exhibits dose-independent hepatotoxic- 
ity in a very small segment of the population, is a true example of 
mechanism-based inactivation of multiple human P450 enzymes.47 The time- and 
inactivation can translate into clinical drug-drug interactions, 
Overall, given the potential for drug-drug interactions via enzyme 
involved in multiple cases of liver 
In certain cases the metabolites are so reactive that they do not 
that formed them and covalently bind to the 
enzyme leading to irreversible inhibition.39,40 Because metabolism 
precludes enzyme inactivation, these compounds fall into the cat-
egory of mechanism-based inactivators. Inactivation can occur 
via coordination of the reactive species with the heme prosthetic 
group (formation of a metabolite-inhibitor (MI) complex) or via 
covalent adduction of the reactive intermediate with heme and/or 
with an amino acid residue on the apoprotein. Cytochrome P450 
inactivation can translate into clinical drug-drug interactions, 
some of which can be potentially deleterious, and can lead to 
the withdrawal of the perpetrator drug. For instance, co-admin-
istration of the calcium-channel blocker and potent P450-3A4 
inactivator mibefradil and simvastatin in patients with hyperten-
sion has been associated with increased cases of myopathy includ-
ing rhabdomyolysis. The biochemical mechanism for the clinical 
drug-drug interactions involves the mechanism-based inactivation of 
the P450-3A4-catalyzed simvastatin metabolism process by mibefradil; 
a consequence which results in elevated plasma concentrations of the 
statin.41,42 Myopathy or rhabdomy-
alysis is a rare side effect common 
to the statin class of compounds and 
is usually associated with high levels of HMG-CoA reductase inhibitory activity in susceptible target tissue. 
Given the potential for such life-
threatening drug-drug interactions, 
the manufacturer of mibefradil 
announced a voluntary withdrawal of 
the drug from the market worldwide. 
Dietary supplements containing 
Piper methysticum (kava) have been 
implicated in multiple cases of liver 
injury in humans following the usage 
of kava-containing products. Its seda-
tives and anxiolytic benefits have been 
reported.45 Consequently, over-the-counter sales of kava herbal 
preparations have been banned in several countries in the European 
Union. Studies on P450 inhibition with kavalactone derivatives 
methysticin and 7,8-dihydromethysticin (Fig. 5), the major 
constituents of kava extract,46 have revealed potent mechanism-based 
inactivation of multiple human P450 enzymes.47 The time- and 
NADPH-dependent formation of the 455-nm absorbing MI com-
pound is consistent with a bioactivation mechanism involving the 
metabolism of the 1,3-benzodioxole group to the corresponding car-
bene intermediate followed by its coordination with the heme iron.47 
Consistent with this finding, the kavalactone kawain (Fig. 5), 
which differs from methysticin in that it does not contain the 
1,3-benzodioxole group is not a P450 inactivator.47 GSH conjugates 
of electrophilic ortho-quinone intermediates obtained via the 
biotransformation sequence (1,3-benzodioxole→catechol→ortho-
quinone) in methysticin and 7,8-dihydromethysticin have also 
been identified in rat and human liver microsomes (Fig. 5), and 
the involvement of these reactive quinonoid intermediates in the immu-
noallergenic hepatotoxic effects of kava extract has been speculated.48 
Overall, given the potential for drug-drug interactions via enzyme 
inactivation, mechanism-based inactivation of major human P450 
enzymes by new compounds is routinely assessed in a drug-
discovery paradigm. 
Reactive metabolites which covalently bind to the DNA have 
a dominant role in the mutagenicity and carcinogenicity. The concept of genotoxic/mutagenic response arising from metabo-
In the 1930s and the 1940s to account 
for the carcinogenicity of chemically inert polycyclic aromatic hydrocarbons, aminoazo dyes, and nitroso compounds.49,50 All 
pharmaceutical companies utilize a standard battery of genetic
toxicology assays to test the mutagenic potential of drug candidates.51 These assays measure several different types of genetic damage in a variety of cell types to increase the probability of detecting a mutagenic response. The endpoints routinely monitored include the induction of gene mutations and chromosomal aberrations in bacteria and mammalian cells, respectively, as well as the production of DNA strand breaks, DNA intercalation, and covalent modification.

An ariclor-1254-induced rat liver S-9/NADPH system has been adopted in these in vitro tests for detecting pro-mutagens capable of forming DNA-reactive metabolites.52 Genetic toxicology assessments have become an integral part of drug safety evaluation and are required by regulatory agencies for drug approvals worldwide. Because a good correlation has been established between in vitro metabolism dependent mutagenic response and the outcome of rodent carcinogenicity evaluations, drug candidates intended for non-life-threatening indications are generally discontinued from development, when they exhibit a positive response in the in vitro assays in the presence of S-9/NADPH. An example of this phenomenon was highlighted with a study on the anti-obesity agent and 5-hydroxytryptamine (5-HT)2C agonist 2-(3-chlorobenzyloxy)-6-(piperazin-1-yl)pyrazine (1; Figure 6).40 The attractive in vitro/in vivo pharmacology and pharmacokinetic attributes of 1 were offset by its S-9/NADPH-dependent genotoxic effects in the bacterial Salmonella Ames assay, which led to its discontinuation from clinical development. Studies with (14C)-1 revealed the irreversible and concentration-dependent incorporation of radioactivity in calf thymus DNA in an S-9/NADPH-dependent fashion confirming that 1 was bioactivated to a DNA-reactive metabolite.

Reactive-metabolite trapping studies in S-9/NADPH incubations containing exogenously added hard and soft nucleophilic trapping agents methoxylamine and GSH, respectively, led to the detection of conjugates of 1 and its downstream metabolites. Structural elucidation of these conjugates by mass spectrometry allowed an insight into the bioactivation pathways leading to the formation of DNA-reactive metabolites. The mass spectrum of the methoxylamine conjugate of 1 was consistent with condensation of amine with an electrophilic, aldehyde metabolite derived from piperazine ring scission in 1 (Fig. 6, Pathway a), whereas, the mass spectrum of the GSH conjugate suggested a bioactivation pathway involving initial aromatic ring hydroxylation on the 3-chlorobenzyl motif in 1, followed by β-elimination to a quinone-methide species that reacted with GSH (Fig. 6, Pathway b). The observation that methoxylamine and GSH reduced mutagenicity suggested that the trapping agents competed with DNA towards reaction with the reactive metabolites.

Perhaps the most important susceptibility factor for type-B adverse drug reactions is genetic variability. Genetic polymorphisms have a strong influence on drug metabolism and may increase risk of toxicity. For example, polymorphism of the N-acetyltransferase (NAT) 2 gene differentiates fast from slow acetylators; the latter have increased susceptibility to toxicity of certain aniline-containing drugs such as isoniazid, sulfamethoxazole, dapsone, and procainamide.53,54 The major route of elimination of these drugs in humans involves N-acetylation of the aniline moiety by NAT2, resulting in the neutral amide metabolites. In a NAT2-deficient population, the aniline motif in isoniazid is hydrolyzed by amidases liberating hydrazine, which is toxic in its own right; likewise, sulfamethoxazole, dapsone, and procainamide are biotransformed by P450 enzymes to yield cytotoxic and protein-reactive metabolites that include N-hydroxyaniline derivatives and the subsequent two-electron oxidation products, i.e., the nitroso intermediates.55,56 The reactivity of the nitroso metabolites of these drugs with GSH and/or proteins in target organs has also been demonstrated.55,56

Genetic polymorphisms in glutathione-S-transferase (GST) isozymes, which catalyze GSH conjugation to reactive metabolites, are also considered risk factors for hepatotoxicity caused by several drugs such as troglitazone and carbamazepine.57,58
It is possible that patients deficient in GST isozymes are most at risk towards liver injury by reactive metabolites of troglitazone and carbamazepine because of ineffective scavenging of the reactive metabolites derived from the oxidative bioactivation of these drugs. There is also a strong possibility that components of ingested foods including herbal supplements can modulate drug metabolism and, therefore, increase idiosyncratic adverse drug reaction risk. For example, chronic alcohol abuse increases the risk of paracetamol hepatotoxicity by inducing P450-2E1, which predominantly catalyzes paracetamol bioactivation to NAPQI.59

Individual group summaries. Most reactive metabolites are formed by phase-I metabolic pathways; however, sometimes phase-II pathways can also generate reactive metabolites. Usually phase-II metabolism, such as the conjugation reactions (glucuronidation and sulfation), increases the polarity of a drug making it more polar and readily excreted from the body. In rare cases, these conjugates can be chemically reactive leading to toxicity. In the following section some reactive metabolites of drugs or xenobiotics are discussed with respect to their reactivity and deleterious effects. Elucidation of bioactivation pathways provides valuable information that can lead to designing safer drug candidates.

Quinones. Quinones represent one of the most frequently generated reactive intermediates. Quinones are known to cause a variety of toxicological effects in vivo including acute cytotoxicity, immunotoxicity, genotoxicity and carcinogenesis.60-63 Quinones can be viewed as Michael acceptors and can cause cellular damage through alkylation of crucial cellular proteins and/or DNA. Quinones are also highly redox active molecules, which can redox cycle with their semiquinone radicals leading to formation of ROS including superoxide, hydrogen peroxide, and ultimately the hydroxyl radical. Production of ROS can cause severe oxidative stress within cells through the formation of oxidized cellular macromolecules, including lipids, proteins, and DNA (Fig. 7).

Benzene, a common solvent used in organic chemistry, is converted to hydroquinone by liver P450, and the subsequent peroxidase-catalyzed oxidation of this metabolite to \( \rho \)-benzoquinone in the bone marrow may explain the induction of leukaemia during chronic exposure to this solvent. The fact that \( \rho \)-benzoquinone is known to form DNA adducts strengthens this hypothesis.64 It has been demonstrated that inhibition of human topoisoenzymes I and II by high concentrations of quinoid metabolites of benzene; however, a remarkably greater sensitivity of the enzymes was detected with phenolic metabolites of benzene in the course of their bioactivation using a peroxidase/H\(_2\)O\(_2\) system. This suggests that free radical intermediates of phenolic oxidation (formed in the presence of peroxidase activity) may contribute to the clastogenic and carcinogenic effects of phenolic compounds through inhibition of topoisoenzymes.65

Etoposide has become one of the most widely used anticancer drugs in the world since its introduction.66 However, etoposide is a somatic and germ-cell mutagen capable of inducing both numerical and structural chromosome aberrations in animals.67-70 Moreover, numerous groups have reported that treatment schedules associated with the impressive efficacy of etoposide are also associated with an increased risk of secondary acute myeloid leukaemia.71-73 This has prompted the removal of this highly effective anti-topoisomerases II agent from some treatment regimens. It is believed that the mechanisms for the extremely high susceptibility of myeloid stem cells to the leukemogenic effects of etoposide is due to etoposide phenoxyl radicals (etoposide-O\(^•\)) formed from etoposide by myeloperoxidase.74,75 Etoposide-O\(^•\) is reduced back to etoposide via oxidation of intracellular thiols (RSH), i.e., glutathione and sulfhydryl groups of proteins. Reduced etoposide is thus readily available as a substrate for myeloperoxidase, at the expense of intracellular thiols, which undergo one electron oxidation to reactive thyl radicals (RS\(^•\)). RS\(^•\) can further react to generate disulfide anion-radicals (RS-S\(^–\)R\(^–\)), which can donate an electron to oxygen. Superoxide anion radical (O\(_2\)\(^–\)) thus produced can form, in the presence of transition metal complexes, the extremely reactive hydroxyl radical (HO\(^•\)). It is believed that accumulation of these radicals may cause damage to cell membrane leading to lipid peroxidation and also damage to cellular genome and other critical biomolecules, ultimately leading to cell death.
inducing mutagenicity and leukaemia (Fig. 8). It is worthwhile to note that several strategies have been developed to decrease the deleterious effects of etoposide in normal cells using nutritional antioxidants such as vitamin C, vitamin E homolog, and the metal-chelator, dextrazoxane. Ameliorations of these deleterious effects were associated with a concomitant alteration of the antioxidant potential.

Remoxipride is an atypical antipsychotic used in the treatment of schizophrenia. It is associated with rare cases of aplastic anaemia. It has been demonstrated that the metabolite of remoxipride, NCQ344, forms a reactive p-quinone, which might be responsible for the aplastic anaemia associated with remoxipride (Fig. 9).

Iminoquinones. Iminoquinones are another class of reactive metabolites formed by the substitution of one of the oxygen atoms of the quinone with nitrogen; they can also act as Michael acceptors. Because nitrogen is less electronegative than oxygen, the reactivity of an iminoquinone will be relatively lower compared to a quinone. Once generated, an iminoquinone can covalently bind to protein nucleophiles and cause toxicity. A number of drugs and chemicals of environmental importance can be converted to reactive quinonimines. The 4-aminoquinoline antimalarial, amodiaquine, was withdrawn from the market because of idiosyncratic agranulocytosis and hepatotoxicity, and this was attributed to its bioactivation to a quinonimine metabolite.80 Iminoquinones preferentially react with thiol nucleophiles. In the case of the drug, acetaminophen, the reactive intermediate NAPQI, once formed, interacts with protein thiols, including that of the plasma membrane Ca\(^{2+}\)-ATPase, causing increased cytosolic calcium concentrations, adverse cytoskeletal effects, and cell death.81 Lumaricoxib is a new COX-2 inhibitor used for the treatment of inflammatory diseases. It is associated with idiosyncratic hepatotoxicity in patients. It has been demonstrated that lumaricoxib is bioactivated to a reactive quinone (Fig. 10), which might be responsible for causing idiosyncratic hepatotoxicity.82

Quinonemethide. Quinonemethide is a class of reactive metabolite in which one of the oxygens of a quinone is substituted by carbon. Because of this, the double bond is polarized and can react in a Michael fashion with nucleophiles. The reactivity of the quinone methide depends on the other substituents. A series of o-methoxy-4-alkylphenols were used to investigate the electrophilicity and toxicity of quinonemethide intermediates. It was observed that the reactivity of the corresponding quinone methide was influenced by the presence and nature of substituents on the benzylic carbon; increased steric hindrance of the exocyclic methylene group decreased the rate of nucleophilic attack.

Moreover, the quinone methides formed from o-methoxy-4-alkylphenols of intermediate reactivity were the most cytotoxic, presumably because quinone methides that do not exhibit this optimum reactivity are either too stable to react with critical cellular nucleophiles or so reactive with solvent so as to preclude reaction with critical cellular nucleophiles.83 Acolbifene is a selective estrogen receptor modulator developed for the treatment of breast cancer. It has been demonstrated that acolbifene can be bioactivated to electrophilic quinonemethide and diquinonemethide reactive intermediates, which could cause further toxicity (Fig. 11). It has also been shown that these reactive metabolites bind with the deoxynucleosides causing DNA damage.

Arene oxides and epoxides. Arene oxides are another class of reactive intermediates formed by numerous drugs that are associated with idiosyncratic drug reactions. Cytochrome P450 enzymes are capable of catalyzing this biotransformation in aromatic compounds. In the case of alkenes, the corresponding epoxides are formed. Both arene oxides and epoxides are reactive and are capable of reacting with nucleophilic proteins. The major in vivo detoxification pathways for these arene oxides involve enzymes such as epoxide hydrolase and glutathione-S-transferase. An example of an epoxide is a metabolite of the drug carbamazepine. Carbamazepine is an anticonvulsant drug associated with a variety of idiosyncratic drug reactions such as skin rashes, aplastic anaemia, hepatitis, and generalized anticonvulsant hypersensitivity syndrome. Carbamazepine is biotransformed to a weakly
reactive 10, 11-epoxide by P450-3A4/P450-2C8 (Fig. 12). This is deactivated by epoxide hydrolase to dihydrodiols. 86

Zomepirac is an anti-inflammatory drug (NSAID), which is associated with severe and fatal anaphylactic reactions because of which it was withdrawn from the market in 1983. It has been shown that zomepirac is bioactivated to arene oxide reactive intermediates, which could possibly cause these adverse reactions (Fig. 13). 87

**Glucuronidation.** Glucuronidation is a process by which a drug or other substrate, which usually contain OH groups i.e., phenols, carboxylic acids, and alcohols, are cleared and detoxified. In the case of diclofenac, the carboxylic acid is converted to a glucuronide. This glucuronide is reactive and slowly reacts with protein amino groups leading to covalent binding. 88,89 In some cases, the acyl glucuronide also undergoes ring opening and an Amadori rearrangement that can lead to the formation of irreversible protein binding (Fig. 14). Carboxylic acids can also form Co-A esters, which are reactive because the Co-A group is a good leaving group. These esters may also contribute significantly to the covalent binding associated with carboxylic acids.90

**Sulfation.** Sulfation is another phase-II pathway in which the SO3- functional group is transferred to the substrate through a sulfotransferase enzyme. The usual substrates for this type of reaction are hydroxyl or phenolic groups. Substrates containing nitrogen may also undergo this bio-transformation. Usually these sulfate conjugates are nontoxic and can be excreted from the body. But in some cases, the sulfate group in the conjugate is reactive and can be displaced by a nucleophile or act as a leaving group to form a cation,91 that can covalently bind to a protein leading to toxicity. An example is the bioactivation of an antifungal compound called N-(3,5-dichlorophenyl) succinimide (NDPS), which is known to cause nephrotoxicity. It was shown that its hydroxyl metabolite N-(3,5-dichlorophenyl)-2-hydroxysuccinimide (NDHS) is bioactivated to an activated O-sulfate that is likely responsible for the toxicity (Fig. 15). 92

**Detection and screening for reactive metabolites.** Given the lack of availability of preclinical models as reliable predictors of idiosyncratic adverse drug reactions and the absence of relevant clinical safety biomarkers, it is currently impossible to accurately predict which new drugs will be associated with a significant incidence of idiosyncratic adverse drug reactions. Under the assumption that reactive metabolites, as opposed to the parent molecules from which they are derived, can be responsible for the pathogenesis of certain toxicities, most pharmaceutical companies have implemented assays to evaluate a compound’s potential to undergo bioactivation with the goal of eliminating or minimizing reactive-metabolite formation by rational structural modification of the problematic chemical series.
**Avoiding structural alerts.**

The initial step to avoid the formation of reactive metabolites is to identify functional groups that are known to form reactive metabolites and avoid these functional groups in the structure of drug candidates. For example, functional groups such as aromatic amines are known to form reactive nitroso species.\(^{93}\) Another example is the hydrazine functional group that can form reactive carbocation species.\(^{94}\) A good review of structural alerts is found in the publication of Kalgutkar et al.\(^{38,95}\) Of course elimination of reactive metabolite formation will be of no benefit if it also eliminates the therapeutic effects of the drug.

**Testing for GSH conjugates.**

One of the methods to detect the formation of electrophilic intermediates is to look for GSH conjugates of the drug. GSH is a major scavenger of reactive metabolites and hence the detection of a GSH conjugate is an indication of the formation of a reactive intermediate. Formation of GSH conjugates can be detected by mass spectrometry, which, in turn, provides insight into the reactive metabolite structure.\(^{96,97}\) With the possible exception of acyl glucuronides and cyclic iminium ions, most reactive metabolites are generally short-lived and are not usually detectable in circulation. Their formation can often be inferred from stable conjugates obtained via reaction with the endogenous anti-oxidant GSH. The presence of the soft nucleophilic sulfhydryl group in GSH ensures efficient conjugation with soft electrophilic centers on reactive species (e.g., Michael acceptors, epoxides, arene oxides, and alkyl halides) yielding stable sulfhydryl conjugates.\(^{38,98}\)

Qualitative in vitro assessment of reactive-metabolite formation usually involve ‘trapping’ studies conducted with NADPH-supplemented human liver microsomes and GSH; analysis of the resulting metabolites by mass spectrometry is employed to characterize the structure of GSH conjugates, which, in turn, provides insight into the reactive metabolite structure.

![Figure 10. Bioactivation of lumarioxib to a reactive p-iminoquinone.](image1)

![Figure 11. Bioactivation of acolbifene to reactive p-quinonemethide and p-diquinonemethide metabolites.](image2)
Considering that drug-metabolizing enzymes other than cytochrome P450 [e.g., monoamine oxidases, aldehyde oxidase, alcohol dehydrogenases, myeloperoxidase, uridine 5'-diphosphoglucuronosyl transferase (UGT), and sulfotransferases] are also capable of catalyzing bioactivation, due consideration must be given to the use of alternate metabolism vectors (e.g., S-9 fractions, hepatocytes, neutrophils, monocytes, etc.), which support the activity of these enzymes. This is especially important in cases where multiple enzymatic and/or chemical steps may be involved in the production of the reactive metabolite. It is noteworthy to point out that not all reactive metabolites can be trapped with GSH. Hard electrophiles including DNA-reactive metabolites (e.g., electrophilic carbonyl compounds) will preferentially react with hard nucleophiles such as amines (e.g., semicarbazide and methoxylamine), amino acids (e.g., lysine), and DNA bases (e.g., guanine and cytosine) affording the corresponding Schiff bases.40

Likewise, the cyanide anion, N-acetyllysine and methoxylamine are 'hard' nucleophiles that can be used to trap hard electrophiles such as electrophilic iminium species that are generated via metabolism of tertiary amines.99,100 Usually GSH conjugate screening is performed on samples generated in vitro (using microsomes or hepatocytes), because in vivo, the conjugates might be transported to bile and destroyed by gut bacteria. This is more likely for high molecular weight drugs. Since non-microsomal drug metabolizing enzymes are also capable of catalyzing the bioactivation processes, due consideration must be given to the use of metabolic systems other than liver microsomes such as liver cytosol, the S-9 fraction, or hepatocytes. In the case of drugs causing hematological toxicity such as agranulocytosis or bone marrow toxicity, the myeloperoxidase enzyme system is likely to be more appropriate.101

In the case of drugs that form short-lived free radical intermediates,102 the reactive intermediates can often be trapped using free radical trapping agents such as α-phenyl-N-t-butyl nitrite, which is commonly used to trap nitrogen free radicals.101 Other spin trapping agents such as 2-methyl-2-nitroso-propane (MNP) to trap carbon-centered free radicals,5,5-dimethyl-pyrroline-N-oxide (DMPO) to trap hydroxy, carbon-centered, and phenyl radicals and 5-diethoxophosphoryl-5-methyl-1-pyrroline-N-oxide (DEPMPO) to trap S-centered radicals such as glutathionyl (GS•) and the sulfite radical anion (SO3•) have also been used. The trapped radicals can then be studied using electron spin resonance (ESR). Drugs that possibly form free radicals such as aminoglutethimide,108 procainamide,109 and phenytoin,18 can be detected using the above trapping agents.

**Enzyme inactivation studies.** As mentioned above, in certain cases the metabolites are so reactive that they do not escape the enzyme that formed them and covalently bind to the enzyme leading to irreversible inhibition.39 This is referred to as mechanism-based inactivation as mentioned above, and in the case of P450 enzymes, it may result from irreversible alkylation of an active site amino acid or the heme prosthetic group or a combination of both alkylation of heme inactivates P450, whereas amino acid alkylation does not always result in loss of catalytic activity. Inactivation of P450 enzymes often translates into clinically important drug-drug interactions. Enzyme kinetic studies can provide clues to the existence of mechanism-based inhibition.

**Covalent binding studies.** An important method for detecting and quantifying reactive metabolite formation is the use of radiolabelled drug to study the irreversible binding of the drug. Measurement of the amount of in vitro metabolism dependent covalent binding to biological tissue is possible if radiolabeled drug is available.39 The assay provides quantitative estimates of radioactivity irreversibly bound to tissue but does not correctly provide information about the nature of covalently modified proteins as discussed below. Covalent binding studies can be performed in vivo as well. Either tissue or blood/plasma can be examined for the degree of covalent binding. However, covalent binding may require multiple dosing to establish the true impact of the compound. Reactive metabolites formed after the first dose may be efficiently

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**Figure 12.** Bioactivation of carbamazepine to a reactive carbamazepine-10, 11-epoxide intermediate.

**Figure 13.** Bioactivation of zomepirac to a reactive arene oxide intermediate.
trapped by GSH and eliminated from the body. Once GSH is depleted, the extent of covalent binding with cellular macromolecules may increase rapidly, resulting in toxicity. This is certainly the case with paracetamol where its reactive metabolite can cause direct hepatotoxicity upon an overdose, and yet paracetamol is rarely associated with idiosyncratic adverse drug reactions. This is because, at usual therapeutic doses, the phenolic group in paracetamol undergoes phase-II glucuronidation and sulfonation, resulting in a small amount of NAPQI formed; most of which is scavenged by GSH before it binds to macromolecules. The hypothesis has been strengthened based on studies in mice which have shown that significant covalent binding does not occur until over 60–80% of the paracetamol overdose has been eliminated from the liver with concomitant reduction in GSH levels.37

An example of this overall approach is highlighted with studies on the potassium-channel opener, maxipost (BMS-204352) (Fig. 16), which undergoes P450-mediated bioactivation in rats, dogs, and humans to generate a reactive ortho-quinone-methide intermediate, which covalently binds to protein in vivo in animals and humans.110-111 Acidic hydrolysis of plasma collected after intravenous administration of (14C)-BMS-204352 to rats and human led to the characterization of a unique lysine conjugate of des-fluoro des-O-methyl BMS-204352 (Fig. 16).

Recent studies examining P450-mediated covalent binding of 18 drugs (nine hepatotoxins and nine non-hepatotoxins) to liver microsomes, S-9, and/or hepatocytes show no correlation between extent of covalent binding and toxicity.112,113 Indeed, covalent binding study can also make it possible to determine to which proteins the reactive metabolite is bound but this method can also give false negatives if the wrong metabolism system is used. For example the drug trimethoprim is bioactivated by neutrophils or myeloperoxidase, which is the major oxidative enzyme in these cells. So this enzyme system was used to investigate the bioactivation potential of the drug. Trimethoprim is also associated with liver toxicity and hence its investigation of bioactivation by P450 was also carried out. So it is important to use the right enzyme system depending upon the site of toxicity. Although there are major advantages to an in vitro system, the
potent drug, even if it is efficiently converted to a reactive metabolite, the amount will still be too low to represent a significant liability. It is noteworthy to point out that there are no examples of drugs that are dosed at <20 mg/day that cause idiosyncratic adverse drug reactions (whether or not these agents are prone to bioactivation). There are many examples of two structurally related drugs that possess identical toxicophore susceptible to bioactivation, but the one administered at the lower dose is safer than the one given at a higher dose. An illustration of this concept is evident with the antidiabetic thiazolidinedione drugs troglitazone (200–400 mg/day), rosiglitazone, and pioglitazone (<10 mg/day). Troglitazone was withdrawn from the United States market after numerous reported cases of liver failures requiring immediate liver transplantation or leading to death. In contrast, rosiglitazone and pioglitazone are devoid of the hepatotoxicity associated with troglitazone. Another consideration is the structure of the drug and presumed reactive species and how hard it would be to eliminate reactive metabolite formation and still have an active drug. In some cases such as proton pump inhibitors, the pharmacological activity depends on covalent binding of the drug to the enzyme; therefore, it would be impossible to eliminate covalent binding. Other risk versus benefit considerations in using this data are discussed below.

**Immunological studies.** It is also important to know what proteins are modified by a reactive metabolite. Although radiochemical methods can be used, it is more common to use immunological methods to determine which proteins are modified. This involves synthesis of an immunogen (i.e. reactive metabolite adduct conjugated to protein), against which antibodies are raised via immunization of animals with the immunogen. The antiserum is screened for anti-drug antibodies using an enzyme-linked immunosorbent assay. The antibody then can be used to identify haptenized biomacromolecules via Western blot analysis. The bands can then be cut out and the modified proteins identified by mass spectrometry. While this method can be a very powerful technique for the identification and characterization of cellular constituents that have undergone covalent modification by a
reactive metabolite, it would be impractical to use as a screening tool because an antibody would have to be generated to each drug candidate. It is also not quantitative.

Unfortunately, without a valid animal model, it is practically impossible to determine whether a specific reactive metabolite is responsible for a given idiosyncratic drug reaction. Therefore, we are left with trying to infer causality. Given their unpredictable nature, idiosyncratic drug reactions are generally not detected until the drug is released onto the market because clinical trials involve a limited number of subjects. If a drug is found to be associated with an unacceptable risk of serious idiosyncratic drug reactions it will cause a huge financial loss to the pharmaceutical company involved because drug development is a very expensive process. The current cost of drug development has been pegged at $1.3 billion US as per a 2009 report. Even though one can minimize the ability of drugs to cause bioactivation, it is hard to predict which compounds will cause idiosyncratic drug reactions because not all covalent binding is associated with the same risk.

The existing in vitro tests are not able to mimic the complexity of in vivo biological systems, and our present mechanistic understanding of idiosyncratic drug reactions is quite superficial. Therefore, animal models are an essential tool for mechanistic studies. They might also be used to screen compounds in development with similar structures to predict which are most likely to cause idiosyncratic drug reactions. There are currently only two practical animal models that appear to involve the same mechanism as the idiosyncratic drug reactions that occur in humans. They are nevirapine-induced skin rash, and D-penicillamine-induced autoimmunity. Development of further animal models is necessary to understand the mechanisms underlying idiosyncratic drug reactions. It is unlikely that significant progress will be made in preventing idiosyncratic drug reactions until we have a better mechanistic understanding of these adverse reactions.

There are also other animal models of idiosyncratic drug reactions such as propylthiouracil-induced autoimmunity in cats; however, cats are not a practical species to work with. Another model is halothane-induced liver injury in guinea pigs; however, in this model halothane exposure never leads to significant damage and is milder on re-exposure rather than being worse as occurs in humans. A good discussion of these animal models is available in the review by Shenton et al. Nevertheless, there are not many models of idiosyncratic drug reactions and developing more animal models would significantly contribute to a better understanding of underlying mechanisms associated with idiosyncratic drug reactions and ultimately help in the development of safer drugs.

Risk Versus Benefit

Testing of drug candidates for the formation of the reactive metabolites would likely result in safer drugs entering development. It can also result in false positives and false negatives. Nevertheless a decision has to be reached whether to advance compounds to development. Until we develop a better understanding of the risk of toxicity arising from the formation of reactive metabolites, a strategy for identifying and potentially minimizing their formation via rational and iterative medicinal chemistry efforts seems logical in certain cases. It is noteworthy to point out that reactive-metabolite formation is only one aspect of the overall risk/benefit assessment for advancing a drug candidate into development. Consequently, bioactivation data (reactive metabolite trapping and/or covalent binding) needs to be placed in a broader context with due consideration given to the following points (Fig. 17):

1. Is the drug intended to address a previously unmet medical need or a lifethreatening disease?
2. Is the drug candidate intended to provide proof-of-mechanism for a novel target?
3. Is the drug intended for acute or chronic use?
4. Is the clinical dose predicted to be low?
5. What is the intended patient population (e.g., would it be given to immunocompromised patients or patients with impaired liver functions)?
6. Are there alternate chemical series with comparable pharmacologic and pharmacokinetic attributes, wherein bioactivation liability is minimized or eliminated?
7. Is there an alternative (higher affinity but innocuous) route of metabolism within the drug candidate that minimizes bioactivation liability associated with the compound?
8. Is metabolism the exclusive route of elimination? What is the likelihood of nonmetabolic elimination processes (e.g., renal and/or biliary excretion of unchanged parent) in humans?

If the benefits outweigh the risk, the compounds can be advanced cautiously. In conclusions, a critical point of this review is the possibility that the reactive metabolites must be considered as “structural alerts” or “toxicophores” that should be avoided in drug development. Then the question is whether drug candidates that can form reactive metabolites must be totally avoided? There is no simple answer to this question. At least two major factors seem to be important: the dose of the drug and the amount of covalent binding. Any drug that is taken at a total dose of 20 mg/day or less is unlikely to be associated with a high incidence
of idiosyncratic drug reactions in humans.

Sometimes the marketed drug may contain a structural alert or toxiphore but still not cause a significant incidence of idiosyncratic drug reactions. Again, total exposure to a reactive metabolite is important. One possible reason for this is that the toxiphore is not the primary site of metabolism and therefore not much of the potential reactive metabolite is formed. Even if bioactivation is the major pathway in vitro, a microsomal incubation does not contain all of the metabolic enzymes, and there may be other major clearance pathways in vivo that do not lead to a reactive metabolite. An example of this is raloxifene; in vitro using microsome system, the major pathway is bioactivation of the phenolic metabolite leading to quinone intermediate whereas in vivo the principal mode of clearance is through the glucuronidation of phenolic metabolites rather than the bioactivation. It is associated with low incidence of idiosyncratic drug reactions presumably because it is associated with metabolites rather than the bioactivation. It is associated with the major pathway is bioactivation of the phenolic metabolite leading to quinone intermediate whereas in vivo the principal mode of clearance is through the glucuronidation of phenolic metabolites rather than the bioactivation. It is associated with low incidence of idiosyncratic drug reactions presumably because of the protective effect of glucuronidation.39,126 Another reason could be that the principle route of clearance is through a non-metabolic pathway such as renal elimination.38

Examples of strategies that could be followed in drug design to minimize the metabolic liability associated with reactive metabolite formation are: (a) Replacement of the structural alert with substituents that are resistant to metabolism or can be metabolized to nonreactive species; (b) Blocking the functional groups that are known to undergo bioactivation by a functional group that does not undergo activation; (c) Incorporating a bulky substituent close to the site of metabolism so that metabolism could not occur at the site of metabolic activation. Of course elimination of reactive metabolite formation will be of no benefit if it also eliminates the therapeutic effects of the drug and, therefore, it is essential that the pharmacological effects of drug candidates be tested at each step in the optimization of the structure.

Finally, it is now widely appreciated that reactive metabolites, as opposed to the parent molecules from which they are derived, are responsible for the pathogenesis of some idiosyncratic adverse drug reactions, it is essential to determine exactly how they induce idiosyncratic adverse drug reactions, and what factors determine which reactive metabolites are most likely to cause idiosyncratic adverse drug reactions, and which patients are at highest risk. This requires the development of animal models and the identification of which target proteins are most important for the induction of idiosyncratic adverse drug reactions. Knowledge gained through these processes will be useful to unravel the mysteries of the fascinating research area of idiosyncratic or bizarre adverse drug reactions. This will, in turn, help us to design and bring safer drugs to the market.

Acknowledgements

Editorial assistance by Prof. Kenneth Maiese is acknowledged.
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