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Reactive Metabolites in the Biotransformation of Molecules Containing a Furan Ring

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Abstract

Many xenobiotics containing a furan ring are toxic and/or carcinogenic. The harmful effects of these compounds require furan ring oxidation. This reaction generates an electrophilic intermediate. Depending on the furan ring substituents, the intermediate is either an epoxide or a *cis*-enedione with more ring substitution favoring epoxide formation. Either intermediate reacts with cellular nucleophiles such as protein or DNA to trigger toxicities. The reactivity of the metabolite determines which cellular nucleophiles are targeted. The toxicity of a particular furan is also influenced by the presence of competing metabolic pathways or efficient detoxification routes. GSH plays an important role in modulating the harmful effects of this class of compound by reacting with the reactive metabolite. However, this may not represent a detoxification step in all cases.

Keywords

furan; metabolism; reactive metabolites; epoxide; enedione

1 Introduction

Furan-containing compounds are abundant in food, synthetic and herbal medicines, industrial processes and the environment.[1–5] Many but not all of these compounds are toxic, causing concerns when human exposure is likely (Table 1). Small changes in structure affect which organ is targeted as well as what toxicity is observed. The harmful effects result from cytochrome P450 (P450) catalyzed furan ring oxidation.[1,6] This oxidation leads to the formation of a reactive metabolite which alkylates proteins and, in some cases, DNA. Adduct formation is thought to be an important trigger to the toxic effects with the reactive intermediate's chemical nature playing an influential role in which cellular targets are hit. Because furan is a structure alert for medicinal chemists and risk assessors,[7,8] understanding how structure influences the formation, lifetime and targets of the reactive intermediate is critical for the design of safe drugs and the determination of which furan-containing compounds may represent a human health risk.

The structure of the reactive intermediate resulting from furan ring oxidation is somewhat ambiguous. Two structures have been proposed: an epoxide or a *cis*-enedione (Scheme 1). Furan oxidation by P450 enzymes is thought to proceed through one of two general mechanisms.[9,10] The first involves the direct formation of an epoxide. The other involves the addition of the high valent iron(IV)-oxospecies to the π-system of the furan ring to produce a tetrahedral intermediate or cationic σ complex that can rearrange to yield either an

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epoxide (**1**) or a zwitterionic intermediate (**2**, Scheme 1). Either intermediate **1** or **2** can rearrange to form a *cis*-enedione (**3**, Scheme 1).[10]

Evidence for the involvement of furanyl epoxide intermediates has been sought in several indirect ways since they are difficult to isolate for chemical characterization. The potential for their formation can be determined through the reaction of the parent compound with oxidizing agents that generate epoxides, such as dimethyldioxirane.[11] Transient formation of furanyl epoxides have been reported for a few furan-containing compounds, including methfuroxam (2,4,5-trimethyl-*N*-phenyl-3-furancarboxamide)[12] and menthofuran [(*R*)-3,6-dimethyl-4,5,6,7-tetrahydrobenzofuran] (Table 1).[13] Both compounds have multiple substitutions on the furan ring. Therefore, the epoxide generated is sterically hindered, possibly stabilizing the reactive intermediate. No epoxide intermediates have been detected for a number for furans, including furan[11,14] and teucrin A.[15] For these compounds, the products of furanyl oxidation are *cis*-enediones. [11,14,15] The inability to detect the epoxide intermediates may be due to their extreme instability and ease at which they rearrange to a *cis*-enedione.

Additional support for the participation of an epoxide in the toxicity of furanyl compounds comes from epoxide hydrolase inhibition studies performed in vitro with 1,2-epoxy-3,3,3-trichloropropane (TCPO). The results of these experiments have been compound dependent. For example, TCPO increased the protein binding of furosemide 2-fold[16] whereas it had no effect on the protein binding of 4-ipomeanol[17] or 2-(*N*-ethylcarbamoylhydroxymethyl)-furan.[18] The inability of TCPO to inhibit protein binding of an epoxide can be interpreted several ways. First, it may mean that there is no epoxide intermediate. However, a negative result does not rule out the involvement of an epoxide in the formation of protein or DNA damage. It is possible that it is not a substrate for epoxide hydrolase or that it is too reactive to be detoxified by epoxide hydrolase.

Trapping experiments indicate that *cis*-enedione intermediates are reactive metabolites of many furans. Inclusion of semicarbazide in microsomal incubations of a number of furan-containing compounds results in the trapping of the reactive metabolite as either a *bis*-semicarbazone or a pyridazine derivative.[14,19] Glutathione (GSH) trapping reactions also are consistent with the involvement of a *cis*-enedione intermediate with preferential formation of a 1,4-addition product as opposed to that resulting from GSH addition to the carbon adjacent to the furanyl oxygen (Scheme 1). All these observations do not preclude initial epoxide formation since it may rapidly rearrange to an enedione prior to reaction with nucleophiles.

This article reviews the involvement of reactive metabolites in the metabolism of a variety of compounds containing furan rings. It is focused on furan-containing compounds where there have been multiple studies exploring the characteristics of the reactive intermediate and its role in the compound's toxicity. In general, the nature of the reactive intermediate is controlled by the substitution on the furanyl ring, with more substitution favoring an epoxide intermediate. In addition, not all furan-containing molecules are toxic despite extensive oxidation of the furan ring. In these cases, the potentially toxic metabolite is rapidly neutralized by further metabolism or it is so reactive that it does not alkylate many targets beyond the enzyme catalyzing the oxidation reaction. Therefore, the structural features of the parent compound are important determinants as to whether the presence of the furanyl ring represents true toxic potential.

2 Furan

Furan is the parent compound for this class of toxic compounds. It is an important industrial chemical.[20] A product of incomplete combustion, it is also present in the environment as a component of smog, wood smoke, tobacco smoke and car exhaust and has been detected in food and beverages.[20–24] Furan is a potent liver toxicant and carcinogen in rats and mice.[25] It also induces cholangiocarcinomas in rats.[25] Because of its carcinogenic potency in animal models and the ubiquitous presence of furan in the environment, furan is listed as a possible human carcinogen (class 2B) by the National Toxicology Program[26] and the International Agency for Research on Cancer. [20]

The mechanism of tumor induction by furan is unknown. The prevailing view is that furan is a nongenotoxic carcinogen. Furan is not mutagenic in mutagenesis assays.[25,27–29] In addition, it does not elicit a DNA repair response[30] and induces a toxic response followed by compensatory cell proliferation,[30] similar to what has been observed with other nongenotoxic liver carcinogens.[31,32] However, a genotoxic mechanism cannot be excluded. There is evidence that furan induces liver tumors in mice via a genotoxic mechanism since furan induced unique mutations in activated oncogenes.[33] In addition, low levels of DNA adducts have been detected in liver DNA from [^{14}C]furan-treated rat.[34] Furthermore, furan induced liver DNA damage as detected by the comet assay.[29,34,35] Finally, a metabolite of furan is mutagenic in a number of model systems[28,36] and reacts with DNA.[37–40]

Cytotoxicity is likely to contribute to the hepatocarcinogenic properties of furan. It causes toxicity in livers of rodents at doses as low as 0.12 mg/kg.[25,41–43] A recent report indicates that furan targets cytosolic and mitochondrial proteins involved in energy production, redox regulation and protein folding, providing a possible mechanistic link between protein adduct formation and cytotoxicity resulting from the disruption of normal homeostasis.[44] Furan is a strong inducer of cell proliferation in both mouse and rat liver as a consequence of its toxic activity.[30,41,42,45,46] Genes involved in stress response, DNA damage and cell proliferation are up-regulated in rat liver following furan exposure (4 daily doses of 2, 4, 8, 12 or 16 mg/kg or 1, 3, 7, or 14 doses of 4 or 40 mg/kg or 3 months of 30 mg/kg).[35,47,48] Genes related to cell cycle regulation and DNA repair were down regulated.[35] Alterations in cell cycle and apoptotic genes were observed in the livers of rats treated daily with 0.1 mg/kg for four weeks.[49] Toxicity and proliferation precedes the development of cholangiofibrosis and subsequent cholangiocarcinomas in rats.[46,48,50] These studies support a mechanism of carcinogenesis in which chronic toxicity with secondary cell proliferation triggers cancer in furan-exposed rodents.

It is well established that furan's toxic effects require metabolism. Furan is transformed into a protein-binding intermediate via a P450-dependent process both *in vivo* and *in vitro*.[51,52] Furan depletes GSH and reduces cell viability at μM concentrations in freshly isolated hepatocytes.[53] Furan also depletes ATP in isolated hepatocytes and uncouples oxidative phosphorylation both *in vitro* and *in vivo*.[54] These effects are much reduced in the presence of P450 2E1 inhibitors such as 1-phenylimidazole and enhanced by acetone pretreatment (induction of P450 2E1), paralleling the effects of inhibitors and inducers of furan metabolism.[55]

The initial product of P450-catalyzed oxidation of furan is *cis*-2-butene-1,4-dial (BDA, Scheme 2).[14,56,57] The primary P450 enzyme responsible for the conversion of furan to BDA is P450 2E1.[57,58] While it is conceivable that there is an epoxide formed at the P450's active site, there is no evidence that this intermediate has a significant lifetime. The epoxide is not a detectable product of furan oxidation by dimethyldioxirane.[11,14] Semicarbazide trapping experiments in microsomes indicate that BDA is a reactive metabolite of furan with

the major product detected being *cis*-2-butene-l,4-dia1 *bis*-semicarbazone (**4**, Scheme 2).[14] GSH-trapping experiments generated both mono- and *bis*-GSH reaction products (**5** and **6**, respectively, Scheme 2).[57] The regiochemistry of the *bis*-GSH reaction products formed from reaction of GSH with the P450-generated metabolite was similar to that observed with synthetic BDA.[57] This observation suggests that the primary product of furan oxidation is BDA and not furanyl epoxide; the latter metabolite is expected to generate primarily the 2-substituted reaction product whereas the 3-substituted reaction product dominates in the reaction of GSH with BDA.[56] *S*-(2-Furanyl)-glutathione was not detected as a metabolite of furan.

Characterization of the *in vivo* rat metabolites of furan indicates that BDA plays a central role in its overall metabolism (Scheme 3). Oxidation of furan to BDA is likely the first step in the overall conversion of furan to its major metabolite, carbon dioxide; this metabolite represent 26% of a 8 mg/kg dose in rats.[51] All of the characterized urinary metabolites are derived from the reaction of BDA with cellular nucleophiles. [59–63] The mono-GSH-BDA reaction product **5** and its downstream metabolites **7** and **8** result from the reaction of BDA with GSH (Scheme 3).[59,60] *R*-2-Acetylamino-6-(2,5-dihydro-2-oxo-1H-pyrrol-1-yl)-1-hexanoic acid (**9**) is derived from the reaction of BDA with lysine to form pyrrolinone adducts.[60,61] A variety of urinary metabolites are derived from the cysteine-BDA-lysine cross-*S*-[1-(5-amino-5-carboxypentyl)-1*H*-pyrrol-3-yl]-L-cysteine (**10**).[60–62] Biotransformation of the cysteine portion of **10** generated metabolite **11**.[60,62] Metabolites **12**–**19** result from cysteine *N*-acetylation as well as metabolism of the lysine part of the cross-link (Scheme 3).[60–62] *N*-Acetylcysteine-BDA-spermidine cross-links **20** and **21** have also been detected in the urine from furan-treated rats (Scheme 3).[63] Similarly, the identified biliary metabolites are derived from GSH-BDA reaction products such as **5** and downstream metabolites of **6** (GSH-BDA-glutamate, CysGly-BDA-GSH, CysGly-BDAglutamate, and Cys-BDA-GSH).[64] Metabolite **14** was also detected in bile.[64]

The cysteine in the cysteine-BDA-lysine cross-links observed in vivo has several possible sources: free or protein bound cysteine or GSH. Studies in rat hepatocytes indicate that a major source of the cysteine-BDA-lysine cross-link metabolites is a GSH-BDA-lysine cross-link, *S*-[1-(5-amino-5-carboxypentyl)-1*H*-pyrrol-3-yl]-glutathione (**22**, Scheme 4).[61] This observation indicates that once BDA is formed, it reacts with GSH to form 2-(*S*-glutathionyl)butanedial (**23**), which then reacts with lysine to form GSH-BDA-lysine cross-links (**22**, Scheme 4). Both α- and ε-amino groups of lysine react with GSH-BDA to form cross-links and both products (**22a** and **22b**) are observed in hepatocytes.[61] Interestingly, the ratio of **22a** to **22b** drops over time, indicating a further enrichment of the ε-amino cross-link (**22b**).[61] This shift is consistent with an increased contribution of degraded GSH-BDA-protein lysine cross-links since GSH-BDA-lysine protein cross-links will only occur with the ε-amino group of lysine as the α-amino group is involved in a peptide bond. Subsequent metabolism of the GSH and lysine portions of this cross-link results in the observed urinary metabolites, **11**–**19** (Scheme 3). Consistent with the source of these metabolites being GSH-BDA-protein cross-links, immunoblot analysis with anti-GSH antibodies demonstrated that GSH is covalently cross-linked to liver proteins in a furan- and metabolism-dependent manner.[61] It is not surprising to consider that a major portion of the urinary and biliary metabolites are degraded protein adducts since 13% of an 8 mg/kg dose was covalently bound to liver proteins 24 h after treatment.[51]

Characterization of other hepatocyte metabolites indicated that **23** is an important reactive intermediate in furan metabolism. In addition to lysine, **23** reacted with other cellular amines including glutamine, ornithine, putrescine, and spermidine generating GSH-BDA-amine cross-links **24**–**26** (Scheme 4).[61,63] Based on these observations, it is clear that reaction of BDA with GSH does not completely deactivate BDA as previously suggested.[56] Earlier

studies indicated that GSH significantly blocked P450 catalyzed protein binding of [^{14}C]furan in microsomes.[52] It was hypothesized that GSH is a good trap for the reactive metabolite because it has both a thiol and an amino group and, therefore, is able to neutralize all the reactive characteristics of BDA.[56] However, the abundance of GSH-BDA-amine cross-links indicates that the intramolecular reaction to form the mono-GSH reaction product **5** is not fast enough to protect against alkylation of other cellular nucleophiles. In fact, our data indicate that **23** might have a sufficiently long half-life that it may migrate across membranes and alkylate amine groups distant from the site of its formation.[61] The alkylation of cellular nucleophiles by **23** likely contributes to the overall toxic properties of furan.

BDA itself is toxic and mutagenic in bacteria and mammalian cell lines.[28,36] It reacts readily with the exocyclic and endocyclic nitrogens of dGuo, dAdo and dCyd to form relatively stable diastereomeric oxadiazabicyclo(3.3.0)octaimine adducts (**27**, **28**, and **29**; Scheme 5).[37,38] The presence of the masked aldehyde in all of the BDA-derived adducts indicate that these adducts retain electrophilic reactivity. The dGuo and dAdo adducts rapidly rearrange under physiological conditions to generate substituted etheno adducts with a reactive aldehydic group (**30** and **31**, Scheme 5).[39] The dCyd and dAdo adducts were detected in bacteria treated with mutagenic concentrations of BDA.[40] However, they were not detected in liver DNA isolated from furan-treated rats.[34,65] Studies with [^{14}C]furan in rats indicate that the levels of furan-derived DNA damage is low;[34,51] a dose of 0.1 mg/kg or 2 mg/kg [^{14}C]furan (20 mCi/mmol) generated 1.7 ± 0.7 adducts/10^8 nucleotides and 3.3 ± 2.1 adducts/10^7 nucleotides, respectively, in liver DNA as determined by accelerator mass spectrometry.[34] This radioactivity did not co-elute with standards for **27**, **28**, or **29**.[34] The chemical structure of these DNA adducts was not determined. The levels of protein adduct formation far exceed the levels of DNA damage,[51] indicating that furan's reactive metabolites preferentially react with protein. BDA may be so reactive that only low quantities of this metabolite reach nuclear DNA, explaining the low levels of BDA-derived DNA damage in furan-treated rats. In addition, reaction of BDA with GSH to form **23** may also reduce the availability of BDA for reaction with DNA.

Based on these observations, we can start to make some predictions about what reactive metabolites are important to the overall toxic and mutagenic effects of furan and develop molecular mechanisms for how furan exerts its toxic effects. BDA has been considered the ultimate reactive metabolite of furan. This chemical is very reactive; it readily alkylates protein, polyamine and DNA nucleophiles *in vitro*.[38–40,56] However, a number of the cellular reaction products are derived from the reaction of GSH-BDA (**23**) with various amines such as lysine, protein amino groups and polyamines. Further studies are required to determine the relative contribution of the reactive metabolites BDA and **23** to the overall toxic and carcinogenic properties of furan.

3 2-Substituted Furans

3.1 2-Methylfuran

2-Methylfuran has been detected in many foods, coffee and cigarette smoke.[21,22,66] It induces liver necrosis and pulmonary bronchiolar lesions in rats following a single i.p. injection (≥ 100 mg/kg).[67] The toxic effects of long-term exposure to 2-methylfuran have not been investigated. The toxicity of 2-methylfuran requires metabolism. Pretreatment of rats with the P450 inhibitor *N*-octylimidazole blocked its toxicity and the P450 inducer phenobarbital enhanced its toxicity.[67] Pretreatment of rats with 3-methylcholanthrene had no effect.[67]

Metabolism led to the covalent binding of 2-methylfuran to protein and DNA *in vivo*.[67] The level of [^{14}C]2-methylfuran binding was highest in the liver; significantly lower levels of binding were also observed in kidney, lung and blood.[67] Pretreatment of rats with phenobarbital increased the covalent binding of [^{14}C]2-methylfuran to DNA and proteins in all tissues whereas the P450 inhibitor, *N*-octylimidazole, reduced covalent binding. Similar observations were reported when [^{14}C]2-methylfuran was incubated with either rat liver or lung microsomes.[68] These results suggest the involvement of a reactive metabolite in the *in vivo* toxicity of 2-methylfuran.

The initial product of P450 catalyzed oxidation of 2-methylfuran is 4-oxo-2-pentenal (**32**, Scheme 6). This enedione metabolite is a potent inhibitor of 2-methylfuran metabolism.[68] It was trapped as the *bis*-semicarbazone derivative **33** in microsomal incubations of 2-methylfuran (Scheme 6).[10] The inclusion of semicarbazide in the microsomal incubations inhibited 80% of the 2-methylfuran-derived protein binding indicating that 4-oxo-2-pentenal is a likely candidate for the reactive metabolite formed *in vivo*.[10,68] Interestingly, GSH and cysteine blocked 80% and 95% of the protein binding whereas *N*-acetylcysteine (NAC) or lysine were only able to block 40–50% of the binding. This observation indicates that both a thiol and an amino group may be required for efficient blocking of protein binding by the proposed reactive metabolite, 4-oxo-2-pentenal. However, the GSH conjugate of 4-oxo-2-pentenal was not stable enough for chemical characterization.[10]

2-Methylfuran may be activated as a result of GSH conjugation. Pretreatment of rats with L-2-oxothiazolidine-4-carboxylate (OTZ), an inducer of liver GSH, enhanced the liver toxicity of 2-methylfuran as well as increased the amount of radiolabeled 2-methylfuran covalently bound to liver proteins.[69] OTZ decreased the amount of liver DNA binding of this compound.[69] These observations are consistent with protein, not DNA, adduction as a trigger for toxicity. They also indicate that 4-oxo-2-pentenal is likely responsible for DNA adduct formation. *In vitro*, it generates 1,N^2-substituted adducts with dGuo (**34** and **35**, Scheme 7).[70,71] It is possible that a GSH-aldehyde reaction product is responsible for protein adduct formation, similar to what has been proposed for furan.[61] Characterization of the urinary and/or hepatocyte metabolites would provide some insight into the involvement of a GSH-aldehyde reaction product in this process. Attempts have been made to characterize the urinary metabolites of 2-methylfuran but these efforts were complicated by difficulties in separation as well as by the large number of metabolites detected.[69] One of the urinary metabolites was thought to be a cysteinyl derivative.[69] More in-depth characterization of the metabolites of 2-methylfuran are warranted.

3.2 Furosemide

Furosemide (4-chloro-2-(furan-2-ylmethylamino)-5-sulfamoylbenzoic acid, Scheme 8) is a 2-substituted furan that is a diuretic agent used to treat hypertension and edema. At therapeutic doses, furosemide is not toxic in humans. However, it has been associated with hypersensitivity and jaundice.[72,73] High intraperitoneal doses of furosemide (≥ 200 mg/kg) generated necrosis in the midzonal and centrilobular areas of the liver in mice.[74,75] Furosemide-treated rats or hamsters showed little evidence of hepatotoxicity.[75,76] In mice, necrosis was preceded by the formation of covalently modified proteins.[74] The toxicity does not involve dysregulation of mitochondrial function[77] or depletion of GSH.[74,77] Furosemide is toxic to both mouse and rat hepatocytes, with the toxicity accompanied by a slow depletion of GSH[78,79] and protein thiols.[79] The difference between the *in vitro* and *in vivo* observations is likely explained by the rapid elimination of the compound *in vivo*, reducing its conversion to the reactive metabolite.[79]

P450-mediated oxidation of furosemide initiates toxicity in mice. Pretreatment with the P450 inhibitors piperonyl butoxide, cobalt chloride, α-napthylisothiocyanate or 1-

aminobenzotriazole, reduced the hepatotoxicity as well as the covalent binding of the drug to liver proteins.[74,76] Pretreatment of mice with phenobarbital shifted the necrosis to the midzonal region and increased the incidence but not severity of necrosis.[76] Consistent with the induction of furosemide metabolism, phenobarbital pretreatment increased the rate of furosemide clearance and slightly enhanced the extent of furosemide covalently bound to liver proteins.[76]

Studies with microsomal preparations confirmed that P450 enzymes are involved in the generation of a protein-reactive furosemide metabolite.[16,76] Rat liver microsomal preparations were more active than mouse liver microsomes in converting furosemide to a protein-binding intermediate; both species were much more active than human liver microsomes.[75,80] This binding required oxygen and NADPH and was inhibited by a NADPH-cytochrome c reductase antibody or a carbon monoxide atmosphere. Microsomes from mice pretreated with piperonyl butoxide or cobalt chloride were less effective at converting furosemide to a protein-binding metabolite.[16,76] Microsomal protein binding was enhanced by pretreatment with phenobarbital but not 3-methylcholanthrene.[16]

Strong support for an epoxide intermediate in furosemide metabolism was the observation that the epoxide hydrolase inhibitor, TCPO, significantly enhanced the microsomal protein binding of furosemide.[16] Consistent with this hypothesis, the furan ring is required for the formation of a protein-reactive metabolite; the reduced analog, [^{35}S]tetrahydrofurosemide, did not bind to microsomal proteins.[16] Furthermore, dual labeling studies established that the furan portion of furosemide but not the sulphamoylanthranilic acid segment of the molecule is covalently attached to protein both *in vitro* and *in vivo*.[16,74,76]

Thiols are good trapping agents for the reactive metabolite; GSH, cysteine or cysteamine reduced microsomal binding by >90%.[75,76,80] The toxicity of this drug in hepatocytes was blocked by NAC.[78] Consistent with the trapping of a reactive intermediate, several GSH conjugates are formed when GSH is included in microsomal mixtures.[80] The chemical structure of these metabolites was not determined. Inclusion of NAC and *N*-acetyl-lysine (NAL) in microsomal incubations led to the formation of a double NAC/NAL furosemide adduct whose mass spectral characteristics were supportive of a pyrrole ring structure with the amino group of NAL providing the pyrrole nitrogen (**36**, Scheme 8).[75] This latter observation suggests the formation of a γ-ketoenal intermediate (**37**) following oxidation of the furan ring.

Attempts to synthesize the epoxide or γ-ketoenal metabolite by oxidizing furosemide with dimethyldioxirane led to the detection of 9-chloro-2,6-dioxo-9-sulfamoyl-1,2,4a,6-tetrahydro-1H-benzo[d]pyrido[2,1-b][1,3]oxazine (**38**) which converted to 4-chloro-2-(3′-hydroxypyridinium-1′-yl)-5-sulfamoylbenzoic acid (**39**, Scheme 8).[81] There was no evidence of an epoxide or γ-ketoenal intermediate in this process. This compound resulted from initial intramolecular condensation of the amino group with the aldehyde of the presumed γ-ketoenal intermediate. This pyridinium ion was detected in rat liver microsomal incubations of furosemide in the presence of the required cofactors.[81]

Characterization of the *in vivo* and hepatocyte metabolites of furosemide in rats and mice indicated that there are three major routes of metabolism.[75] They include acyl glucuronidation to form **40**, *N*-dealkylation to generate 2-amino-4-chloro-5-sulfamoylbenzoic acid (**41**), and furan ring oxidation to produce a GSH conjugate **42** and a γ-ketocarboxylic acid metabolite, 4-chloro-2-(4-carboxy-2-oxobutylamino)-5-sulfamoylbenzoic acid (**43**, Scheme 9). Metabolites **42** and **43** were formed through P450 mediated pathways since their levels were reduced by the P450 inhibitor, 1-aminobenzotriazole.[75] P450 enzymes 2C11, 2E1, 3A1 and 3A2 are involved in the overall

metabolism of furosemide in rats.[82] Wistar rats metabolize furosemide more rapidly than CD-1 mice but have significantly less covalent binding of the compound to hepatic liver proteins.[75] In these studies, the products of *N*-dealkylation (**41**) and furan ring oxidation (**43**) were the major biliary metabolites in rats representing 21 and 22% of the excreted dose, respectively.[75] The acyl glucuronide **40** accounted for 13% of the excreted dose. In mice, furosemide represented roughly 90% of the excreted dose. The remaining 10% was attributed to roughly equal amounts of metabolites **40**, **41** and **43**.[75] The GSH conjugate was not detected in mice.

These same metabolites were observed in hepatocytes from CD1 mice and Wistar rats.[75] Rat hepatocytes were more active than mouse hepatocytes in converting furosemide to metabolites, with the major pathway being acyl glucuronidation (~60% of the recovered radioactivity). The extent of glucuronidation in mouse hepatocytes was roughly one half that observed in rats. Similar to the *in vivo* results, the GSH conjugate **42** was not observed in mouse hepatocytes. The levels of P450-dependent metabolism were similar in both species.

Collectively, these observations indicate that epoxide and γ-ketoenal intermediates are important in the bioactivation of furosemide to a toxic metabolite. The structures of the γ-ketocarboxylic acid metabolite may require the formation of an γ-ketoenal intermediate. Either intermediate will lead to the formation of the NAC/NAL adduct and the pyridinium metabolite observed in microsomal preparations.

Species differences in toxicity between rat and mouse may be explained by the relative turnover of parent drug to stable metabolites: 66% turnover in rats as compared to 10% turnover in mice.[75] This is predicted to increase the exposure of the mouse liver to furosemide. The lack of hepatotoxicity in humans results in part from a reduced ability to generate the protein-reactive intermediate.[75,80] The cause of the decreased protein binding is not known; it could be either low furan oxidase activity or increased detoxification of the reactive intermediate. Furthermore, glucuronidation is the dominant pathway of metabolism in humans,[83–85] reducing the amount of drug available for oxidation. In addition, the efficacious dose in humans (1–2 mg/kg) is well below the doses required for toxicity in rodents (>200 mg/kg). Finally, the reactive metabolite of furosemide may be inactivated by intramolecular trapping; however, it is not known if this metabolite is generated *in vivo*.

3.3 Prazosin

Prazosin (2-[4-(2-furoyl)piperazin-1-yl]-6,7-dimethoxyquinazolin-4-amine) is an antihypertensive agent that is also used as a treatment for post-traumatic stress disorder.[86] This drug has been in use since 1976 with no evidence of safety issues despite the presence of the 2-substituted furan ring. Furan ring oxidation is a major *in vivo* pathway of metabolism for this drug,[86] which indicates that furanyl ring oxidation does not necessarily lead to toxicity. The most abundant metabolite detected in urine and feces of prazosin-treated rats is the ring-opened carboxylic acid derivative **44** (Scheme 10).[86] GSH conjugation to the furanyl group is a minor pathway.

Studies in liver microsomes confirmed that furan ring opening is a major metabolic pathway of prazosin.[19] Both the acid **44** and alcohol **45** products of the ring-opening reaction were observed (Scheme 10). Levels of these two metabolites were significantly reduced when semicarbazide was included in the incubation mixture.[19] The intermediate was trapped as a pyrazidine product **46** which is consistent with the involvement of a γ-ketoenal intermediate **47** (Scheme 10). GSH trapping led to the formation of GSH reaction products with molecular ions at *m/z* 709.[19] This mass is consistent with the addition of GSH to a γ-keto-α,β-unsaturated alcohol **48**. These data indicate that the γ-ketoenal intermediate is rapidly reduced to an alcohol in microsomes. A recent report indicates that P450s are good catalysts

for the reduction of α,β-unsaturated aldehydes.[87] Therefore, the swift reduction/oxidation of the reactive metabolite is likely responsible for the absence of toxicity observed with prazosin despite the extensive metabolism of the furanyl ring.

4 3-Substituted Furans

4.1 3-Methylfuran

3-Methylfuran is a component of indoor and outdoor air pollution,[4,5] as well as a volatile mold metabolite.[5] It has been detected in food[88] as well as in the exhaled breath from smokers and passive smokers.[89,90] When inhaled, 3-methylfuran causes acute pulmonary toxicity in mice (≥ 14 μmol/L for 1 h),[91–94] rats (148 μmol/L for 1 h) and hamsters (322 μmol/L for 1 h).[91] It also induces acute olfactory necrosis in rats[92] and mice[93] and kidney toxicity in mice.[93] When administered intraperitoneally, it is a lung but not liver or kidney toxicant.[94,95] Exposure to 3-methylfuran by inhalation (344 μmol/L for 2 h once a week for 10 weeks) did not led to any adverse effects 10 months or 2 years later.[96]

The toxicity of 3-methylfuran requires metabolism by P450 enzymes. The general P450 inhibitor, piperonyl butoxide, blocked both pulmonary macromolecular binding and necrosis induced by the inhalation of 3-methylfuran.[97] Binding of 3-methylfuran to lung microsomal proteins required NADPH and O^2 and was inhibited by carbon monoxide or piperonyl butoxide.[10,97] Inclusion of semicarbazide in hepatic and pulmonary microsomal incubations trapped the proposed reactive metabolite, 2-methyl-*cis*-2-butene-1,4-dial (**49**), as a *bis*-semicarbazone derivative **50** (Scheme 11) and protected against the protein binding of 3-methylfuran *in vitro*,[10] supporting the hypothesis that 2-methyl-*cis*-2-butene-1,4-dial is the reactive metabolite responsible for the toxic effects of this compound.

4.2 4-Ipomeanol

4-Ipomeanol [1-(3′-furyl)-4-hydroxy-1-pentanone, Scheme 12] and its structural analogs, ipomeanine [1-(3′-furyl)-1,4-pentanedione], 1-ipomeanol [1-(3′-furyl)-1-hydroxy-4-pentanone] and 1,4-ipomeadiol [1-(3′-furyl)-1,4-pentanediol] are cytotoxicants produced by moldy sweet potatoes.[98] Ipomeanine and 1,4-ipomeadiol are also metabolites of 4-ipomeanol.[99] They are lung specific toxicants in laboratory animals.[98,100,101] 4-Ipomeanol is a liver toxicant in humans.[102–104]

The toxicity of these compounds requires P450 oxidation of the furan ring.[100,105] Tissue selective P450 expression likely contributes to the species differences in target organ toxicity caused by 4-ipomeanol. P450 4B1 catalyzes the oxidation of 4-ipomeanol in rodent lungs.[106–108] The human homolog of this enzyme is functionally defective,[109] explaining why 4-ipomeanol is not a pulmonary toxicant in humans. Human liver P450s 1A2, 2C19, 2D6 and 3A4 are all able to catalyze the oxidation of 4-ipomeanol to a reactive metabolite;[106,108] the activities of these enzymes are low in human lung.[110]

Consistent with the proposal that metabolism is important for the toxicity of 4-ipomeanol, a shift in target organ toxicity to the liver was observed in rats when they were pretreated with 3-methylcholanthrene.[100] 3-Methylcholanthrene induces liver but not lung microsomal metabolism of 4-ipomeanol.[105] A similar shift in organ specificity is not observed with phenobarbital pretreatment despite increased liver microsomal metabolism of 4-ipomeanol.[105] This seemingly contradictory response can be explained by the likely simultaneous induction of detoxification enzymes; phenobarbital, but not 3-methylcholanthrene, induced a large increase in urinary level of ipomeanol-4-glucuronide in rats.[111]

Oxidation of 4-ipomeanol led to the formation of protein and DNA adducts.[17,106,112] The structures of these adducts have not been characterized. Consistent with the hypothesis that metabolism drives the toxicity of this chemical, levels of protein binding were higher in lung versus liver microsomes from rats.[112] Phenobarbital and 3-methylcholanthrene pretreatment increased microsomal covalent binding of 4-ipomeanol in rat liver microsomes but not lung microsomes, paralleling the effect of these inducers on its microsomal metabolism.[112] 4-Ipomeanol is a mechanism-based inhibitor of P450 3A4 (Table 2) but not the other P450s known to metabolize this furan.[108,113] The stoichiometry of binding between the radioactive substrate and enzyme was ~1.5:1. GSH provided only minor protection (10%) against inactivation indicating that the metabolite that inhibits the enzyme is very reactive.

The chemical structure of the reactive intermediate is not clearly established. Addition of the epoxide hydrolase inhibitor TCPO had little effect on covalent binding,[17] suggesting that an epoxide intermediate may not participate in this reaction. GSH protects against protein binding and toxicity *in vitro* and *in vivo*.[17,100,112] Correspondingly, GSH conjugates have been detected in GSH-enriched microsomal incubations of 4-ipomeanol.[99,108,112,114,115] Inclusion of cytosol does not influence the levels of GSH-conjugates indicating that the reactive metabolite is either very reactive or it is not a good substrate for cytosolic GSTs.[112]

Chemical characterization of the GSH-trapped metabolites demonstrates a complex picture. Buckpitt and Boyd reported the observation of two GSH reaction products in rat liver and lung microsomes incubated in the presence of 4-ipomeanol, NADPH and GSH.[112] These metabolites lacked UV absorbance, indicating that the furan chromophore of the parent compound had been disrupted but the structures of these products were not determined. Alvarez-Diez and Zheng detected one microsomal GSH-4-ipomeanol reaction product whose MS data were consistent with the addition of one molecule of GSH to an epoxide or enedione intermediate (*m/z* 492).[114] Baer *et al.* did not observe the mono-GSH reaction product. However, they reported two *bis*-GSH-reaction products whose MS data were consistent with the formation of 4-ipomeanol mediated cross link between the thiol of one GSH and the amino group of another (**51** or **52**, Scheme 12).[108] Chen *et al.* demonstrated that the reactive metabolite of 4-ipomeanol can react with either one or two molecules of GSH to form multiple mono- or *bis*-GSH reaction products **51**–**56** (Scheme 12).[99] The structure of these products can be rationalized by either initial 1,2- or 1,4-addition to the proposed enedial intermediate **57**. These addition products then react with the amino group of the same or different GSH molecule to form pyrrole cross-links. Based on the balance of product formation, 1,2-addition is the major pathway. Similar findings were reported for the trapping of the reactive intermediate with NAC and NAL where the major product was a 2-(*S*-*N*-acetylcysteinyl)-4-(4-hydroxypentanoyl)-pyrrole adduct of NAL (**58**, Scheme 13).[108] These products could also be formed by thiol attachment of an epoxide intermediate. Presumably this reaction would occur primarily on the carbon adjacent to the furanyl oxygen ring. Model reactions with the proposed enedial intermediate have been stymied by the difficult synthesis of this possible reactive intermediate.[99]

4.3 L-739,010

L-739,010, [1*S*,5*R*]-3-cyano-1-(3-furyl)-6-(6-[3-(3α-hydroxy-6,8-dioxabicyclo[3.2.1]-octanyl)]pyridin-2-yl-methoxyl)naphthalene, is a direct inhibitor of 5-lipoxygenase. While the plasma half-life of this drug is about 4h, the plasma half-life of radioactivity after i.v. treatment with the [^{14}C]labeled drug was 2.7 and 4 days in rats and rhesus monkeys, respectively.[116] The radioactivity was covalently associated with plasma proteins. While L-739,010 caused a dose-dependent elevation in alanine transaminase levels in dogs, suggesting that it induced hepatotoxicity,[116] histopathological analysis showed no indication of liver toxicity.[117] It initiated lipidosis in the gall bladder.[117]

Binding of L-739,010 to proteins requires metabolism. Incubation of L-739,010 with liver microsomes results in the NADPH-dependent loss of parent compound without significant formation of detectable metabolites.[116,118] There were species differences in the rate of protein binding (monkey > rat > human > dog).[116,118] Most of the unaccounted-for-material was covalently bound to microsomal proteins.[116] P450 3A enzymes are implicated as the critical enzyme for this process since protein adduct formation was induced by phenobarbital and dexamethasone and was inhibited by the P450 inhibitors triacetyloleandomycin and diethyldithiocarbamate.[116,119] Inclusion of GSH and methoxyamine reduced the covalent binding by ~50% and ~80%, respectively.[116]

Furanyl ring oxidation to a reactive enedial (**59**) is an important step for the covalent binding of L-739,010 to proteins (Scheme 14). Inclusion of methoxyamine led to the formation of five methyloxime adducts.[116,118] The mono- and *bis*-methyloxime adducts of the furan ring-opened metabolite of L-739,010 were observed (**60** and **61**, respectively). The presence of the mono-methyloxime adduct suggests that one of the aldehydes is more reactive than the other. The major product was the *Z* isomer of the 1-*O*-methyloxime-2-butene-4-alcohol derivative of L-739,010 (**62**). This product results from the reduction of the enedial metabolite of L-739,010 to an alcohol. The fifth methyloxime adduct was to the 6,8-dioxabicyclo[3.2.1]octanyl portion of the molecule.[116,118] Furan oxidation was the dominant pathway in rat liver microsomes.[116] The absence of any detectable metabolites in the absence of trapping agents indicates that the enedial intermediate **46** or possible epoxide precursor is very reactive with protein nucleophiles.

4.4 Teucrin A

The 3-substituted furan, teucrin A, is the major germander neoclerodane diterpenoid present in germander extracts.[120] Germander has been used as an herbal antiseptic and anticholeretic medicine as well as a bitter flavoring of wine and spirits.[120] In the early 1990s, there were numerous reports of cytolytic hepatitis resulting from germander capsule or tea consumption.[120–127] A number of the cases showed signs of an immunoallergic type of hepatitis since a rechallenge caused early recurrence of the toxicity. Germander-derived human liver toxicity was replicated in the mouse and shown to be caused by furan-containing neoclerodane diterpenoids.[128] A major member of this group, teucrin A, produced the same midzonal hepatic necrosis in mice as extracts enriched in these compounds.[129]

Metabolism of the furan ring was required for teucrin A-induced hepatotoxicity. Toxicity was enhanced by P450 3A inducers and blocked by P450 3A inhibitors.[128,129] It was accompanied by the depletion of intracellular GSH and alkylation of protein thiols.[129,130] GSH depletion enhanced the toxic effects of both teucrin A and teucrin A containing extracts.[128–130] These data indicate a GSH-reactive metabolite is likely responsible for the liver toxicity. Consistent with the formation of a reactive metabolite, teucrin A becomes covalently associated with hepatocellular proteins in a dose-dependent manner.[130] The tetrahydrofuran analog of teucrin A was not a hepatotoxicant in mice, providing strong evidence that oxidation of the furan ring was critical for toxicity.[129]

Several pieces of data are consistent with a furanyl epoxide of teucrin A as the reactive metabolite (**63**, Scheme 15). First, teucrin A's hepatotoxicity was reduced in animals pretreated with either butylated hydroxyanisole or clofibrate, two compounds that induce epoxide hydrolase.[128] Additional support was obtained *in vitro*. P450 3A4 converted teucrin A into a single uncharacterized metabolite which was converted into two uncharacterized products when epoxide hydrolase was included in the incubation mixtures.[131] In these reactions, epoxide hydrolase was inactivated in a time dependent manner. These observations are consistent with P450 3A4-catalyzed formation of a teucrin A furanyl

epoxide intermediate, which is hydrolyzed by epoxide hydrolase. Since serum antibodies from patients who chronically consumed germander tea recognized human epoxide hydrolase both modified and unmodified with teucrin A, it has been postulated that the P450 3A4- and teucrin A-dependent modification of epoxide hydrolase triggers autoantibody formation.[131] This mechanism could explain the immunoallergic nature of the toxicity in some patients.

Attempts to generate the furanoepoxide of teucrin A were unsuccessful.[15] The product of dimethyldioxirane oxidation of teucrin A is the *cis*-2-ene-dialdehyde derivative of teucrin A **64**, not the epoxide (Scheme 15). This observation does not rule out the possible intermediacy of an epoxide in the P450 catalyzed oxidation of teucrin A because it is expected to rapidly rearrange to the enedial. Reaction of the synthetic enedial of teucrin A with model protein nucleophiles, NAC and/or NAL, led to adducts (Scheme 15).[15] Reaction with NAL led to *N*-alkyl-3-pyrrolin-2-one reaction products with the more sterically hindered product, *N*-alkyl-3-alkyl-3-pyrrolin-2-one (**65**) as the major product. Reaction of the enedial with NAC led to the formation of unstable products as well as a stable *N*-acetyl-S-2-furanyl-L-cysteine adduct (**66**). When the enedial was incubated with NAC and NAL, a number of products were observed with the major product being a 1,2,4-subsubstituted pyrrole (**67**). Small amounts of tetrasubstituted pyrroles were also observed. In all cases, epimerization occurred at the C12 of teucrin A, providing support for the involvement of enedial and hydroxyenal tautomers in the reactions of this compound with nucleophiles.

Protein adduction by teucrin A appears to be an important trigger of its toxicity. Specific protein targets were identified using antibodies generated against teucrin A enedial-treated peptides.[132] The major teucrin A targets in rat livers were mitochondrial and endoplasmic reticulum associated proteins in addition to enzymes involved in cell maintenance and cellular metabolism. A number of the endoplasmic reticulum proteins were xenobiotic metabolizing enzymes such as P450 enzymes (P450s 2A1, 2A2, 2C7, 2D1 and 2D3), UDP-glucuronosyl transferase 1-1 and epoxide hydrolase. GSH *S*-transferase was a cytosolic target of teucrin A. The widespread alkylation of mitochondrial proteins may suggest that teucrin A causes its toxicity by triggering mitochondrial dysfunction. The toxicological significance of these reactions remains to be determined.

5 2,3-Substituted Furans

5.1 Menthofuran

Pennyroyal oil is used as a flavoring agent or fragrance component as well as an herbal medicine for abortion and menses induction.[133] High doses of pennyroyal oil used for the latter purpose cause hepatoxicity and death in humans.[134,135] Menthofuran is a minor constituent of pennyroyal oil as well as a primary metabolite of R-(+)-pulegone in rat, the major terpene constituent of pennyroyal oil.[136–139] Menthofuran is a liver and lung toxicant in mice[133,140] and a liver toxicant in rats.[141] It induces midzonal hepatoxicity in Swiss-Webster mice[140] and centrilobular necrosis in BALB/c mice.[133]

Like other furans, menthofuran-induced toxicity requires P450-mediated oxidation. The toxicity was increased by pretreating animals with phenobarbital[140,142] and blocked by pretreatment with piperonyl butoxide,[140] indicating the importance of P450 enzymes in the metabolic activation of menthofuran. 3-Methylcholanthrene did not influence menthofuran toxicity in rats.[142] The metabolism of [^{14}C]menthofuran converts it into a protein-binding metabolite *in vivo*.[140] Interestingly, while phenobarbital pretreatment caused a doubling of the liver toxicity, it slightly reduced the levels of menthofuran covalently bound to liver proteins.[140] The reasons for this discrepancy are not understood. Liver GSH levels are only

modestly reduced following menthofuran exposure.[133] Depletion of GSH with diethylmaleate or buthionine sulfoximine had no effect on menthofuran toxicity.[133,141]

Incubation of [^{14}C]menthofuran with rat, mouse or human liver microsomes led to the formation of protein adducts.[140] This binding required NADPH and was blocked by the inclusion of semicarbazide. This binding was reduced by the pretreatment of mice with piperonyl butoxide and enhanced by the pretreatment of mice with phenobarbital.[140] All these data support the involvement of P450 enzymes in the formation of a reactive metabolite.

A metabolite with the mass expected for the γ-ketoenal metabolite, 2-*Z*-(2′-oxo-4′-methylcyclohexylidene)propanal (**68**) was detected by GC-MS as a metabolite of menthofuran in rat liver microsomes (Scheme 16).[143,144] This metabolite was converted to 5,6,7,8-tetrahydro-4,7-dimethyl-7*H*-cinnoline (**69**) upon inclusion of semicarbazide in rat, mouse or human liver microsomes,[140,143,144] indicating that **68** or some precursor to **68** is the protein-binding metabolite of menthofuran. 2-Hydroxymenthofuran (**70**) was also detected as a product of P450 catalyzed oxidation of menthofuran.[145] This metabolite rearranges to diastereomeric mintlactones **71**, additional products of microsomal menthofuran oxidation.[140,145] 2-Hydroxymenthofuran did not react with semicarbazide to form **69**.

Oxygen-18 and ^{2}H-labeling studies provide support for initial epoxide formation in the P450 catalyzed formation of 2-hydroxymenthofuran.[145] The epoxide intermediate can either directly rearrange to form **70** or γ-ketoenal **68** and/or react with water to form the 2,3-diol which then can dehydrate to form either **70** or rearrange to the γ-ketoenal **68** (Scheme 16). Support for this mechanism was obtained in chemical model studies.[13,145] NMR studies of the chemical oxidation of menthofuran by dimethyldioxirane at low temperatures indicated that the epoxide **72** is the initial oxidation product and it undergoes a subsequent sigmatropic rearrangement to the γ-ketoenal **68**.[13] When the dimethyldioxirane reaction mixture was quenched with water, 2-hydroxymenthofuran and the mintlactones were formed.[145] When semicarbazide was present in the quenching solution, 5,6,7,8-tetrahydro-4,7-dimethyl-7*H*-cinnoline (**69**) was observed.[145] Based on this mechanism, either the epoxide **72** or the γ-ketoenal **68** could be responsible for the protein binding and toxic effects of menthofuran. Further research is required to address this question.

P450s 1A2, 2A6, 2C19 and 2E1 are catalysts for the oxidation of menthofuran in humans.[145] P450 2E1 was the most efficient catalyst of this reaction. Menthofuran is a mechanism based inactivator of P450 2A6, but not P450 1A2, 2E1 or 2A13.[146,147] The kinetic parameters of this inactivation are displayed in Table 2. The inactivation of P450 2A6 was not blocked by GSH or NAC. However, GSH, methoxylamine and semicarbazide but not NAL protected against adduction to NADPH-P450 reductase.[146] Covalent binding to P450 2A6 was not influenced by these trapping agents,[146] indicating that the reactive metabolite responsible for the inactivation does not escape the enzyme's active site.

Chemical characterization of the *in vivo* metabolites of menthofuran indicates that the oxidation of the furan ring is an important pathway *in vivo*.[143,144,148,149] *In vivo* metabolites of [2-^{14}C]menthofuran in rats indicates that the furan ring is a target for oxidation and the reactive intermediate in this process, furanoepoxide or γ-ketoenal, reacts with water, GSH or taurine.[148,149] Subsequent metabolism of these products generates a complex mixture of metabolites.[148]

Menthofuran is a metabolite of pulegone and a number of studies indicate that the metabolism of pulegone to menthofuran followed by the subsequent oxidation of

menthofuran's furanyl ring is responsible for the toxic effects of pennyroyal.[137,141,143–145,150] Incubation of pulegone with mouse liver microsomes in the presence of semicarbazide led to the formation of 5,6,7,8-tetrahydro-4,7-dimethyl-7H-cinnoline (**69**) which is consistent with the P450 catalyzed conversion of pulegone to menthofuran which subsequently is oxidized to an γ-ketoenal intermediate (Scheme 16).[151] The presence of semicarbazide reduced the protein binding of [^{14}C]pulegone,[151] indicating that **68** or **72** are involved in the covalent binding of pulegone to proteins. The importance of this pathway to the toxic effects observed in humans consuming high doses of pennyroyal is supported by the detection of menthofuran-modified proteins in liver samples from an individual who died from the consumption of pennyroyal oil.[135] However, pennyroyal and *R*-(+)-pulegone resulted in extensive depletion of GSH.[133] Moreover, GSH depletion enhanced the toxicity of these two treatments.[133] These results are different from what has been reported for menthofuran which does not deplete GSH nor is its toxicity enhanced by GSH depletion.[133,141] There are likely other pathways contributing to pennyroyal toxicity in addition to the toxicity triggered by menthofuran.

5.2 Furanocoumarins

5.2.1 Bergamottin—Bergamottin is the parent compound for a variety of furanocoumarins present in grapefruit juice.[152–157] Consumption of grapefruit juice resulted in increased bioavailability of a variety of drugs, which results in altered drug activity as well as toxicity.[158] This interaction is thought to occur via irreversible inhibition of intestinal P450s, resulting in increased absorption of the drug.[158] All of the furanocoumarins in grapefruit juice are mechanism-based inhibitors of P450 3A4 as well as other P450s.[154–157,159–161] The furan ring is required for inactivation.[157]

The inactivation of P450s by bergamottin is time- and concentration-dependent and requires NADPH.[154,159,160] The kinetic parameters for the inactivation are displayed in Table 2. GSH does not block the irreversible inhibition suggesting that the reactive metabolite does not escape the active site prior to inactivation.[154] The stoichiometry of bergamottin binding to the apoprotein is ~0.5:1.[159] The P450 reduced carbon monoxide spectrum was decreased and the native heme destroyed for all inactivated P450s.[154,159] The reactive intermediate reacts with the apoprotein; no products of heme alkylation were observed.[154,159]

Mass spectral analysis of the inactivated enzyme indicated the addition of bergamottin plus 2–3 oxygen atoms.[159] Metabolism studies performed with P450 2B6 and P450 3A5 in the presence of GSH indicated that oxidation of the furan double bond leads to the formation of a GSH conjugate (**73**) in which GSH has added to an epoxide intermediate (**74**, Scheme 17).[161] The corresponding dihydroxymetabolite resulting from the reaction of an epoxide intermediate with water was not detected. The main site of oxidation is the geranyloxy side chain which is readily hydroxylated by P450s. Based on these observations, it was proposed that bergamottin is first oxidized on the geranyloxy side chain and then undergoes oxidation of the furan ring to generate the reactive intermediate which results in enzyme activation.[159] Lin *et al*. proposed either a furanoepoxide or γ-ketoenal intermediates as possible structures for the reactive metabolite.[159]

5.2.2 L-754,394—L-754,394 (*N*-[2(*R*)-hydroxy-1(*S*)-indanyl]-5-[2(*S*)-[((1,1-dimethylethyl)amino)-carbonyl]-4-[(furo[2,3-*b*]pyridin-5-yl)methyl]piperazin-1-yl]-4(*S*)-hydroxy-2(*R*)-(phenyl-methyl) pentanamide) is an experimental anti-AIDS drug that is a potent and selective inhibitor of HIV-1 protease. Preclinical pharmacokinetic studies indicated that it was a mechanism based inhibitor of P450.[162]

Studies in rat, dog, monkey and human liver microsomal preparations demonstrated that L-754,394 caused an NADPH-dependent and time-dependent reduction in the CO-binding

spectrum of P450.[162,163] P450 2C11 and P450 3A1/2 are the targets in rat liver microsomes with the activity of P450 2C11 decreased by 60–70% in liver microsomes from rats given a single iv dose of 10 mg/kg.[162] L-754,394 is a mechanism based inhibitor of human P450 3A4[163–165] as well as P450 2D6 (Table 2).[163] The low partition ratio (≤ 4) indicates that L-754,394 is a potent inactivator of P450 3A4. The binding stoichiometry was 1.08 ± 0.5 moles drug covalently bound/moles P450 inactivated in studies performed with recombinant P450 3A4.[165] While GSH, NAC and methoxyamine did not protect against L-754,394-mediated P450 inactivation, these trapping agents modestly reduced (15–25%) the NADPH-dependent binding of [^{14}C]L-754,394 to microsomal proteins.[164] The epoxide hydrolase inhibitor, TPCO, did not affect microsomal protein binding when present during the microsomal incubations.[164] These data indicate that the metabolic intermediate in the inactivation process is very reactive. Studies performed with recombinant P450 3A4 indicate that very little of the reactive metabolite escapes the enzyme's active site.[165]

The furan ring is required for the inactivation; the dihydrofuran derivative and the analog that lacks the furan ring were not irreversible inhibitors of P450.[164] Chemical characterization of the microsomal metabolites of L-754,394 provides evidence for a furanopyridine epoxide intermediate **75** (Scheme 18). However, further experimentation is required to eliminate the involvement of a γ-ketoenal intermediate **76**.[165] Mass spectral characterization of the trapped GSH/NAC reaction products indicates that they are GSH/NAC adducts of a mono-oxygenated L-754,394 metabolite with the proposed structure **77** as shown in Scheme 18.[164] In rat and monkey liver microsomes, a metabolite was detected whose MS was consistent with a dihydrodiol **78** which was converted into an *O*-methyloxime **79** when methoxyamine was included in the incubation mixtures (Scheme 18).[164]

Attempts to characterize the L-754,394-P450 adduct were complicated by its instability to protein purification conditions. The covalent modification was unstable to protein purification and hydrolysis conditions.[165] However, Lighting *et al* demonstrated that the radioactivity was specifically associated with the apoprotein and that the radioactivity released from the protein during CNBr generation of peptides had a retention time similar to the dihydrodiol metabolite **78**.[165] The peptide fraction associated with L-754,394-derived radioactivity was tentatively identified as an active site peptide. Subsequent LC/MS studies performed with the intact adducted P450 3A4 indicated that the inactivation of P450 was accompanied by the addition of 685 ± 3 Da. This mass increase indicates that the mass of the apoprotein has increased by the weight of two oxygen atoms and L-754,394, not by the addition of one oxygen atom and L-754,394 as expected for enzyme inactivation by a furanopyridine epoxide or γ-ketoenal intermediate.[166] These data suggest that L-754,394 undergoes a double oxidation or an oxidation plus addition of water in the inactivation reaction. Therefore, simple reaction of a protein nucleophile with an oxidized intermediate of L-754-394 is ruled out as a possible mechanism of enzyme inactivation. Further experiments are necessary to elucidate the mechanism of L-754,394-mediated irreversible inhibition of P450 3A4.

6 Conclusions and Future Directions

Several common themes are highlighted in this literature review. One is that the toxicity of furan ring containing compounds requires oxidation of the furan ring. The target organ toxicity exhibited by this group of compounds is often driven by tissue-specific expression of the P450 enzymes responsible for the oxidation of the furan ring. There are at least three factors that impact the overall toxicity of a particular furan-containing compound. First, the involvement of other metabolic pathways can influence its toxicity. Furans that are more actively metabolized through other pathways are less toxic than those compounds whose

primary metabolic pathway involves furan ring oxidation. For example, furosemide is relatively non-toxic since its major route of metabolism is glucuronidation, not furan ring oxidation. Second, rapid detoxification of the reactive intermediate results in a less toxic compound. This explains why prasozin is a safe drug despite the fact that furan ring oxidation is a major pathway of metabolism; rapid reduction of the α,β-unsaturated system of the intermediate results in an nontoxic metabolite. Third, the reactivity of the furan ring oxidized metabolite will influence the cellular targets and, consequently, the overall toxicity of the compound. The most reactive metabolites result in enzyme inhibition such as bergamottin and L-754,394. The less reactive migrate beyond the site of formation and alkylate targets in other subcellular locations.

Structure also determines the relative stability of the intermediate in furan ring oxidation. Less substituted furans generate less stable epoxides and are more likely to rearrange to a reactive enedione intermediate. The strongest evidence for the intermediacy of an epoxide was obtained with more substituted furans such as menthofuran, bergamottin and L-754,394. In addition, the more rigid the furan ring, the longer the lifetime of the epoxide intermediate.

GSH plays an important role in modulating the toxicity. It readily reacts with the reactive intermediates to form GSH conjugates, in most cases, in the absence of glutathione *S*-transferases. For most furans, this reaction protects cells against the harmful effects of the furan; consistently, GSH depletion enhanced the toxicity of these compounds. However, GSH may enhance the toxicity for the less substituted furans. 2-Methylfuran was more toxic when GSH levels were elevated. The mechanism behind this enhancement has not been investigated. Furan, one of the most toxic in this chemical class, is converted to many GSH-BDA-amine cross-links. Whether GSH plays a role in the toxicity of furan is a question still needs investigation.

Future studies will define how these reactive intermediates cause their toxicity. It is clear that the molecular structure of the reactive intermediate influences the location and types of cellular nucleophiles targeted. Alkyl substitutions on the enedione reactive intermediate alter the rate at which it reacts with cellular nucleophiles and has some influence on its toxicity.[167] For example, the reactive intermediate(s) in furan bioactivation primarily target proteins whereas the addition of a methyl group results in significant formation of DNA as well as protein adducts.[34,51,67,69] The cellular targets will determine the toxicological outcome; DNA adduct formation is critical for cancer initiation whereas protein alkylation is important for toxicity. A catalog of how furan structure alters the cellular targets will provide insights into the toxic mechanisms of this class of chemicals and will provide valuable information for the design of safe drugs as well as aid in human risk assessment when exposed to furan-containing compounds.

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Abbreviations

BDA	*cis*-2-butene-1,4-dial
NAC	*N*-acetylcysteine

NAL	*N*-acetyl-lysine
OTZ	L-2-oxothiazolidine-4-carboxylate
TCPO	1,2-epoxy-3,3,3-trichloropropane

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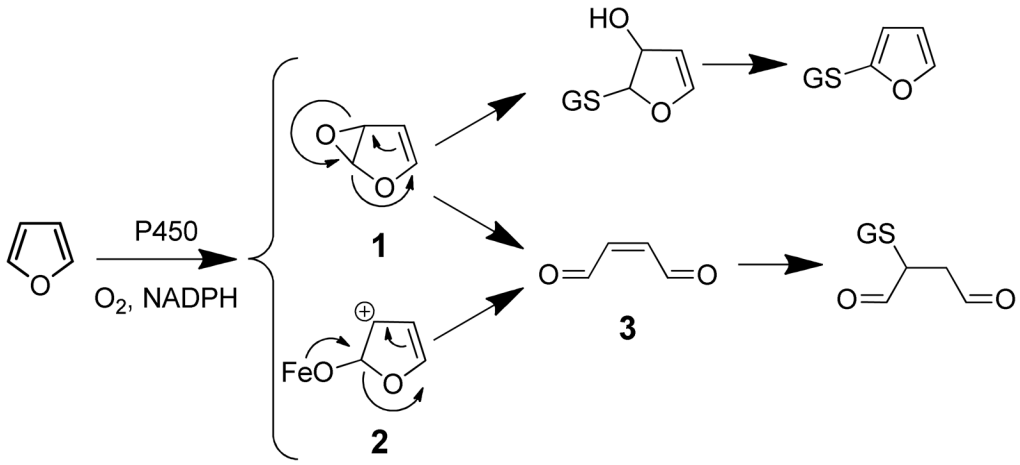
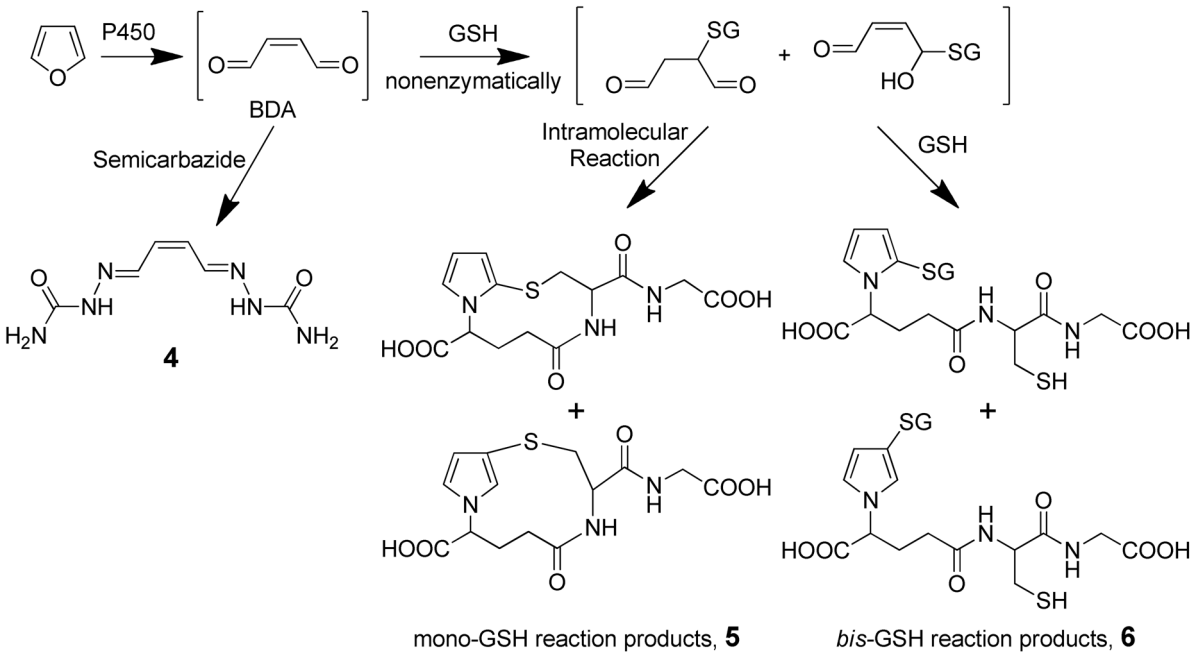
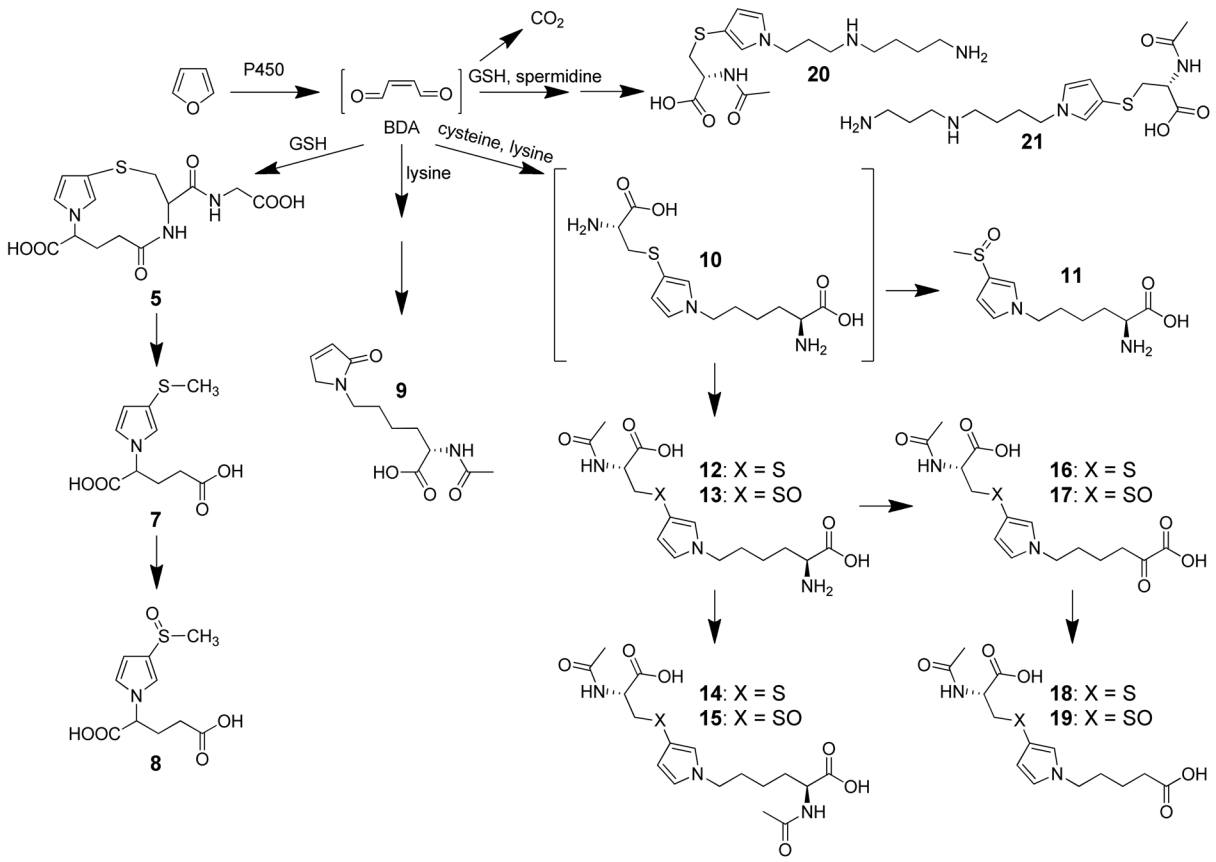


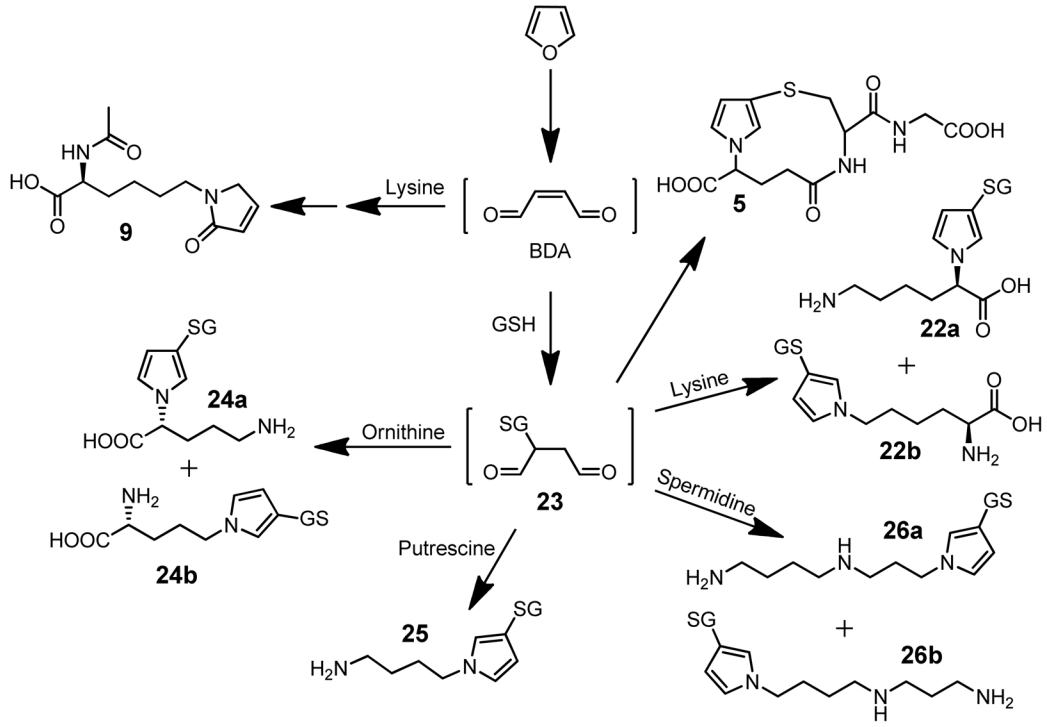
Scheme 1.
Oxidation of furan by P450 enzymes to either an epoxide **1** or a cis-enedione **3** metabolite and their reaction with GSH.[9,10]



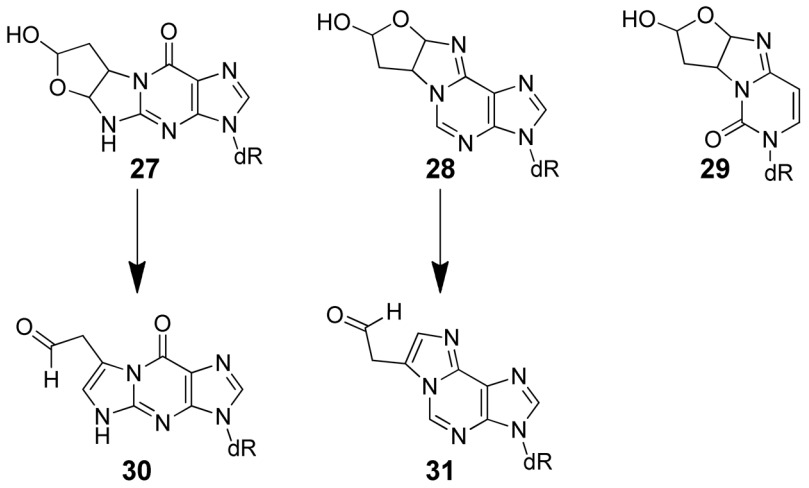
Scheme 2.
Trapping of the reactive metabolite formed in the P450-catalyzed oxidation of furan in microsomes.[14,57]



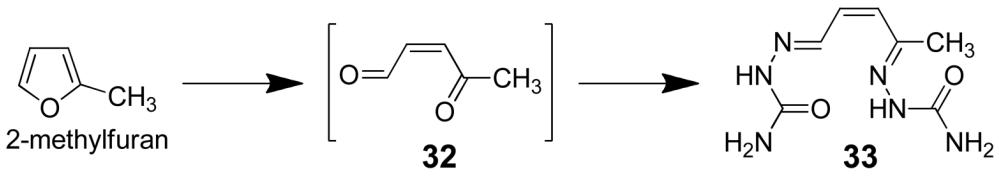
Scheme 3.
Pathways of *in vivo* metabolism of furan.[51,59–62]



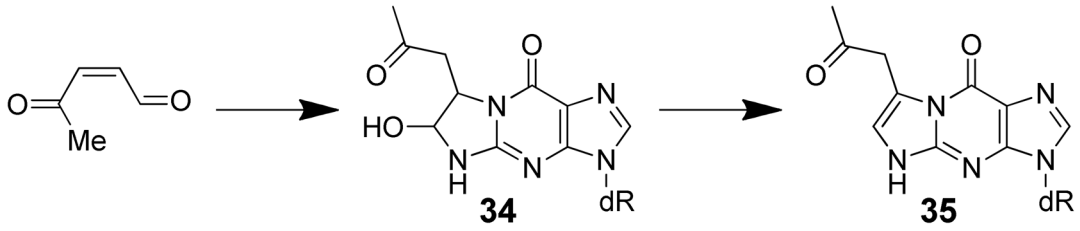
Scheme 4.
Metabolites formed from furan in rat hepatocytes.[61,63]



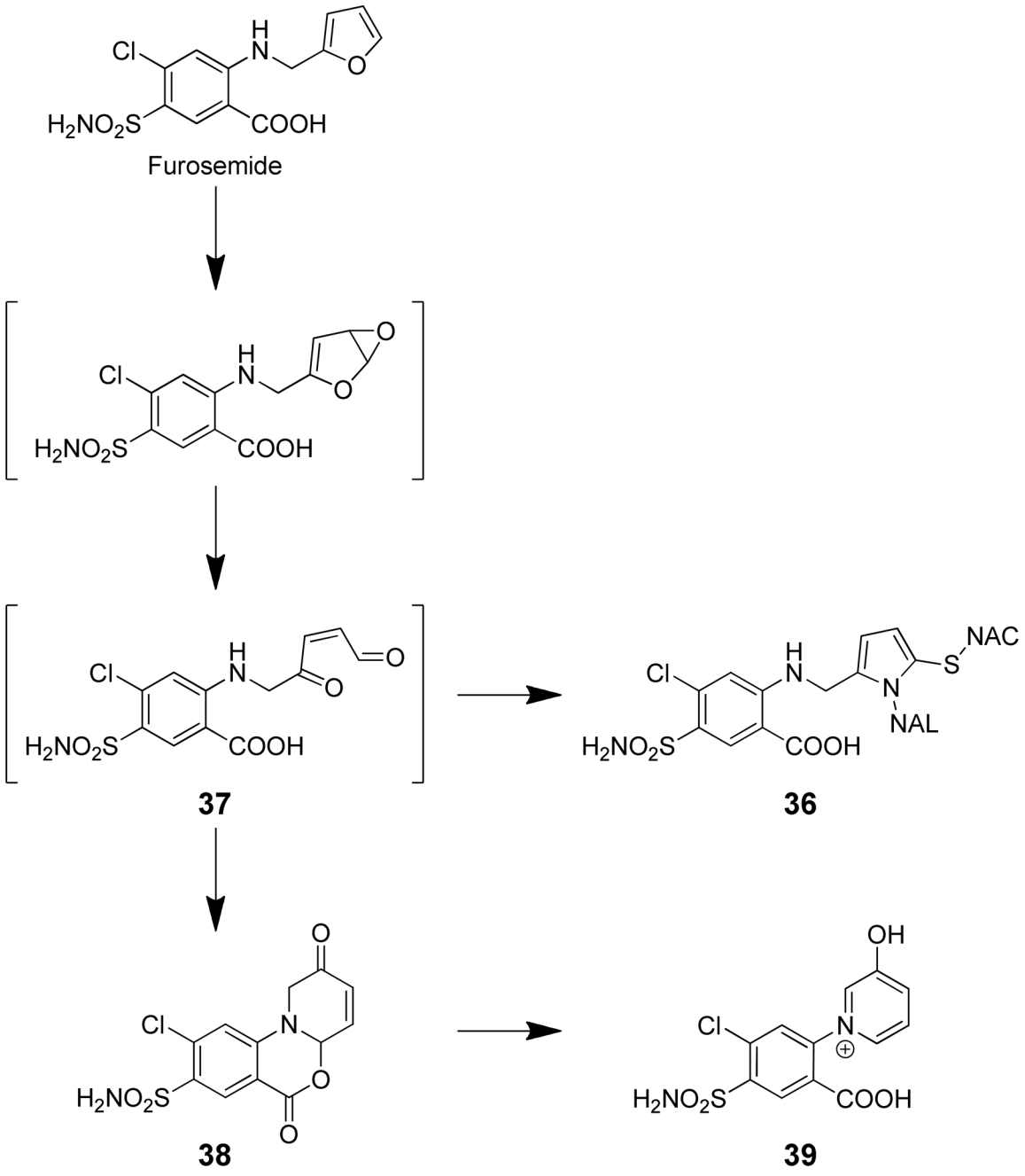
Scheme 5.
Structure of BDA-derived DNA adducts.[37–39]



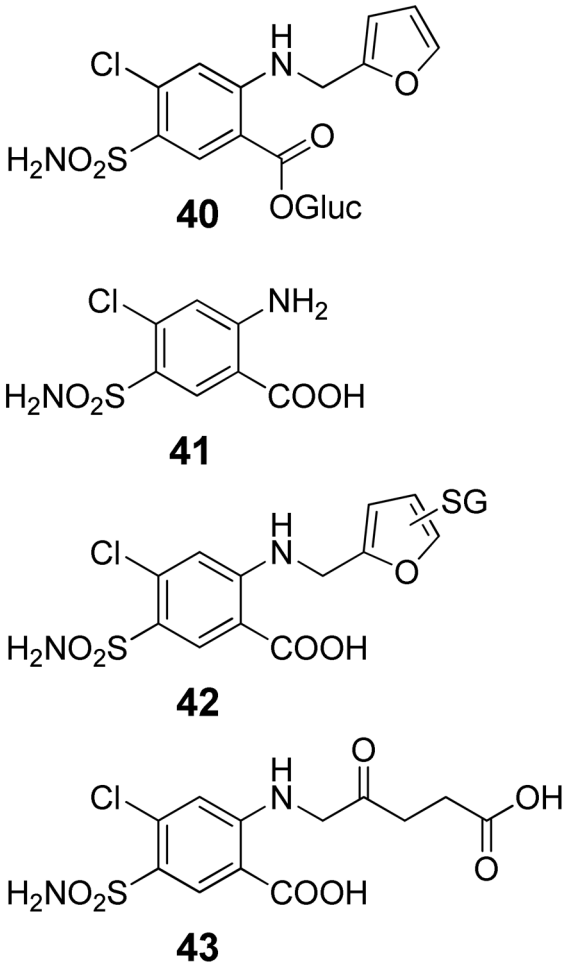
Scheme 6.
Trapping of the reactive metabolite formed in the P450-catalyzed oxidation of 2-methylfuran in microsomes.[10]



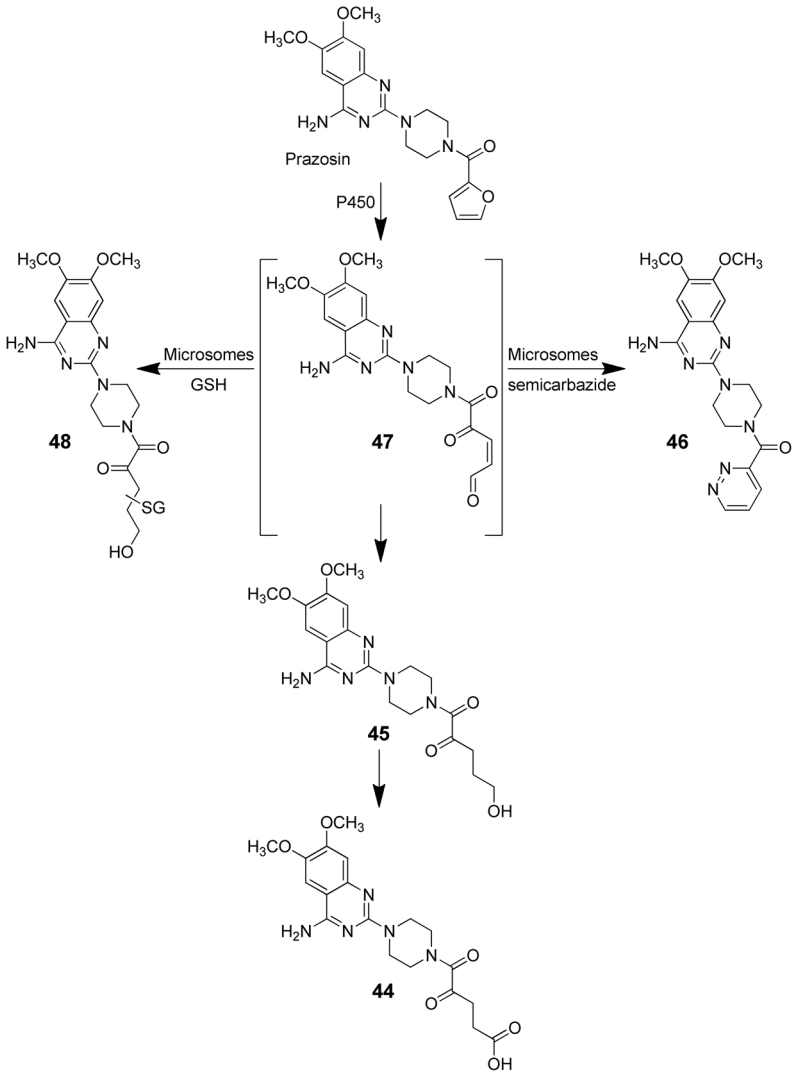
Scheme 7.
Reaction of 4-oxo-2-pentenal with 2′-deoxyguanosine. [70,71]



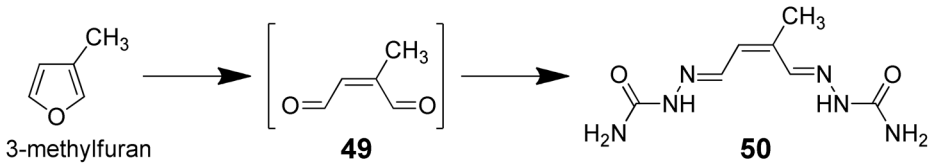
Scheme 8.
Microsomal metabolism of the furan ring of furosemide.[75,81]



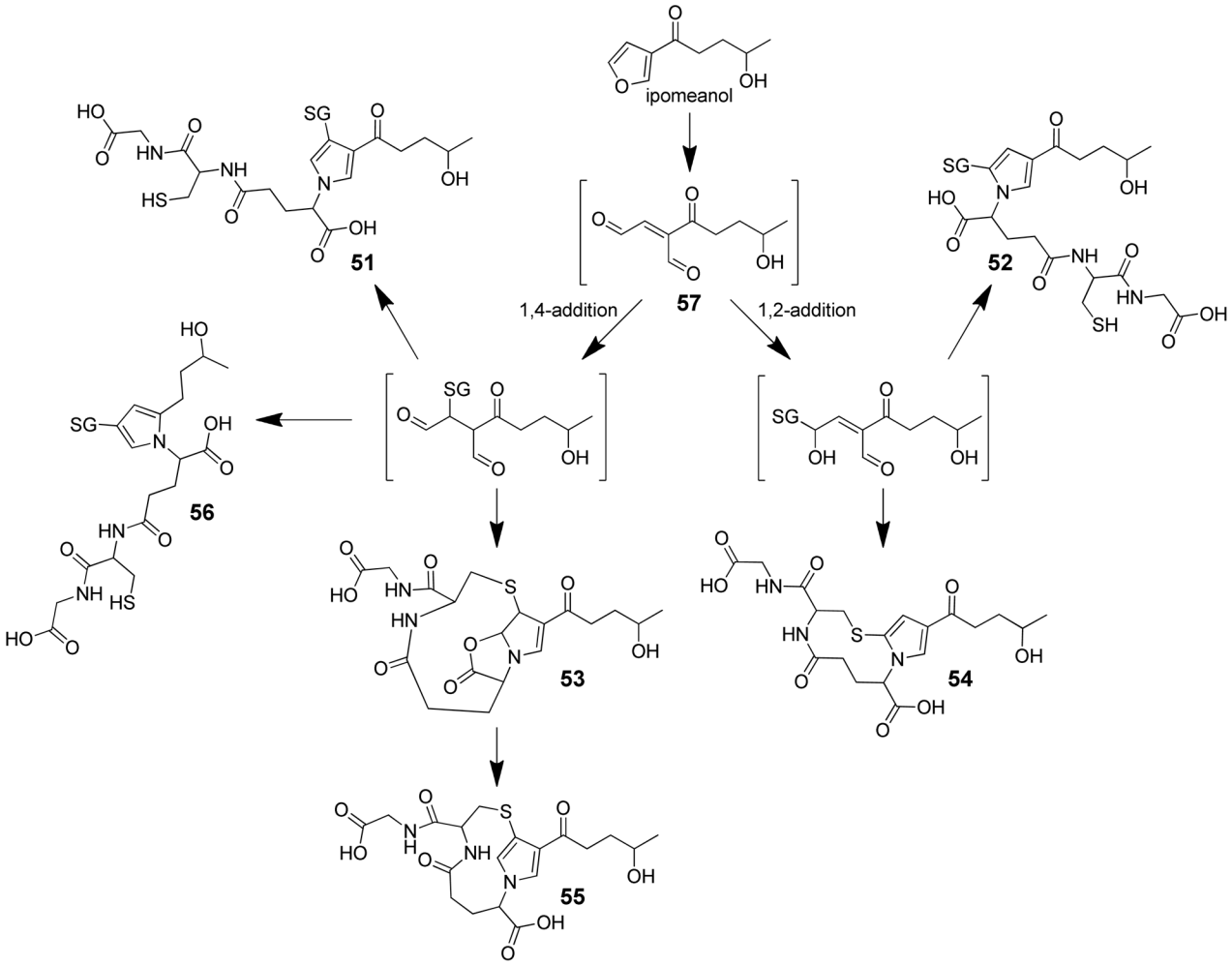
Scheme 9.
Biliary and hepatocyte metabolites of furosemide.[75]



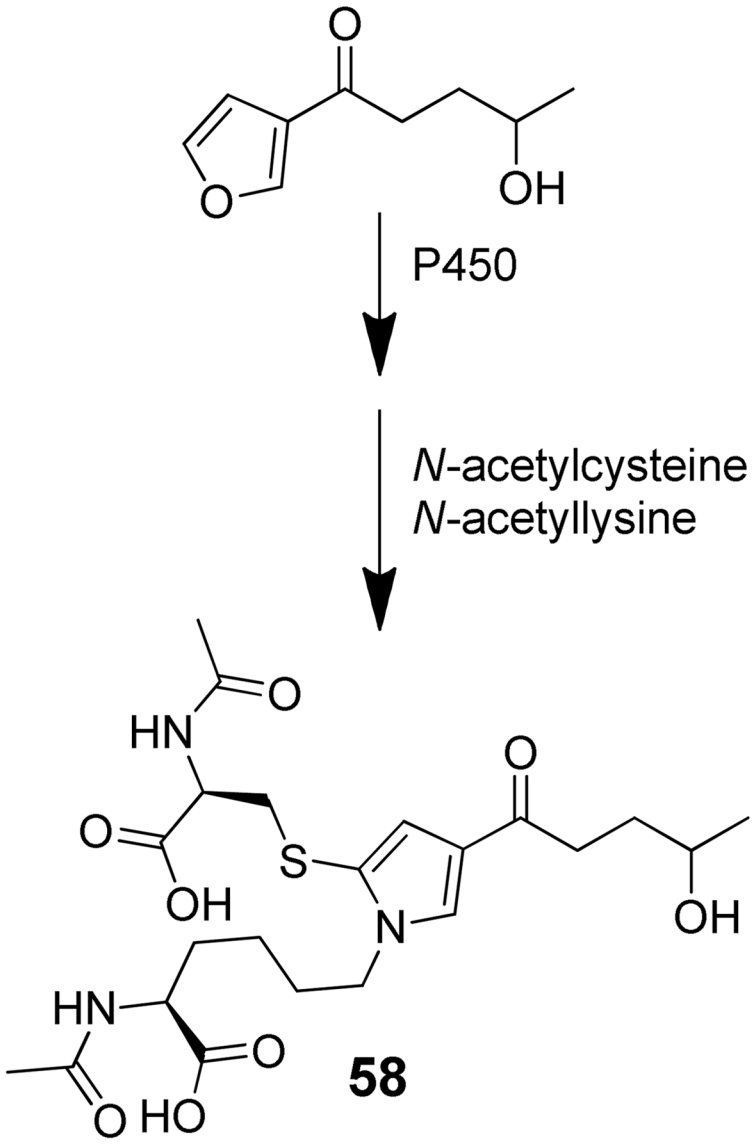
Scheme 10.
Metabolites derived from furanyl ring oxidation of prazosin.[19,86]



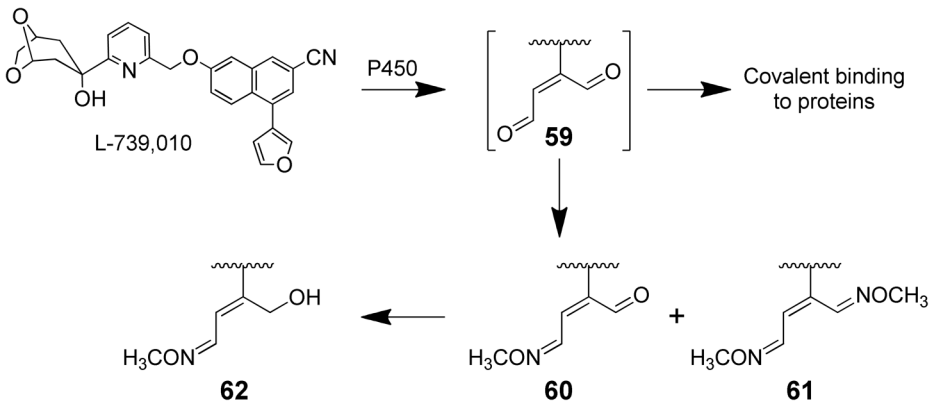
Scheme 11.
Trapping the reactive metabolite of 3-methylfuran as a *bis*-semicarbazone.[10]



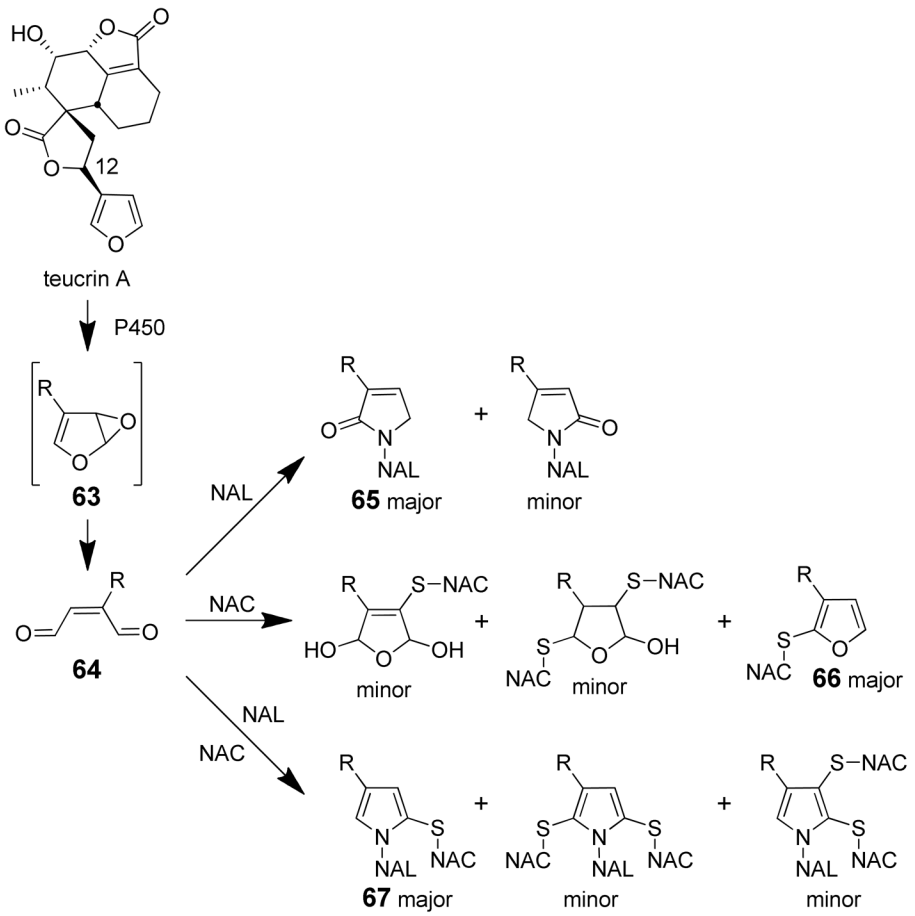
Scheme 12.
Structures of the GSH reaction products formed in GSH-fortified microsomal incubations of 4-ipomeanol.[99,108,114]



Scheme 13.
Trapping of reactive intermediate formed in *N*-acetylcysteine (NAC)/*N*-acetyllysine (NAL) fortified microsomal incubations of 4-ipomeanol.[108]

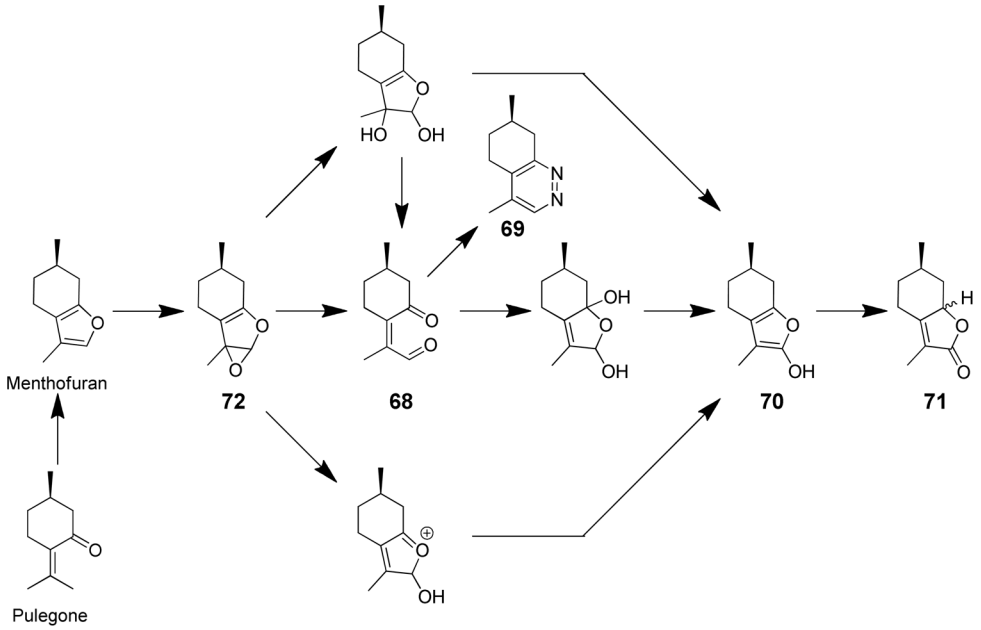


Scheme 14.
Products formed during the microsomal oxidation of L-739,010 in the presence of semicarbazide and required co-factors.[116,118]

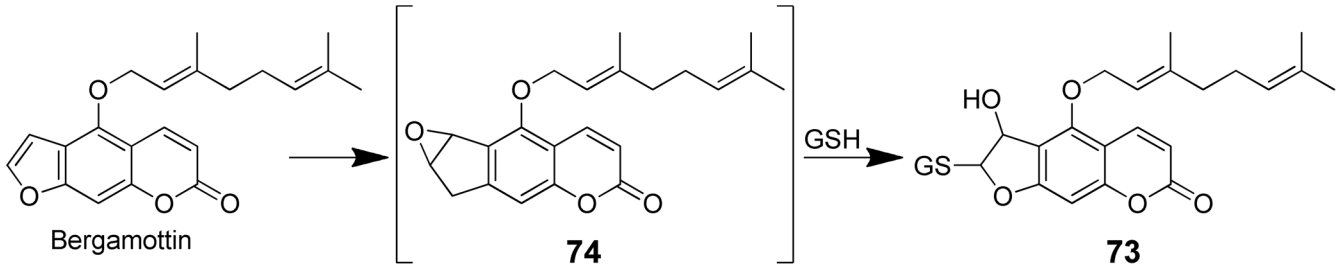


Scheme 15.

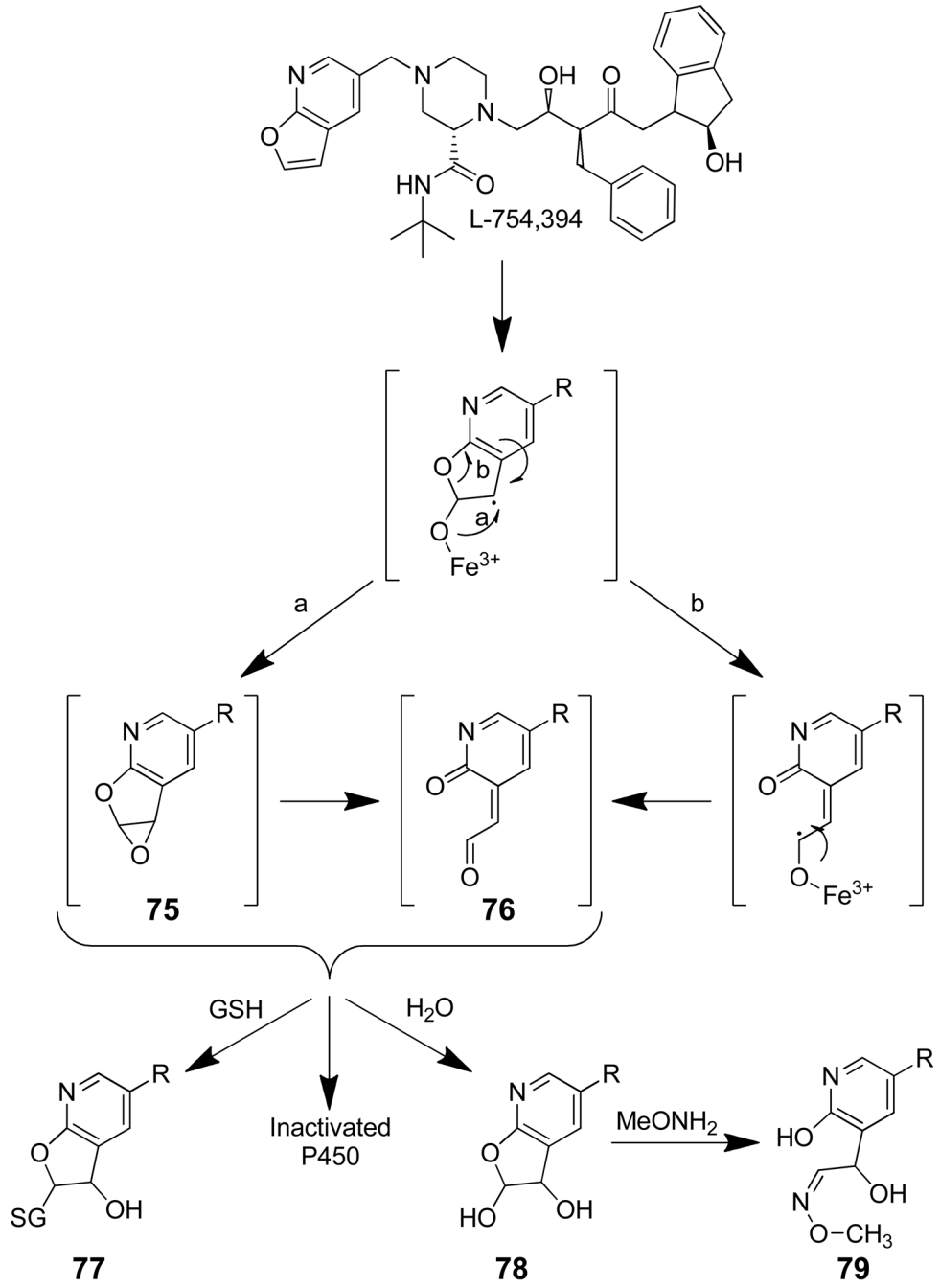
Reactive intermediates in teucrin A metabolism and the reaction products between teucrin A enedial and *N*-acetylcysteine (NAC) and/or *N*-acetyl-lysine (NAL).[15]



Scheme 16.
Mechanisms of metabolite formation from the proposed epoxide intermediate of menthofuran.[140,143–145]



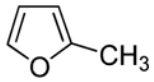
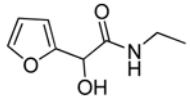
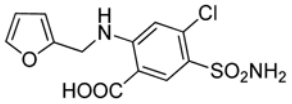
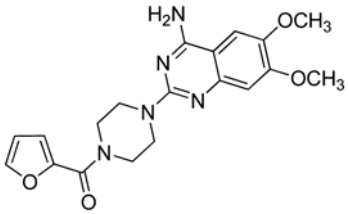
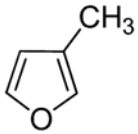
Scheme 17.
Metabolism of bergamottin to a reactive metabolite.[161]

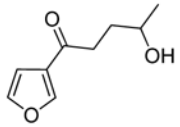
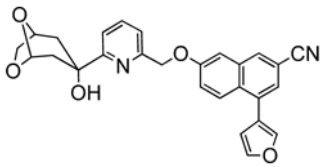
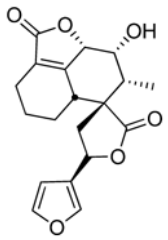
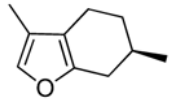
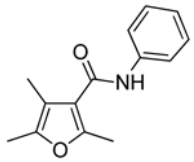


Scheme 18.
Products of furanyl ring oxidation of L-754,394 in the presence and absence of trapping agents.[164]

Table 1

Representative Furan-containing Compounds: Their source, toxicity and the nature of the reactive intermediate

Compound	Source	Toxicity	Reactive Intermediate
 Furan	Present in processed foods, smog, wood smoke, tobacco smoke and car exhaust[20–24]	Rat and mouse liver toxicant and carcinogen when dosed orally[25,41–43]	enedialdehyde[14,57,58]
 2-Methylfuran	Present in foods, coffee and tobacco smoke.[21,22,66]	Rat lung and liver toxicant when dosed i.p.[67]	enedione[10]
 2-(*N*-ethylcarbamoyl-hydroxymethyl)-furan	Synthetic	Rat lung toxicant when dosed orally[18]	Unknown furanyl epoxide unlikely[18]
 Furosemide	Pharmaceutical	Not toxic to humans at therapeutic dose Associated with hypersensitivity and jaundice in a few cases.[72,73] Not toxic to rats or hamsters[75,76] Mouse liver toxicant at high i.p. doses[74,75]	Furanyl epoxide[16] and enedione[75]
 Prazosin	Pharmaceutical	Not toxic	enedione[19]
 3-Methylfuran	Present in indoor and outdoor air pollution,[4,5] food,[88] and tobacco smoke.[89,90] Mold metabolite.[5]	Lung toxicant when inhaled or given by i.p.[91,93–97]	enedione[10]

Compound	Source	Toxicity	Reactive Intermediate
 4-Ipomeanol	Produced by moldy sweet potatoes.[98]	Lung toxicant in lab animals[98,100,101] Liver toxicant in humans[102–104]	unclear
 L-739,010	Synthetic	No overt toxic properties.[117] Binds to proteins.[116,118]	enedione[116,118]
 Teucrin A	Germander extracts[120]	Germander extracts are human and mouse liver toxicant.[120–128] Mouse liver toxicant[129]	Furanyl epoxide[128,131] and/or enedione[15]
 Menthofuran	Pulegone, pennyroyal oil[136–139]	Mouse liver and lung toxicant[133,140] Rat liver toxicant.[141] Pennyroyal oil is human toxicant[134,135] Mechanism based inactivator of P450[146,147]	Furanyl epoxide[13] and/or enedione[141,141,143,144]
 Methfuroxam	Fungicide		furanyl epoxide[12]

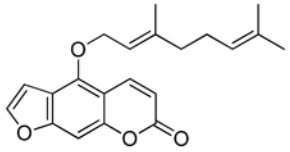
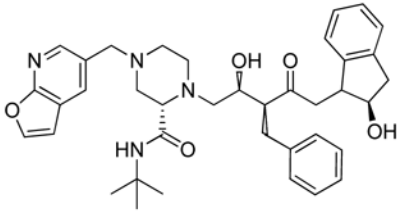
Compound	Source	Toxicity	Reactive Intermediate
 Bergamottin	Grapefruit juice[152–157]	Mechanism based inactivator of P450[154–157,159–161]	unclear
 L-754,394	Synthetic	Mechanism based inactivator of P450.[162]	unclear

Table 2

Kinetic parameters for the inhibition of P450s by furan-containing compounds

Chemical	Enzyme	K_i (μM)	k_{inact} (min^{-1})	Partition ratio[a]
4-Ipomeanol	Recombinant P450 3A4[113]	20	0.15	257
Menthofuran	Recombinant P450 2A6[146]	0.84	0.25	3.5 ± 0.6
	Recombinant P450 2A6[147]	2.2	1	30
	P450 2A6 activity in HLM[146]	2.5	0.22	
Bergamottin	Recombinant P450 3A4[154,159]	7.7	0.3	~27
	Recombinant P450 2B6[159]	5.2	0.087	~2
	Recombinant ΔP450 2B6 K262R[160]	8.2	0.23	
	Recombinant P450 3A5[159]	20	0.045	~20
L-754,394	P450 3A4 activity in HLM[163]	7.5	1.62	1.4
	P450 2D6 activity in HLM [163]	32	0.18	40
	recombinant P450 3A4[165]	7.5		4

[a] nmoles product formed per nmoles enzyme inactivated