Peroxidases: a role in the metabolism and side effects of drugs

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Current safety screening of drug candidates or new chemical entities for reactive metabolite formation focuses on the role of cytochrome P450. However, peroxidases also have a major role in drug metabolism, and peroxidase-catalyzed drug oxidation could lead to reactive metabolite formation, resulting in oxidative stress and cytotoxicity. Here, the different classes of human peroxidases are summarized and the molecular mechanisms of peroxidase-catalyzed drug metabolism are discussed. In addition, evidence is presented that indicates a role of these enzymes in drug toxicity.

Despite extensive animal testing and safety monitoring during clinical trials, >10% of new drugs that were approved between 1975 and 2000 either had to be withdrawn from the market or received a 'black box' warning [1]. The development of numerous other drugs has also been halted before or during trials because of safety concerns. Possible idiosyncratic drug toxicity might only appear in late-stage clinical trials, or even later. This idiosyncratic toxicity does not involve pharmacological properties of the drug, for example, no simple dose–response relationship, delayed onset (6–90 days) and rechallenge with the drug often produced severe consequences. One hypothesis is that the adverse reaction in these cases is immune-mediated and results from the modification of a protein by reactive metabolites, which, instead of inducing resistance or tolerance, creates an 'immunotoxic or immunogenic protein' and/or causes cell damage that stimulates the immune system [2]. Currently, it is thought that cytochrome P450 (CYP) is responsible for much of the reactive metabolite formation and, therefore, candidate drugs or new chemical entities (NCEs) are screened for drug–protein covalent binding when the drug is incubated with human liver microsomes and NADPH [1]. However, major target tissues for adverse reactions include the bone marrow (e.g. agranulocytosis) or skin (early rash sign), which are likely to be associated with peroxidase-catalyzed drug metabolic activation [3,4].

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The peroxidase family

The peroxidases are heme-containing enzymes that catalyze the one-electron oxidation of a variety of oxidizable xenobiotics using hydrogen peroxide (H_2O_2). The native enzyme contains a heme, usually ferriprotoporphyrin IX, with four pyrrole nitrogens bound to FeIII. The fifth coordination position on the proximal side of the heme is usually the imidazole side chain of a histidine residue. The sixth coordination position is vacant in the native enzyme on the distal side of the heme. The following cycle of reactions are involved in the oxidation of xenobiotics (Equations 1–3):

$$\text{Peroxidase} + \text{ROOH} \rightarrow \text{Compound I} + \text{ROH} \quad \text{[Eqn 1]}$$

$$\text{Compound I} + \text{XOH} \rightarrow \text{Compound II} + \text{XO}^{\bullet} + \text{H}^{+} \quad \text{[Eqn 2]}$$

$$\text{Compound II} + \text{XOH} \rightarrow \text{Peroxidase} + \text{XO}^{\bullet} + \text{H}_2\text{O} \quad \text{[Eqn 3]}$$

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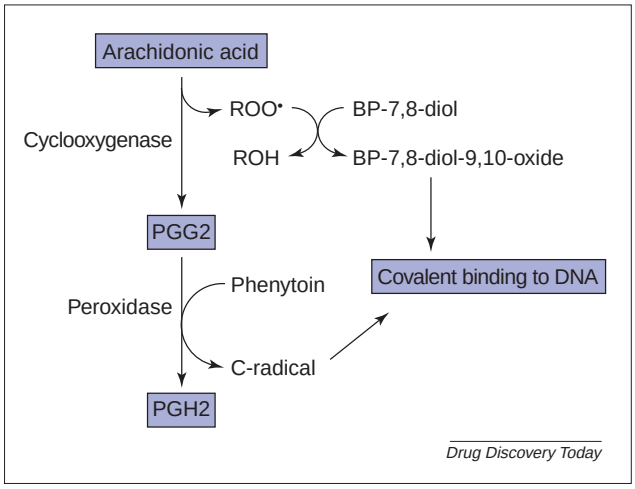

FIGURE 1

Activation of substrates by prostaglandin-endoperoxide H synthase enzymes. The cyclooxygenase and peroxidase catalytic sites contribute to the activation of substrates, for example, phenytoin is oxidized to a carbon radical by the peroxidase moiety [73]. Peroxyl radicals are formed at the cyclooxygenase catalytic site that can introduce an oxygen atom across an isolated double bond, as in the activation of benzopyrene-7,8-diol. Abbreviation: GS•, thiyl radical.

In the first step, peroxidases catalyze the oxidation of hydroperoxides (lose two electrons) to form the corresponding alcohol and a spectroscopically detectable higher oxidation state called Compound I. A diverse range of physiological or xenobiotic molecules with numerous cellular functions are oxidized by compound I. Spectral properties, including electron paramagnetic resonance (EPR) spectra, suggest that compound I comprises a Fe=O linkage, which is probably produced by the transfer of an electron from the porphyrin ring to form a porphyrin radical cation, for example, Por$^{\bullet+}$FeIV=O, or from the protein (usually a tyrosine residue) to form a phenoxyl radical. By contrast, compound II is well defined as a FeIV=O species in which the iron is one oxidation state higher than its resting FeIII state.

Peroxidases can be classified into two types according to their interaction with the substrate phenylhydrazine. The phenyl radical formed via the phenyldiazene intermediate binds to the heme-iron of human or animal catalase, hemoglobin, prostaglandin-endoperoxide H synthase (PGHS) and CYP, leading to inactivation of the enzyme. However, the covalent binding of the phenyl radical formed by the animal peroxidases myeloperoxidase (MPO), eosinophil peroxidase (EPO) and lactoperoxidase (LPO) to the δ-meso heme edge and the protein causes inactivation, which indicates that phenylhydrazine access to the heme-iron is restricted by the distal histidine and that phenylhydrazine is oxidized at the heme edge [5]. Thus, presumably, the phenyl radicals formed by human peroxidases cannot recombine with iron-bound oxygen radicals and are more likely to diffuse away and cause intracellular oxidative stress than the phenyl radicals formed by CYP or catalase.

Here, the differences in the molecular mechanisms of drug metabolism catalyzed by peroxidases are discussed and the substrate specificity and halogenating specificity are described, as well as the location of the various human peroxidases. In addition, evidence for peroxidase-catalyzed drug-induced agranulocytosis (bone marrow toxicity), hepatitis (liver toxicity) and skin rashes, and also the possible involvement of peroxidases in intestinal toxicity, uterine cancer, atherosclerosis and teratogenicity, are described. Finally, supporting evidence from recent transgenic mice research is discussed.

The human peroxidase family

The catalytic oxidation states of MPO, EPO and uterine, thyroid and gastric peroxidases, their ancestry, physiological role and intracellular location, have been reviewed previously [4]. The focus here is the role of PGHS peroxidase (PGHSP), MPO, EPO and catalase, and their drug metabolism and side effects. Although CYPs have peroxidase and peroxygenase activity, they have no known amino acid sequence homology with the peroxidases and are therefore discussed in a subsequent section.

Prostaglandin-endoperoxide H synthase peroxidase

PGHSP catalyzes a two-electron reduction of the 15-hydroperoxyl group of prostaglandin G2 (PGG2) to form prostaglandin H2 (PGH2) using a physiological donor (Figure 1): this reaction also activates the cyclooxygenase activity of the enzyme, which, once initiated, can operate independently of peroxidase activity [6]. Aspirin irreversibly inhibits the cyclooxygenase activity of PGHSP-I and PGHSP-II by acetylation of Ser530 and Ser516, respectively, but does not inhibit the peroxidase activity [7]. Although the physiological donor has yet to be identified, xenobiotics, including drugs, can act as a donor and are consequently oxidized (Figure 1). At the same time, the donor protects the PGHSP from inactivation by the hydroperoxide cofactors. 15-Hydroperoxyeicosatetraenoic acid is the most active hydroperoxide cofactor, but H_2O_2 can substitute for PGG2 in this peroxidase activity [4]. PGHSP has an important role in the activation of carcinogenic aromatic and heterocyclic amines resulting in extra hepatic tissue carcinogeneis (Table 1) [8]. Cosubstrates that are oxidized to carbon- or sulfur-centered free radicals can also trap molecular oxygen to form a peroxyl radical, which, in turn, can oxidize xenobiotics, including phenylbutazone, sulfate, retinoic acid, methylindole and indoleacetic acid [9].

Myeloperoxidase

MPO forms 2–5% of the dry weight of the human neutrophil, and is also present in monocytes and, to a lesser extent, tissue macrophages. Human neutrophils have much higher MPO levels and oxidant-generating capacity than rodents, which could suggest that peroxidase-catalyzed drug metabolism has a bigger role in humans

TABLE 1

Metabolic activation by prostaglandin-endoperoxide H synthase

Colon, kidney, bladder, breast and lung carcinogens (source or use)[a]
Benzidine (dyes)
4-Aminobiphenyl (smoking)
Benzopyrene-7,8-diol (smoking)
MeIQx (barbecued meat or fish)
IQ (barbecued meat or fish)
PhIP (barbecued meat or fish)
MOCA (curing agent)
Phenol (benzene metabolite)
o-Phenylphenol (fungicide)
Drug toxicity[b]
Phenylbutazone
Diethylstilbestrol
Phenytoin
Thalidomide
Cyclophosphamide

[a]Information adapted from [8]. [b]Information adapted from [4,9,73,74]. Abbreviations: IQ, 2-amino-1-methyl-6-phenylimidazo [4,5-*b*] pyridine; MeIQx, (+)benzo[*a*]pyrene-7,8-diol,2-amino-3,8-dimethylimidazo [4,5-*f*]quinoxaline; MOCA, 4,4′-methylene*bis*(2-chloroaniline); PhIP, 2-amino-3-methylimidazo [4,5-*f*]quinoline.

than rodents, which are the major species used for drug toxicity studies [10]. Human MPO and H_2O_2 (generated by activated NADPH oxidase) in neutrophils, Kupffer cells and bone marrow $CD34^+$ myeloid progenitor cells are thought to have an antimicrobial role [11,12].

MPO is unique among all peroxidases in catalyzing chloride oxidation to produce hypochlorite, which accounts for 20–70% of the H_2O_2 liberated by activated neutrophils. Hypochlorite is likely to be produced in the phagosomes, although the phagosomal chloride concentration is not known and appreciable amounts of hypochlorous acid (HOCl) are released into the plasma. Compound I performs a one-electron oxidation of a wide variety of aromatic amines (e.g. anilines and benzidines [13]) but is unique in catalyzing a two-electron oxidation of chloride to form hypochlorous acid (Equation 4). An acidic pH causes a loss of the distal His95 ligand of the heme that is already distorted by a sulfonium ion linkage with Met243, thereby facilitating the binding of chloride ions [14].

$$\text{MPO Compound I} + Cl^- + H^+ \rightarrow \text{MPO} + \text{HOCl} \quad \text{[Eqn 4]}$$

Eosinophil peroxidase

EOP generates HOCl at plasma chloride levels but is also the most active peroxidase for generating hypobromite (OBr^-). Bromide at plasma concentrations (20–100 μM) also markedly increased the HOCl activation and toxicity of acetaminophen, 4-aminophenol and *p*-phenetidine towards hepatocytes through the formation of hypobromous acid (HOBr; Equation 5) [15].

$$Br^- + HOCl \rightarrow HOBr + Cl^- \quad \text{[Eqn 5]}$$

The high peroxidase activity in rat intestine has been attributed to invading eosinophils rich in peroxidase, which provide an antibacterial system in the intestinal mucus. The intestinal peroxidase was purified and shown to have similar (including immunological) properties to EPO. Whereas invading EPO could be responsible for the majority of the total intestinal peroxidase activity, only 3% could be attributed to an intestinal epithelial cell peroxidase [16]. Much of the uterine peroxidase activity has been attributed to EPO [17].

Catalases

The catalases belong to a third peroxidase superfamily and are the products of a constitutive, housekeeping gene found in all cells that catalyzes H_2O_2 disproportionation detoxication to form O_2 and H_2O. Catalase peroxidase activity at lower H_2O_2 concentrations oxidizes small substrates, such as methanol, formaldehyde, formate, ethanol, NADPH, nitrite, hydroxylamine, 5-aminosalicylate, azide, aminotriazole and phenylenediamine derivatives [18], as well as the hydrazine drugs phenylhydrazine and phenelzine [5]. Catalase might be inactivated by aminotriazole radical-mediated heme or protein adduct formation [19]. *Mycobacterium tuberculosis* catalase-peroxidase (KatG) is also responsible for oxidizing the antituberculosis drug isoniazid to isonicotinyl radicals, which covalently bind NAD(H) and prevent mycolic acid synthesis, thereby causing Mycobacterium death: this is important because isoniazid-resistant tuberculosis has been attributed to KatG mutations [20]. Brain catalase peroxidase activity has also been implicated in the neurological effects of ethanol [21]. The α-hydroxyethyl radical metabolite formed from ethanol metabolism, as demonstrated with electron spin resonance (ESR) using a-phenyl-*N*-tert-butylnitrone–5,5-dimethyl-1-pyrroline-*N*-oxide spin traps, oxidized glutathione (GSH), ascorbate and vitamin E [22]. In addition to covalently binding to cytochrome c, Tyr48, Tyr67, Tyr74 and Trp59 [23], the α-hydroxyethyl radical also formed bonding interactions with CYP2E1, and this adduct appeared on the cell surface and inactivated various antioxidant enzymes [24]. The protein-bound radical also formed antibodies *in vivo*, which were cytotoxic towards hepatocytes isolated from ethanol-treated rats [25].

The human cytochrome P450 family: peroxidase and peroxygenase activity

Similarities exist between the peroxidases and CYP in that both form compounds I and II, which have a FeIV=O oxidizing center. However, there is no known homology between the protein sequences of peroxidases and CYP.

TABLE 2

Enzyme biomarkers: enzyme inactivation by peroxidase-catalyzed drug oxidation

Drugs that inactivate enzymes when oxidized by H_2O_2–peroxidase	Refs
Chlorpromazine	[77]
Mefenamic acid	[78]
Phenylbutazone	[79]
Indomethacin	[80]
Piroxicam	[34]
Tenoxicam	[34]
Adriamycin	[81]
Enzyme biomarkers inactivated	
Cholinesterase	[77]
Creatine kinase	[78–80]
Glyceraldehyde dehydrogenase	[79]
Alcohol dehydrogenase	[34]
Succinate dehydrogenase	[81]

Furthermore, the proximal side of the peroxidase heme active center contains a histidine ligand and a distal histidine that is involved in the hemolytic scission of H_2O_2, whereas the CYP heme comprises a cysteine thiolate ligand and a distal oxygen-binding site that is lined primarily with non-polar groups. Only chloroperoxidase, a fungal peroxidase, resembles CYP by having a similar cysteine ligand (part of a CYP three residue stretch), which enables it to catalyze peroxidase, catalase and CYP reactions. In peroxidases, substrates are limited to electron transfer reactions at the heme edge and, therefore, form substrate radicals that have restricted access to the FeIV=O center of compounds I and II. An oxygen atom is not transferred to substrates, and hydrogen abstraction, as well as addition to double bonds, is compatible only with a radical oxygen structure. By contrast, the CYP edge is not accessible and substrates must bind directly adjacent to the FeIV=O center, enabling substrate hydroxylation via insertion of oxygen.

Microsomal CYP and NADPH act as a monooxygenase in catalyzing the hydroxylation of a variety of aromatic substrates that are not oxidized by peroxidase (Equation 6). However, the monooxygenase activity cannot catalyze the one-electron oxidation of peroxidase substrates (e.g. phenols, biphenols, aminophenols, hydrazines and phenylenediamines).

$$RNH_2 + O_2 + NADPH + H^+ \rightarrow RNHOH + H_2O + NADP^+ \quad \text{[Eqn 6]}$$

In the absence of oxygen or NADPH, CYP can use hydroperoxides to catalyze a one-electron oxidation of classical peroxidase substrates ('peroxidase' activity). The unique proximal heme-iron ligand is a cysteine thiolate rather than a histidine nitrogen, which enables CYP to transfer the ferryl oxygen to its substrates instead of simply abstracting an electron, as is the case with peroxidases. Therefore, in various CYP-catalyzed hydroxylation reactions, oxygen, NADPH and CYP reductase can be replaced by hydroperoxides, including physiological molecules, suggesting that the peroxygenase activity involves a short circuit of the oxygen activation step and that monooxygenase activities were mediated by the same ferryl-oxidizing species [26]. However, this supposition is still to be resolved by computational chemistry. Although monooxygenase activity predominates under physiological conditions, CYP peroxidase activity probably has a role in detoxifying intracellular hydroperoxides or H_2O_2 [27]. During cellular oxidative stress, CYP peroxidase could catalyze other one-electron oxidative reactions, for example, *N*-oxidation and *N*- and *O*-demethylation of drugs [28].

Peroxidase-catalyzed drug metabolic activation

It has been hypothesized that peroxidase-catalyzed drug oxidation causes radical production, protein binding and oxidative stress cytotoxicity and thereby contributes to reactive metabolite formation and cell death. The evidence for this hypothesis includes the finding that peroxidase catalyzes the oxidation of drugs, carcinogens or xenobiotics to form radicals that cooxidize biomolecules (e.g. GSH, DNA, NADH, ascorbate or unsaturated fatty acids) and form reactive oxygen species [26,29]. Furthermore, hepatocyte cytotoxicity that is induced by phenothiazines or nonsteroidal anti-inflammatory drugs (NSAIDs) was markedly enhanced by nontoxic concentrations of extracellular H_2O_2–peroxidase, and intracellular GSH, lipid and ascorbate were cooxidized and reactive oxygen species formed [30,31]. Other drugs for which the cytotoxicity was markedly enhanced included troglitazone and tolcapone, which were recently withdrawn from the market as a result of hepatotoxicity (including idiosyncratic hepatotoxicity) [32]. This enhanced hepatocyte cytotoxicity assay might mimic *in vivo* liver inflammation animal models in which H_2O_2–peroxidase-releasing immune cells (e.g. neutrophils) infiltrate the liver [33]. Phenothiazine drug-induced hepatotoxicity *in vivo* was also markedly increased by endotoxemia-induced inflammation [33]. Therefore, it would be useful to screen NCEs or candidate drugs for their ability to induce oxidative stress using this 'hepatocyte inflammation model' as an enhanced cytotoxicity assay (Figure 2).

In vitro protein binding by peroxidase-formed phenothiazine or NSAID drug radicals also resulted in enzyme inactivation and could prove useful for screening NCEs. Enzymes that are inactivated by various drugs when oxidized by H_2O_2–peroxidase are listed in Table 2. The inactivation of these enzymes has been attributed to protein thiol or tryptophan oxidation and adduct formation by drug radicals [34]; some of these target enzymes could be useful as radical biomarkers.

Proteins, such as chymotrypsin, serum albumin or low-density lipoprotein (LDL), can also be oxidized directly by the peroxidase heme edge or myoglobin and H_2O_2 to form

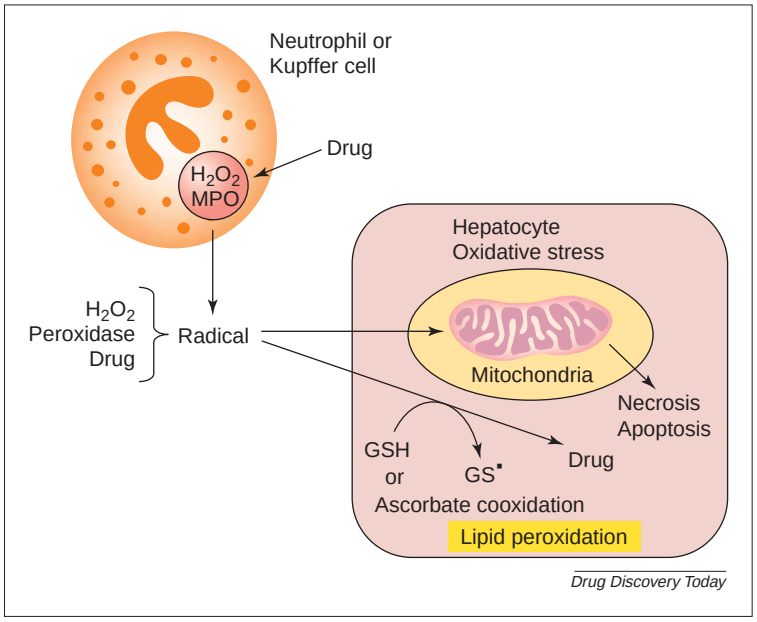

FIGURE 2

The inflammatory cell–hepatocyte model for hepatitis. Neutrophils and Kupffer cells, which are resident liver macrophages, contain MPO, and form H_2O_2 on activation of plasma membrane NADPH oxidase during the inflammatory response. The large increase in liver susceptibility to drugs has been attributed to the infiltrated cells of the immune system or activated resident Kupffer cells [33]. It is suggested that the H_2O_2–peroxidase of the infiltrated cells catalyzes the oxidation of drugs (or their CYP metabolites) to form reactive pro-oxidant radicals that are toxic to hepatocytes. Abbreviation: BP, benzopyrene.

protein tyrosine phenoxyl radicals, which can crosslink proteins and enzymes, leading to inactivation, or oxidize other biomolecules, for example, linoleic acid [35]. Phenolic xenobiotics (PhOH) or tyrosine oxidized by peroxidase form phenoxyl radicals ($PhO^{\bullet}$), which bind covalently to protein and induce lipid peroxidation [35–37]. They would also be expected to increase protein tyrosine nitration by $NO_2^{\bullet}$, the nitrite one-electron oxidation metabolite of peroxidases [38] (Equations 7–9). Protein oxidation and nitration is associated with inflammatory diseases and drug toxicity [39].

$$PhO^{\bullet} + Protein - TyrH \rightarrow Protein - Tyr^{\bullet} + PhOH \quad [Eqn\ 7]$$

$$Protein - Tyr^{\bullet} + NO_2^{\bullet} \rightarrow Protein - Tyr - NO_2 \quad [Eqn\ 8]$$

$$H_2O_2 + NO_2^- \xrightarrow{Peroxidase} NO_2^{\bullet} + HO^- + H_2O \quad [Eqn\ 9]$$

Myeloperoxidase-containing bone marrow cells: the radical hypothesis

Xenobiotics, or their metabolites, that accumulate in the plasma or bone marrow might also be cooxidized or chlorinated by activated leukocytes. The leukemias induced by chronic exposure to etoposide (a phenolic topoisomerase inhibitor) have been attributed to MPO–H_2O_2-catalyzed one-electron oxidation and *O*-demethylation [40]. The leukemias induced by chronic exposure to benzene have also been attributed to the MPO–H_2O_2-catalyzed one-electron oxidation of the phenolic metabolites formed by hepatic CYP [41].

HL-60 cells are a bone marrow-derived leukemia cell line that contain MPO and NADPH oxidase and form H_2O_2 when activated. Incubation of HL-60 cells with etoposide and H_2O_2 led to the oxidation of cellular glutathione to thiyl radicals (as demonstrated by spin-trap adduct formation), whereas cellular lipid did not undergo lipid peroxidation [42]. Etoposide phenoxyl radicals were detected by EPR only in GSH-depleted cells. The formation of thiyl and phenoxyl radicals was prevented by decreasing MPO levels with succinyl acetone, a heme synthesis inhibitor. PMC, a water-soluble vitamin E analogue and MPO substrate, has been proposed as a nutritional strategy to prevent etoposide-induced secondary leukemia [40].

The triphenylethylene drug tamoxifen, which is used in metastatic breast cancer treatment or as adjuvant therapy, is one of the safest anticancer drugs, but is a hepatocarcinogen in rats, where peroxidase catalyzed the activation of the major metabolite 4-hydroxytamoxifen and formed DNA and protein adducts [43]. The phenoxyl radical initially formed in HL-60 cells oxidized ascorbate to semidehydroascorbyl radicals, GSH to thiyl radicals and initiated lipid peroxidation [44].

Idiosyncratic agranulocytosis and the hypochlorite–radical hypotheses

Activated neutrophils or MPO–H_2O_2 form DNA adducts with the carcinogens benzidine, methylaminoazobenzene and aminofluorene and could potentially contribute to chemical carcinogenesis [45]. ESR indicated that chlorpromazine and aminopyrine were oxidized by H_2O_2–peroxidase to pro-oxidant radicals, and spin-trapping showed that phenylhydrazine was oxidized to phenyl radicals [46]. The aminopyrine *N*-cation radical formed by peroxidase is a pro-oxidant and is likely to be an oxidized protein [29] (Figure 3), whereas the aminopyrine dication (the two-electron oxidation product formed by MPO–H_2O_2–Cl or HOCl) could covalently bind to protein [47]. HOCl oxidation of other idiosyncratic drugs (e.g. vesnarinone, propylthiouracil, dapsone, sulfonamides and procainamide [4]) also resulted in the formation of protein adducts. The antipsychotic drug clozapine was metabolized by MPO–H_2O_2 to a pro-oxidant radical [48] and protein binding nitrenium ions, which induced neutrophil GSH depletion and caused apoptosis and necrosis [49]. Activated neutrophils or bone marrow cells [50] caused clozapine protein binding but not GSH depletion. Another clozapine structural analogue, fluperlapine, was not oxidized by neutrophils but induced agranulocytosis. However, the major CYP metabolite, 7-hydroxyfluperlapine, was oxidized to a protein-binding iminoquinone in neutrophils [51]. DMP406, another clozapine analogue substitute, was oxidized to an imine similar to that formed from mianserin by activated neutrophils

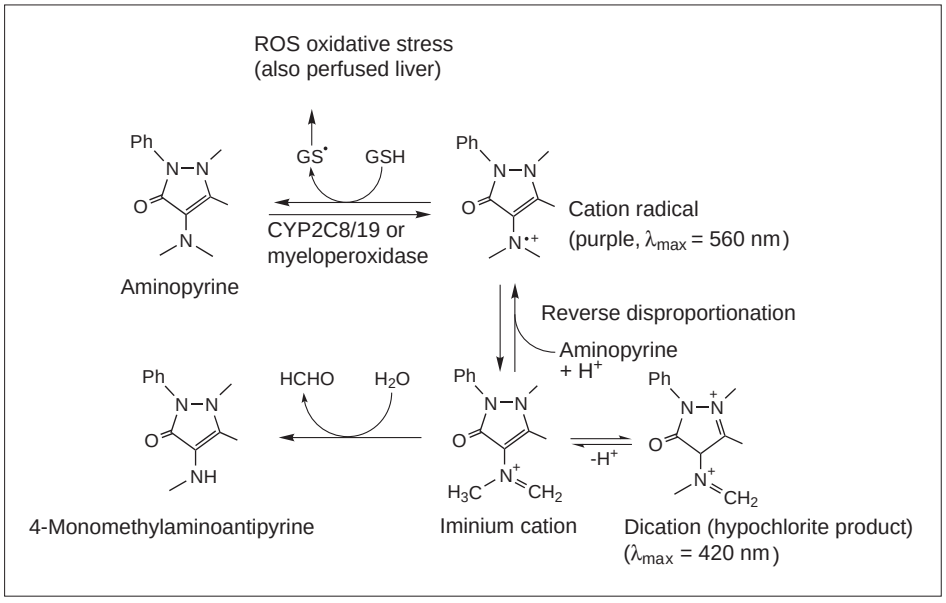

FIGURE 3

Proposed peroxidase-catalyzed aminopyrine activation. Aminopyrine *N*-cation radical formed by peroxidase, readily oxidized GSH, which, in the process, formed reactive oxygen species. The *N*-cation radical was also formed by reverse disproportionation from an iminium cation, the two-electron oxidation product and aminopyrine. The iminium cation is also hydrolyzed to *N*-methylaminoantipyrine and formaldehyde (HCHO) [47]. The aminopyrine dication is the two-electron oxidation product formed by MPO–H_2O_2–Cl or HOCl at acidic pH. Abbreviation: ROS, reactive oxygen species.

or H_2O_2–horseradish peroxidase (HRP) but not by HOCl [52]. The idiosyncratic adverse reactions of the anticonvulsant drugs carbamazepine and phenytoin have also been attributed to protein oxidation by phenoxyl radicals formed by the myeloperoxidase-catalyzed oxidation of their 3-hydroxy and 4-hydroxy metabolites, respectively, and/or iminoquinone formed from 2-hydroxy-carbamazepine by hypochlorite [53].

Ticlopidine (used for inhibiting platelet aggregation) induced agranulocytosis, aplastic anemia and hepatitis, and was oxidized to dehydro-ticlopidine by leukocytes. HOCl or MPO–H_2O_2–Cl also formed dehydro-ticlopidine, and probably a thiophene-S-chloride reactive metabolite, to a greater extent than MPO–H_2O_2. However, CYP inactivation, and possibly hepatitis, was attributed to metabolism by liver microsomes resulting in the formation of a thiophene-S-oxide [54].

Hepatitis and in vivo *evidence for the 'inflammatory hypothesis'*

Drugs, such as chlorpromazine and acetaminophen, which have the potential to cause mild hepatotoxicity, become much more hepatotoxic in the face of a modest ongoing inflammatory response [33]. Drug hepatotoxicity *in vivo* can be prevented by inactivating the Kupffer cells with gadolinium chloride or dextran sulfate, or by superoxide dismutase or tumor necrosis factor inhibitors and/or antibodies. Such hepatic inflammation could be caused by endotoxins released from infective bacteria or gut microflora in response to insults, such as alcohol consumption, liver disease or dietary changes, which might not be noticed by the patient and could partly explain the rareness of idiosyncratic drug reactions (IDRs). Hepatotoxicity can also be increased by activating the Kupffer cells with retinol or endotoxins. Inflammation increases plasma endotoxins and immunomodulators, which activate Kupffer cells and lead to liver infiltration by neutrophils and macrophages, whereas parasite infection causes infiltration by eosinophils, thereby increasing liver MPO or EPO activity by 50–100-fold compared with the low peroxidase activity normally observed in hepatocytes. Activation of these peroxidase-containing cells by drugs or proinflammatory cytokines leads to a burst of oxygen uptake and H_2O_2 formation.

In vivo evidence for peroxidase-catalyzed oxidation of tyrosine to phenoxyl radicals during inflammation is that patients with sepsis have high urinary tyrosine dimers. Furthermore, mice with acute peritoneal inflammation have high peritoneal leukocyte protein dityrosines and carbonylated proteins and trichlorobromotyrosine in the peritoneal fluid [55–57]. Therefore, drug metabolism during hepatic inflammation probably also involves radicals formed by the metabolic activation of drugs by H_2O_2–peroxidases. Recently, it has been shown that drugs associated with hepatotoxic idiosyncrasy became toxic to isolated rat hepatocytes if nontoxic concentrations of H_2O_2–peroxidases were present [26,29–32] (Figure 2). These drugs included troglitazone, a gluconeogenesis inhibitor, and tolcapone, a catechol-*O*-methyltransferase (COMT) inhibitor for L-dihydroxyphenylalanine (DOPA) therapy in Parkinson's disease, which were recently removed from the market because of severe hepatotoxicity. Troglitazone, a vitamin E analogue with a phenolic toxicophore, presumably underwent a peroxidase-catalyzed troglitazone oxidation to form a phenoxyl radical because hepatocyte GSH and lipid oxidation occurred [32]. By contrast, liver microsomal CYP3A4 catalyzed the oxidation of troglitazone to a quinone metabolite that was less toxic than troglitazone [58]. However, only 1 in 10,000 patients on troglitazone experienced a serious hepatotoxic reaction. Tolcapone contained a catechol toxicophore and was also much more toxic in the 'inflammation hepatocyte' model [32]. However, the parent drug was also a powerful mitochondrial uncoupler, thereby implicating that a reactive semiquinone radical or *o*-quinone was formed by peroxidase [59]. The NSAID diclofenac has been successfully used by millions, but an estimated 5000 people developed severe hepatotoxicity after 1–3 months of treatment [60]. Reactive metabolites implicated were quinoneimines formed by CYP [60] or peroxidase-formed pro-oxidant radicals [29]. In addition, isoniazid-induced idiosyncratic

hepatitis and lupus erythromatous disease could involve reductive hydrazinyl radical intermediates and pro-oxidant acetyl radical metabolites formed by oxidative metabolism of isoniazid, catalyzed by MPO–H_2O_2–Cl, HOCl or neutrophils [61,62]. The hepatotoxic antidepressant iproniazid and the anticancer agent procarbazine were also oxidized to isopropyl and methyl radicals, respectively, by hepatocytes and HL-60 cells, which was attributed to CYP and MPO activity, respectively [63]. Phenylbutazone caused hepatotoxicity in 1–5% patients and readily caused cytotoxicity and lipid peroxidation in the inflammation hepatocyte model [32].

Skin rashes

Skin rashes are an early sign of life-threatening IDRs for the HIV antimicrobials sulfamethoxazole, dapsone and nevirapine, and the antiepileptic lamotrigine. Less than 10% of patients taking lamotrigine developed skin rashes before other IDRs (e.g. neutropenia, leucopenia, agranulocytosis and hepatitis), whereas 16% of patients taking nevirapine developed skin rashes and 1% developed hepatitis. Recently, an animal model (Brown-Norway female rats) for nevirapine-induced skin rashes has become available [64]. Although lamotrigine and nevirapine are unlikely to be peroxidase substrates, the phenolic CYP metabolites of these drugs are likely to be substrates, particularly if their glucuronidation rate is slow. If the skin rashes are caused by unstable reactive metabolites (e.g. phenoxyl radicals) then metabolic activation is likely to occur in the skin. A rat skin peroxidase located in the nuclear–mitochondrial fraction has been isolated and characterized [65]. Skin microsomal CYP3A1 and CYP2B1 levels were 4.7% and 2.3%, respectively, of those found in liver microsomes [66]. Epidermal keratinocytes, the most abundant skin cell, also contained peroxidase, CYPs and PGHSP and activated sulfonamides [67]. PGHSP oxidized phenol to phenoxyl radicals, which cooxidized keratinocyte lipids, thiols and ascorbate to their respective radicals [68,69]. MPO also accumulated in the skin as a result of xenobiotic-induced skin irritation and inflammation [10].

Intestinal toxicity, uterine cancer, atherosclerosis and teratogenicity

Intestinal peroxidase or EPO could have a role in NSAID-induced gastrointestinal (GI) toxicity because NSAIDs are readily oxidized to pro-oxidant radicals [29], which could explain why GI toxicity induced by indomethacin has been attributed to oxidative stress [70]. Uterine peroxidase or EPO has been implicated in the uterine cancer induced by the peroxidase substrates 4-hydroxytamoxifen, diethylstilbestrol and estradiol [43,44,71]. Could plasma MPO–H_2O_2 oxidize drugs to radicals that initiate LDL oxidation, as has been shown for plasma amino acid substrates [72], and thereby accelerate plaque formation and atherosclerosis? Oxidatively modified lipoprotein would then be recognized by the macrophage scavenger receptor and phagocytosed, thus increasing the risk of transformation to a foam cell, which initiates plaque formation. Significant amounts of MPO and hypochlorite modified proteins were expressed in human atherosclerotic lesions colocalized with monocytes and macrophages [72]. It is not known whether plasma oxidized LDL levels increase after chronic drug administration, for example, NSAIDs. The teratogenicity or utero death of thalidomide and the anticonvulsant drug phenytoin have been shown to depend partially on activation by embryonic PGHSP and lipoxygenase [73,74].

Transgenic mouse models

PGHS-1 or PGHS-2 knockout mice were more resistant to polycyclic aromatic hydrocarbon (PAH)-induced skin cancer [75], which could suggest PAH activation by PGHSPs. Mice with a targeted disruption of the NADPH oxidase gp91 subunit were much more susceptible to *Candida albicans* and the mice were resistant to early alcohol-induced hepatitis [76]. MPO-deficient mice exposed to polymicrobial sepsis formed considerably lower concentrations of 3-chlorotyrosine and 3-bromotyrosine in their peritoneal inflammatory fluid than exposed wild-type mice, and were also more likely to die from infection than wild-type mice [56]. MPO or EPO knockout mice were also resistant to the protein nitrotyrosine formation induced by inflammation, which was attributed to activated peroxidase-catalyzed nitrite oxidation [39].

Conclusions

Although the evidence for a peroxidase-catalyzed drug metabolic activation is largely based on *in vitro* studies, there is ample evidence that oxidative stress contributes to many pathological conditions and diseases, particularly those associated with aging. Knockout mice research has also provided evidence that endogenous molecules, such as tyrosine radical oxidation products, were not formed in MPO or EPO knockout mice and increased levels of these reactive species were observed in inflammation animal models or patients with sepsis. Research is underway to show that peroxidase-catalyzed drug metabolism is also modulated in the same way as tyrosine oxidation and that antibodies to drug radical haptens are blocked in MPO knockout mice. Drug candidate or NCE screening should include peroxidase-catalyzed pro-oxidant radical formation and protein oxidation, whereas cytotoxicity assays should use oxidative stress biomarkers to determine if this was part of the cytotoxic mechanism. It might not be enough to determine the amount of microsomal protein covalent binding by candidate drugs or NCEs [1]. New peroxidase substrates could be designed that form toxic radicals for use as anticancer agents, anti-infectives or antibiotics. Alternatively, novel peroxidase inhibitors could also be developed for preventing the toxic side effects of drugs used for treating chronic diseases. For example, cyclooxygenase (COX)-1 and COX-2 inhibitors have been used to prevent human colon carcinogenesis

and animal drug toxicity or chemical carcinogenesis [4,8,9]. Finally, individual susceptibility to various cancers, atherosclerosis, neurodegenerative, autoimmune and other diseases has been associated with a common MPO promoter polymorphism (MPO-463GA), which increases MPO levels and carcinogen metabolic activation [4]. However, some controversy exists with these studies and more research is needed to determine whether MPO or other peroxidase polymorphisms contribute to the large variation in the susceptibility of an individual to drug toxic side-effects.

References

1 Evans, D.C. *et al.* (2004) Drug-protein adducts: an industry perspective on minimizing the potential for drug bioactivation in drug discovery and development. *Chem. Res. Toxicol.* 17, 3–16

2 Ju, C. and Uetrecht, J.P. (2002) Mechanism of idiosyncratic drug reactions: reactive metabolite formation, protein binding and the regulation of the immune system. *Curr. Drug Metab.* 3, 367–377

3 Ju, C. and Uetrecht, J.P. (1998) Oxidation of a metabolite of indomethacin (Desmethyldeschlorobenzoylindomethacin) to reactive intermediates by activated neutrophils, hypochlorous acid, and the myeloperoxidase system. *Drug Metab. Dispos.* 26, 676–680

4 O'Brien, P.J. (2000) Peroxidases. *Chem. Biol. Interact.* 129, 113–139

5 Ortiz de Montellano, P.R. and Kerr, D.E. (1983) Inactivation of catalase by phenylhydrazine. Formation of a stable aryl-iron heme complex. *J. Biol. Chem.* 258, 10558–10563

6 Seibold, S.A. *et al.* (2003) Histidine 386 and its role in cyclooxygenase and peroxidase catalysis by prostaglandin-endoperoxide H synthases. *J. Biol. Chem.* 278, 46163–46170

7 Tsai, A.L. *et al.* (2002) Structural characterization of arachidonyl radicals formed by aspirin-treated prostaglandin H synthase-2. *J. Biol. Chem.* 277, 38311–38321

8 Wiese, F.W. *et al.* (2001) Carcinogen substrate specificity of human COX-1 and COX-2. *Carcinogenesis* 22, 5–10

9 Eling, T.E. *et al.* (1990) Prostaglandin H synthase and xenobiotic oxidation. *Annu. Rev. Pharmacol. Toxicol.* 30, 1–45

10 Trush, M.A. *et al.* (1994) Myeloperoxidase as a biomarker of skin irritation and inflammation. *Food Chem. Toxicol.* 32, 143–147

11 Brown, K.E. *et al.* (2001) Immunohistochemical detection of myeloperoxidase and its oxidation products in Kupffer cells of human liver. *Am. J. Pathol.* 159, 2081–2088

12 Schattenberg, D.G. *et al.* (1994) Peroxidase activity in murine and human hematopoietic progenitor cells: potential relevance to benzene-induced toxicity. *Mol. Pharmacol.* 46, 346–351

13 Corbett, M.D. *et al.* (1989) Metabolic activation and nucleic acid binding of acetaminophen and related arylamine substrates by the respiratory burst of human granulocytes. *Chem. Res. Toxicol.* 2, 260–266

14 Yue, K.T. *et al.* (1997) X-ray absorption and resonance Raman spectroscopy of human myeloperoxidase at neutral and acid pH. *Biochim. Biophys. Acta* 1338, 282–294

15 O'Brien, P.J. *et al.* (1991) Formation of biological reactive intermediates by peroxidases: halide mediated acetaminophen oxidation and cytotoxicity. *Adv. Exp. Med. Biol.* 283, 51–64

16 De, S.K. *et al.* (1986) Localization and origin of the intestinal peroxidase–effect of adrenal glucocorticoids. *J. Steroid Biochem.* 24, 629–635

17 Newbold, R.R. *et al.* (1991) Ontogeny of peroxidase activity in epithelium and eosinophils of the mouse uterus. *Teratog. Carcinog. Mutagen.* 11, 267–278

18 Artemchik, V.D. *et al.* (1985) Peroxidase activity of catalase with respect to aromatic amines. *Biokhimiia* 50, 826–832

19 Putnam, C.D. *et al.* (2000) Active and inhibited human catalase structures: ligand and NADPH binding and catalytic mechanism. *J. Mol. Biol.* 296, 295–309

20 Broussy, S. *et al.* (2003) 1H and 13C NMR characterization of hemiamidal isoniazid-NAD(H) adducts as possible inhibitors of InhA reductase of Mycobacterium tuberculosis. *Chemistry* 9, 2034–2038

21 Pastor, R. *et al.* (2002) Ethanol-stimulated behaviour in mice is modulated by brain catalase activity and H2O2 rate of production. *Psychopharmacology (Berl.)* 165, 51–59

22 Stoyanovsky, D.A. *et al.* (1998) Interaction of 1-hydroxyethyl radical with glutathione, ascorbic acid and alpha-tocopherol. *Free Radic. Biol. Med.* 24, 132–138

23 Anni, H. and Israel, Y. (1999) Characterization of adducts of ethanol metabolites with cytochrome c. *Alcohol. Clin. Exp. Res.* 23, 26–37

24 Puntarulo, S. *et al.* (1999) Interaction of 1-hydroxyethyl radical with antioxidant enzymes. *Arch. Biochem. Biophys.* 372, 355–359

25 Clot, P. *et al.* (1997) Plasma membrane hydroxyethyl radical adducts cause antibody-dependent cytotoxicity in rat hepatocytes exposed to alcohol. *Gastroenterology* 113, 265–276

26 O'Brien, P.J. (1988) Radical formation during the peroxidase catalyzed metabolism of carcinogens and xenobiotics: the reactivity of these radicals with GSH, DNA, and unsaturated lipid. *Free Radic. Biol. Med.* 4, 169–183

27 Anari, M.R. *et al.* (1996) The involvement of cytochrome P450 peroxidase in the metabolic bioactivation of cumene hydroperoxide by isolated rat hepatocytes. *Chem. Res. Toxicol.* 9, 924–931

28 Anari, M.R. *et al.* (1997) Hydrogen peroxide supports human and rat cytochrome P450 1A2-catalyzed 2-amino-3-methylimidazo [4,5-f]quinoline bioactivation to mutagenic metabolites: significance of cytochrome P450 peroxygenase. *Chem. Res. Toxicol.* 10, 582–588

29 Galati, G. *et al.* (2002) Idiosyncratic NSAID drug induced oxidative stress. *Chem. Biol. Interact.* 142, 25–41

30 Eghbal, M.A. *et al.* (2004) Peroxidase catalysed formation of cytotoxic prooxidant phenothiazine free radicals at physiological pH. *Chem. Biol. Interact.* 151, 43–51

31 Tafazoli, S. and O'Brien, P.J. (2004) Prooxidant activity and cytotoxic effects of indole-3-acetic acid derivative radicals. *Chem. Res. Toxicol.* 17, 1350–1355

32 Tafazoli, S. *et al.* Oxidative stress mediated idiosyncratic drug toxicity. *Drug Metab. Rev.* (in press)

33 Buchweitz, J.P. *et al.* (2002) Underlying endotoxemia augments toxic responses to chlorpromazine: is there a relationship to drug idiosyncrasy? *J. Pharmacol. Exp. Ther.* 300, 460–467

34 Muraoka, S. and Miura, T. (2004) Inactivation of alcohol dehydrogenase by piroxicam-derived radicals. *Free Radic. Res.* 38, 217–223

35 Giulivi, C. *et al.* (2003) Tyrosine oxidation products: analysis and biological relevance. *Amino Acids* 25, 227–232

36 Hagerman, A.E. *et al.* (2003) Radical chemistry of epigallocatechin gallate and its relevance to protein damage. *Arch. Biochem. Biophys.* 414, 115–120

37 Subrahmanyam, V.V. *et al.* (1990) Peroxidase/hydrogen peroxide–or bone marrow homogenate/hydrogen peroxide–mediated activation of phenol and binding to protein. *Xenobiotica* 20, 1369–1378

38 van Dalen, C.J. *et al.* (2000) Nitrite as a substrate and inhibitor of myeloperoxidase. Implications for nitration and hypochlorous acid production at sites of inflammation. *J. Biol. Chem.* 275, 11638–11644

39 Brennan, M.L. *et al.* (2002) A tale of two controversies: defining both the role of peroxidases in nitrotyrosine formation *in vivo* using eosinophil peroxidase and myeloperoxidase-deficient mice, and the nature of peroxidase-generated reactive nitrogen species. *J. Biol. Chem.* 277, 17415–17427

40 Kagan, V.E. *et al.* (1999) Mechanism-based chemopreventive strategies against etoposide-induced acute myeloid leukemia: free radical/antioxidant approach. *Mol. Pharmacol.* 56, 494–506

41 Goldman, R. *et al.* (1999) Myeloperoxidase-catalyzed redox-cycling of phenol promotes lipid peroxidation and thiol oxidation in HL-60 cells. *Free Radic. Biol. Med.* 27, 1050–1063

42 Kagan, V.E. *et al.* (2001) Pro-oxidant and antioxidant mechanisms of etoposide in HL-60 cells: role of myeloperoxidase. *Cancer Res.* 61, 7777–7784

43 Pathak, D.N. *et al.* (1995) Microsomal and peroxidase activation of 4-hydroxy-tamoxifen to form DNA adducts: comparison with DNA adducts formed in Sprague-Dawley rats treated with tamoxifen. *Carcinogenesis* 16, 11–15

44 Day, B.W. *et al.* (1999) Peroxidase-catalyzed pro- versus antioxidant effects of 4-hydroxytamoxifen: enzyme specificity and biochemical sequelae. *Chem. Res. Toxicol.* 12, 28–37

45 Tsuruta, Y. *et al.* (1985) Peroxidase-mediated irreversible binding of arylamine carcinogens to DNA in intact polymorphonuclear leukocytes activated by a tumor promoter. *Chem. Biol. Interact.* 53, 25–35

46 Kalyanaraman, B. and Sohnle, P.G. (1985) Generation of free radical intermediates from foreign compounds by neutrophil-derived oxidants. *J. Clin. Invest.* 75, 1618–1622

47 Uetrecht, J.P. *et al.* (1995) Oxidation of aminopyrine by hypochlorite to a reactive dication: possible implications for aminopyrine-induced agranulocytosis. *Chem. Res. Toxicol.* 8, 226–233

48 Fischer, V. *et al.* (1991) Possible role of free radical formation in clozapine (clozaril)-induced agranulocytosis. *Mol. Pharmacol.* 40, 846–853
49 Williams, D.P. *et al.* (2000) Induction of metabolism-dependent and -independent neutrophil apoptosis by clozapine. *Mol. Pharmacol.* 58, 207–216
50 Gardner, I. *et al.* (1998) A comparison of the covalent binding of clozapine and olanzapine to human neutrophils *in vitro* and *in vivo*. *Mol. Pharmacol.* 53, 999–1008
51 Lai, W.G. *et al.* (2000) Bioactivation and covalent binding of hydroxyfluperlapine in human neutrophils: implications for fluperlapine-induced agranulocytosis. *Drug Metab. Dispos.* 28, 255–263
52 Iverson, S. *et al.* (2002) Predicting drug-induced agranulocytosis: characterizing neutrophil-generated metabolites of a model compound, DMP 406, and assessing the relevance of an *in vitro* apoptosis assay for identifying drugs that may cause agranulocytosis. *Chem. Biol. Interact.* 142, 175–199
53 Lu, W. and Uetrecht, J.P. (2004) Oxidation of carbamazepine and phenytoin metabolites to free radicals. *Drug Metab. Rev. Abstract* 384 (Suppl. 1), 192
54 Valadon, P. *et al.* (1996) Thiophene sulfoxides as reactive metabolites: formation upon microsomal oxidation of a 3-aroylthiophene and fate in the presence of nucleophiles *in vitro* and *in vivo*. *Chem. Res. Toxicol.* 9, 1403–1413
55 Bhattacharjee, S. *et al.* (2001) NADPH oxidase of neutrophils elevates o,o?-dityrosine cross-links in proteins and urine during inflammation. *Arch. Biochem. Biophys.* 395, 69–77
56 Gaut, J.P. *et al.* (2001) Neutrophils employ the myeloperoxidase system to generate antimicrobial brominating and chlorinating oxidants during sepsis. *Proc. Natl. Acad. Sci. U. S. A.* 98, 11961–11966
57 Gaut, J.P. *et al.* (2002) Myeloperoxidase produces nitrating oxidants *in vivo*. *J. Clin. Invest.* 109, 1311–1319
58 Kassahun, K. *et al.* (2001) Studies on the metabolism of troglitazone to reactive intermediates *in vitro* and *in vivo*. Evidence for novel biotransformation pathways involving quinone methide formation and thiazolidinedione ring scission. *Chem. Res. Toxicol.* 14, 62–70
59 Haasio, K. *et al.* (2002) Effects of entacapone and tolcapone on mitochondrial membrane potential. *Eur. J. Pharmacol.* 453, 21–26
60 Boelsterli, U.A. (2003) Diclofenac-induced liver injury: a paradigm of idiosyncratic drug toxicity. *Toxicol. Appl. Pharmacol.* 192, 307–322
61 Goodwin, D.C. *et al.* (1996) Redox mediation in the peroxidase-catalyzed oxidation of aminopyrine: possible implications for drug-drug interactions. *Chem. Res. Toxicol.* 9, 476–483
62 Hofstra, A.H. *et al.* (1992) Metabolism of isoniazid by activated leukocytes. Possible role in drug-induced lupus. *Drug Metab. Dispos.* 20, 205–210
63 Sinha, B.K. (1991) Cytochrome P-450- and peroxidase-dependent activation of procarbazine and iproniazid in mammalian cells. *Free Radic. Res. Commun.* 15, 189–195
64 Shenton, J.M. *et al.* (2003) Characterization of a potential animal model of an idiosyncratic drug reaction: nevirapine-induced skin rash in the rat. *Chem. Res. Toxicol.* 16, 1078–1089
65 Strohm, B.H. and Kulkarni, A.P. (1986) Peroxidase, an alternate pathway to cytochrome P-450 for xenobiotic metabolism in skin: partial purification and properties of the enzyme from neonatal rat skin. *J. Biochem. Toxicol.* 1, 83–97
66 Zhu, Z. *et al.* (2002) Expression of P450 enzymes in rat whole skin and cultured epidermal keratinocytes. *Biochem. Biophys. Res. Commun.* 297, 65–70
67 Reilly, T.P. *et al.* (2000) A role for bioactivation and covalent binding within epidermal keratinocytes in sulfonamide-induced cutaneous drug reactions. *J. Invest. Dermatol.* 114, 1164–1173
68 Shvedova, A.A. *et al.* (2000) Redox cycling of phenol induces oxidative stress in human epidermal keratinocytes. *J. Invest. Dermatol.* 114, 354–364
69 Schreiber, J. *et al.* (1989) Detection of glutathione thiyl free radical catalyzed by prostaglandin H synthase present in keratinocytes. Study of co-oxidation in a cellular system. *J. Biol. Chem.* 264, 7936–7943
70 Naito, Y. *et al.* (1998) Role of oxygen radical and lipid peroxidation in indomethacin-induced gastric mucosal injury. *Dig. Dis. Sci.* 43, 30S–34S
71 Epe, B. and Metzler, M. (1985) Nature of the macromolecular binding of diethylstilbestrol to DNA and protein following oxidation by peroxidase/hydrogen peroxide. *Chem. Biol. Interact.* 56, 351–361
72 Heinecke, J.W. (1999) Mechanisms of oxidative damage by myeloperoxidase in atherosclerosis and other inflammatory disorders. *J. Lab. Clin. Med.* 133, 321–325
73 Parman, T. *et al.* (1998) Free radical intermediates of phenytoin and related teratogens. Prostaglandin H synthase-catalyzed bioactivation, electron paramagnetic resonance spectrometry, and photochemical product analysis. *J. Biol. Chem.* 273, 25079–25088
74 Parman, T. *et al.* (1999) Free radical-mediated oxidative DNA damage in the mechanism of thalidomide teratogenicity. *Nat. Med.* 5, 582–585
75 Tiano, H.F. *et al.* (2002) Deficiency of either cyclooxygenase (COX)-1 or COX-2 alters epidermal differentiation and reduces mouse skin tumorigenesis. *Cancer Res.* 62, 3395–3401
76 James, L.P. *et al.* (2003) Acetaminophen toxicity in mice lacking NADPH oxidase activity: role of peroxynitrite formation and mitochondrial oxidant stress. *Free Radic. Res.* 37, 1289–1297
77 Muraoka, S. and Miura, T. (2003) Inactivation of cholinesterase induced by chlorpromazine cation radicals. *Pharmacol. Toxicol.* 92, 100–104
78 Muraoka, S. and Miura, T. (2003) Inactivation of creatine kinase during the interaction of mefenamic acid with horseradish peroxidase and hydrogen peroxide: participation by the mefenamic acid radical. *Life Sci.* 72, 1897–1907
79 Miura, T. *et al.* (2001) Phenylbutazone radicals inactivate creatine kinase. *Free Radic. Res.* 34, 167–175
80 Miura, T. *et al.* (2001) Inactivation of creatine kinase during the interaction of indomethacin with horseradish peroxidase and hydrogen peroxide: involvement of indomethacin radicals. *Chem. Biol. Interact.* 134, 13–25
81 Muraoka, S. and Miura, T. (2003) Inactivation of mitochondrial succinate dehydrogenase by adriamycin activated by horseradish peroxidase and hydrogen peroxide. *Chem. Biol. Interact.* 145, 67–75