Role of Cyclic Tertiary Amine Bioactivation to Reactive Iminium Species: Structure Toxicity Relationship

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Abstract: Cytochrome P450-mediated bioactivation of drugs to reactive metabolites has been reported to be the first step in many adverse drug reactions. Metabolic activation of cyclic tertiary amines often generates a number of oxidative products including *N*-dealkylation, ring hydroxylation, α-carbonyl formation, *N*-oxygenation, and ring opening metabolites that can be formed through iminium ion intermediates. Therapeutic pharmaceuticals and their metabolites containing a cyclic tertiary amine structure have the potential to form iminium intermediates that are reactive toward nucleophilic macromolecules. Examples of cyclic tertiary amines that have the potential for forming reactive iminium intermediates include the piperazines, piperidines, 4-hydroxypiperidines, 4-fluoropiperidines and related compounds, pyrrolidines and *N*-alkyltetrahydroquinolines. Major themes explored in this review include bioactivation reactions for cyclic tertiary amines, which are responsible for the formation of iminium intermediates, together with some representative examples of drugs and guidance for discovery scientists in applying the information to minimize the bioactivation potential of cyclic amine-based compounds in drug discovery. Potential strategies to abrogate reactive iminium intermediate formation are also discussed.

Keywords: Bioactivation, cyanide, cyclic tertiary amines, glutathione, iminium ion, P450, reactive metabolite, structural alert.

INTRODUCTION

Over the years, reactive metabolites have been believed to play an important role in the safety profile of pharmaceuticals. Cytochrome P450-mediated bioactivation of drugs to reactive metabolites has been reported to be the first step in many adverse drug reactions [1]. The covalent binding theory has linked toxicity to the formation of reactive metabolites, where bioactivation is a critical step in the process leading to an electrophilic metabolite that can bind to cellular macromolecules, for example, proteins and DNA. The resulting drug−protein adduct may cause partial or complete loss of the biochemical function of the protein or act as a hapten that elicits an immune response and results in cellular and organ damage [2, 3]. Drug induced toxicity remains one of the major reasons for failures of new pharmaceuticals, and for withdrawal of approved drugs from the market [4]. Efforts are being made to reduce attrition of drug candidates, and to minimize their bioactivation potential in the early stages of drug discovery in order to bring safer compounds to the market. Therefore, in addition to potency and selectivity, drug candidates are now selected on the basis of acceptable metabolism/toxicology profiles [5, 6].

Tertiary cyclic amines occur widely in drugs and environmental chemicals. Many naturally occurring and synthetic drugs contain basic tertiary (3^0) amine structures as seen with azetidine, pyrrolidine, piperidine, azepane, piperazine and morpholine rings in their molecules. Metabolic activation of cyclic tertiary amines often generates a number of oxidative products including *N*-dealkylation, ring hydroxylation, α-carbonyl formation, *N*-oxygenation, and ring opening metabolites that can be formed through an iminium ion intermediate [7, 8]. A very nice review on the diversity of oxidative bioactivation reactions on nitrogen containing xenobiotics was provided by Kalgutkar *et al.* [9], the present article is more focused on bioactivation reactions of cyclic tertiary amines and their potential to form reactive metabolites.

The objectives of this review article are to a) provide an update of the recent literature regarding bioactivation of cyclic tertiary amines b) give a brief description of the commonly used *in vitro* techniques for the detection of hard electrophiles such as iminium ions c) provide structural alerts of cyclic tertiary amines, which are quite often responsible for the formation of iminium intermediates and d) provide some examples for drug discovery scientists in applying the information to minimize the bioactivation potential for the formation of reactive metabolites.

METABOLIC PATHWAYS OF CYCLIC TERTIARY AMINES

The most important metabolic biotransformation that cyclic tertiary amines **1** undergo is the cytochrome P450 or monoamino oxidase (MAO) catalyzed ring α-carbon oxidation that generates the corresponding cyclic iminium ion **2** Fig. (**1**). Cyclic tertiary amines also undergo oxidative *N*-dealkylation *via* oxidation of the exocyclic iminium intermediate **3** to afford the corresponding secondary amine and an aldehyde. Unlike the cyclic intermediate **3**, hydrolysis of **2** to the corresponding aminoaldehyde **4** is reversible, giving rise to possible further metabolic processing of **2**. Whereas the iminium intermediates of acyclic tertiary amines are in equilibrium with carbinolamines that dissociate to aldehyde and secondary amine (*N*-dealkylation), carbinolamine dissociation remains reversible for cyclic tertiary amines, increasing the effective lifetime of the iminium species. Iminium ion intermediates **2** are often oxidized by the liver cytosolic enzyme aldehyde oxidase to the biologically less active lactams **5** Fig. (**1**). If special structural features are present in the substrate molecule or if the cyclic iminium intermediate is generated in extrahepatic tissue lacking aldehyde oxidase, these reactive intermediates may undergo alternative chemical transformations that can produce toxic products [10].

Studies on many independently synthesized endocyclic iminium metabolites have demonstrated that most, if not all, the covalent binding activity arises from metabolism beyond the iminium stage of oxidation. Since the cationic iminium species are themselves unlikely oxidation substrates, further metabolism occurs via oxidation of the corresponding endocyclic enamines **6** Fig. (**1**). Enamines are electron rich species and good candidates for metabolic one-electron oxidations mediated by cytochromes. Oxidation of substituted endocyclic enamines **6** can explain the formation of microsomal 3-one metabolites **7** [10].

Interest in the bioactivation mechanisms of cyclic tertiary amines arose because of the Parkinsonian-inducing properties of the nigrostriatal neurotoxin 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine (**8**, MPTP) Fig. (**2**). Hepatic P450s catalyze the oxidative *N*-demethylation and ring α-carbon oxidation of MPTP **8**, the latter reaction leading to the $MPDP^+$ **9**, which is an excellent substrate for hepatic aldehyde oxidase that catalyzes its conversion to the lactam

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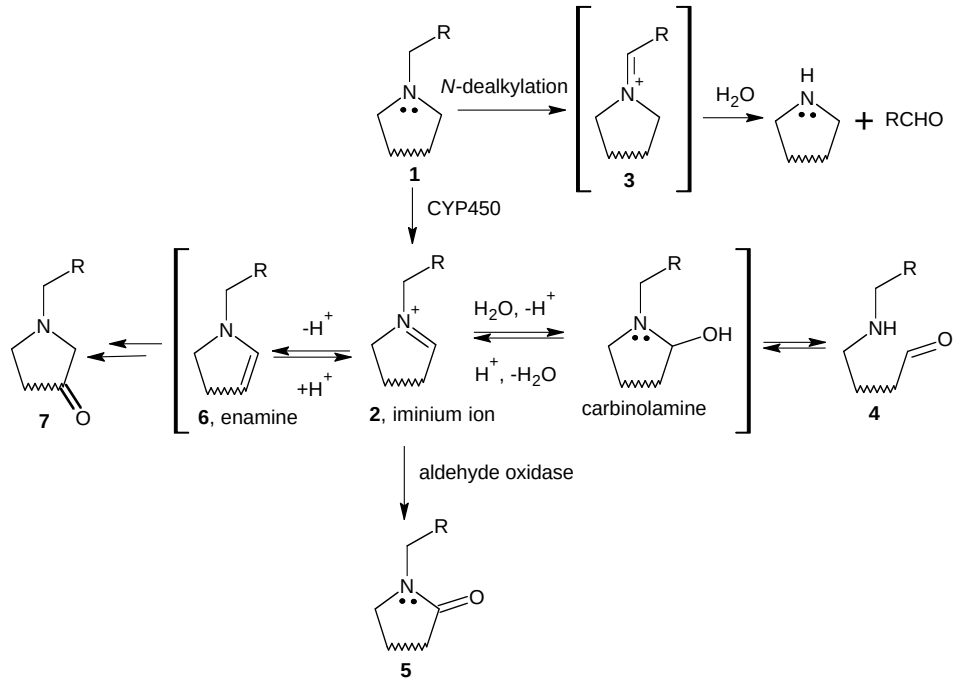

Fig. (1). Metabolic pathways from cyclic tertiary amines *via* iminium ion intermediates.

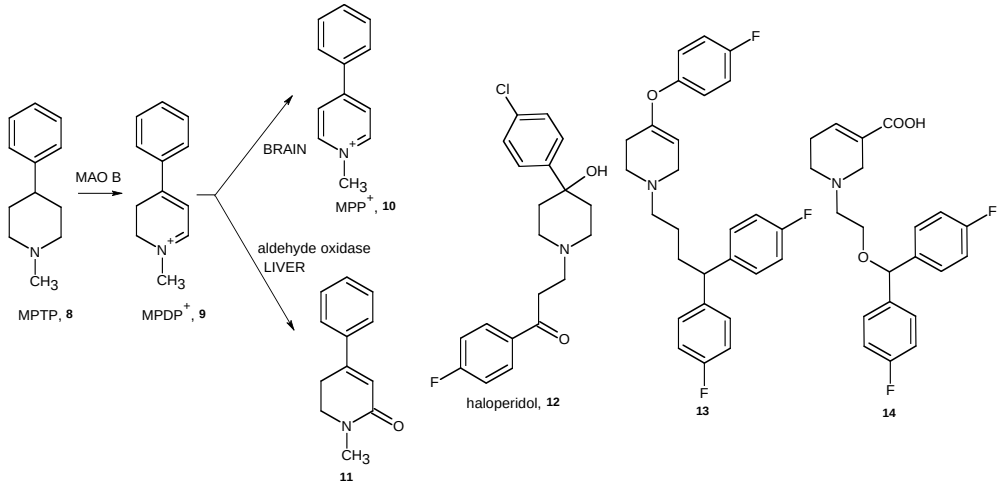

Fig. (2). MAO-B catalyzed metabolism of MPTP. Haloperidol and related analogs of MPP^+-like neurotoxins.

11. Unlike liver homogenates, brain homogenates do not detoxify $MPDP^+$ to **11**, presumably because of the absence of aldehyde oxidase activity in the brain. The principal fate of $MPDP^+$ in brain homogenates is its further two-electron oxidation to MPP^+ **10**, an event that has been thought to be MAO-B catalyzed. The neurotoxicological importance of this oxidation was demonstrated in experiments in which irreversible inhibition of MAO-B inhibitors protected monkeys and mice against MPTP's neurotoxicity. After active transport of MPP^+ into dopaminergic nerve terminals by the dopamine uptake system, MPP^+ localizes in the mitochondrial matrix where it inhibits complex I of the mitochondrial electron transport chain, leading to cessation of oxidative phosphorylation and ATP depletion [11-15]. A compound of interest in this context is haloperidol **12**, a neuroleptic agent that causes severe side-effects including Parkinsonism and tardive dyskinesia that, in many cases, is irreversible [9]. Two other neurotoxic tetrahydropyridines of interest in this context are compounds **13** [16] and **14** [17] presented in Fig. (**2**) [17].

Although cytochrome P450 2D6 is a minor component (~2%) of total cytochrome P450 content in human liver, a significant proportion (~30–40%) of drugs currently in clinical use are metabolized by cytochrome 2D6. Furthermore, the genetic polymorphism associated with CYP2D6-mediated metabolism in humans has heightened awareness of the potential adverse drug reactions following impaired clearance of CYP2D6 substrates in individuals designated as poor metabolizers. Therefore, several efforts have been directed towards a better understanding of the structure-function relationships of CYP2D6 substrates/inhibitors. Kalgutkar examined the relationship between lipophilicity and CYP2D6 affinity of cyclic tertiary (*N*-alkyl-4-phenyl-1,2,3,6-tetrahydropyridines) and quaternary (*N*-alkyl-4-phenylpyridinium) amines. The lack of CYP2D6 inhibition by MPTP **8** Fig. (**2**) and its pyridinium metabolite MPP^+ **9** was due to their hydrophilic nature, since higher *N*-alkyl homologs revealed substantial increases in inhibitory potency against recombinant CYP2D6-mediated bufuralol-1′-hydroxylation. The reasonable correlation between lipophilicity and CYP2D6 inhibition by pyridiniums and 1,2,3,6-tetrahydropyridines was only limited to straight chain *N*-alkyl analogs, since certain *N*-alkylaryl

analogs of lower lipophilicity were better CYP2D6 inhibitors. CYP2D6 substrate properties of straight chain *N*-alkyltetrahydropyridines were also governed by lipophilicity, and *N*-heptyl-4-phenyl-1,2,3,6-tetrahydropyridine was the optimal substrate (K_{mapp} = 0.63 μM). Metabolism studies indicated that the *N*-heptyl analog underwent monohydroxylation on the aromatic ring and on the *N*-heptyl group suggesting that 1,2,3,6-tetrahydropyridines can bind in more than one conformation to the CYP2D6 active site [18]. A study of electrochemistry-electrospray mass spectrometry on the electrochemical oxidation of *N*-alkyl-4-phenyl-1,2,3,4-tetrahydropyridinyl derivatives and the chemical fate of the resulting aminyl radical cation has presented a unique perspective for understanding the metabolic oxidation mechanism for the tetrahydropyridines [19].

DETECTION OF REACTIVE IMINIUM INTERMEDIATES

Reactive metabolites such as electrophilic intermediates or radicals are unstable, formed usually in small amounts, and react rapidly with nucleophiles within proteins and non-proteins, and can, therefore, easily be missed in routine metabolism studies. Hard electrophiles react with hard nucleophiles, such as basic groups in DNA and lysine residues in proteins, while soft electrophiles react with soft nucleophiles such as thiol groups of glutathione and cysteine residue in proteins [20]. The basic methods to trap the reactive intermediates are well established and were used for screening in early drug discovery [21].

One of the most commonly used techniques is a glutathione (GSH) trapping screen with hepatic subcellular fractions in the presence of cytochrome P450 cofactor NADPH [22, 23]. In this method, GSH is used to trap an electrophilic metabolite via the cysteine sulfhydryl group to form a conjugate. The resulting GSH conjugate, when fragmented by collision-induced dissociation (CID), gives a common loss of the pyroglutamic acid moiety from the structure, which can be easily detected and characterized by a neutral loss scan of 129 Da, using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) [24]. Reported reactions with electrophiles include the nucleophilic substitution at saturated and aromatic carbons as well as the oxirane ring, addition to a carbonium and nitrozo ions, and Michael addition [25]. Several structural classes of chemically reactive metabolites such as epoxides, quinones, quinine methides and quinoneimines can be trapped by the addition of GSH in the incubation mixtures. However, there are a few drawbacks associated with the GSH trapping screen. Not all GSH adducts afford the neutral loss of 129 Da upon CID. Aliphatic and benzylic thioether conjugates typically lose glutamic acid (147 Da) from the protonated GSH adducts [26, 27]. Moreover, the GSH trapping screen cannot detect some types of reactive metabolites, for example, acyl glucuronides, aldehydes and iminium ions which generally do not form stable GSH conjugates.

While GSH and its derivatives trap a wide range of reactive metabolites, there are some structural motifs which are not trapped by the soft nucleophile in these compounds. Hard electrophiles such as iminium species and aldehydes require a different approach for their detection. Potassium cyanide is a useful agent for trapping compounds containing alicyclic tertiary amines via a reactive iminium ion and has been shown to be a more sensitive trapping agent than GSH for these classes of compounds. The test compounds are typically incubated in hepatic microsomes with KCN or NaCN Fig. (**3**) and the trapped products are detected by mass spectroscopy, using either full scan MS, CNL or MRM scanning [28].

The metabolism of and tertiary alicylic amines has been studied with radiolabeled cyanide in a radiometry assay [29]. The use of radiolabeled cyanide is necessary if quantitation of these reactive intermediates is required. A quantitative high throughput trapping assay using $K^{13}CN$ has been described by Meneses-Lorente *et al.* The assay involves incubating the test compound with liver microsomes in the presence of $K^{13}CN$. Unreacted trapping agent is removed using solid phase extraction, and the amount of radiolabeled conjugate is determined by liquid scintillation counting [30]. Later, Evans *et al.* reported that the cyanide conjugates could be detected using the neutral loss scan of 27 and 29 Da by LC-MS/MS, using a mixture of containing equal amounts of unlabeled and isotopically labeled cyanide [31, 32]. Recently Argoti *et al.* have reported a relatively high throughput LC-MS/MS screening method for detecting the non-radiolabeled cyanide adduct formed in rat or human liver microsomal incubations with constant neutral loss scan of 27 Da [28].

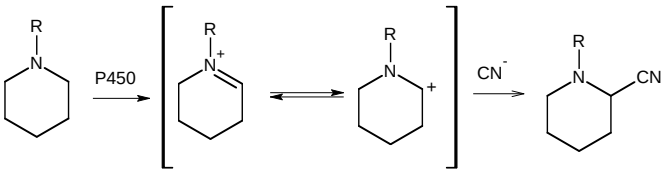

Fig. (3). Trapping of reactive iminium ion by cyanide.

CYCLIC TERTIARY AMINES

Piperazines

The piperazine moiety is a common structural fragment in many drugs with important pharmacological properties. The main groups of drugs incorporating the piperazine moiety are antianginals, antidepressants, antihistamines and antipsychotics. Most of these compounds can be classified as either phenylpiperazines, benzylpiperazines, diphenylmethylpiperazines, pyridinylpiperazines, pyrimidinylpiperazines or tricycles and tetracycles [33]. Quite often the piperazine ring in these compounds is involved in biotransformation reactions leading to potentially toxic reactive iminium intermediates.

An example is MB243, 1,3-disubstituted piperazine derivative **15**, Fig. (**4**) that is a selective melanocortin receptor subtype-4 agonist with potential application in the treatment of obesity and/or erectile dysfunction [34]. It was observed to bind covalently and extensively to liver microsomal proteins from rats and humans. MB243 **15** undergoes CYP450-mediated piperazine bioactivation involving a six electron oxidation of the piperazine ring, attack by GSH, and hydrolysis of the glutamic acid residue to afford a cysteinylglycine conjugate of piperazinone **16**. Attack (aminolysis) by the cysteinylamine moiety resulted in opening of the piperazine ring, leading to a thiazolidine thioaminal intermediate **17**, which, in turn, underwent ring closure to the imidazoline products **18** Fig. (**4**). In keeping with the proposed mechanism, it was found that, while **15** alkylated proteins extensively, simple alkyl substitutions (methyl, isopropyl) α to the N^1-methyl functionality afforded compounds **20** and **21** that did not undergo metabolic activation, and were therefore more attractive as drug candidates [31, 35].

A second example of piperazine ring bioactivation is highlighted by the 5-HT_{2C} receptor agonist **22** Fig. (**5**) that was an attractive candidate for treatment of obesity. The attractive *in vitro*/ *in vivo* pharmacology and pharmacokinetic attributes of **22** were offset by its metabolism-dependent (S-9/NADPH) genotoxic effects in the *Salmonella* Ames assay. Positive findings in the *Salmonella* Ames assay correlate well with the outcome of rodent carcinogenicity testing [36, 37]. The elucidation of a mutagenic mechanism(s) is crucial to the design of follow-on drug candidates devoid of the genetic liability. Compound **22** is bioactivated to reactive, electrophilic intermediates that bind covalently to a DNA base(s). *In vitro* metabolic studies with rat S9/NADPH in the presence of methoxylamine and cyanide led to the detection of amine **24** and cyano **26** conjugates see Fig. (**5**). The mass spectrum of the amine conjugate was consistent with condensation of the amine with an aldehyde metabolite **23** derived from hydroxylation of the secondary piperazine nitrogen-α-carbon bond. The mass spectrum of the cyano conjugate **26** suggested a bioactivation pathway involving *N*-hydroxylation of the secondary piperazine nitrogen followed by two-electron oxidation to generate an electrophilic nitrone **25**,

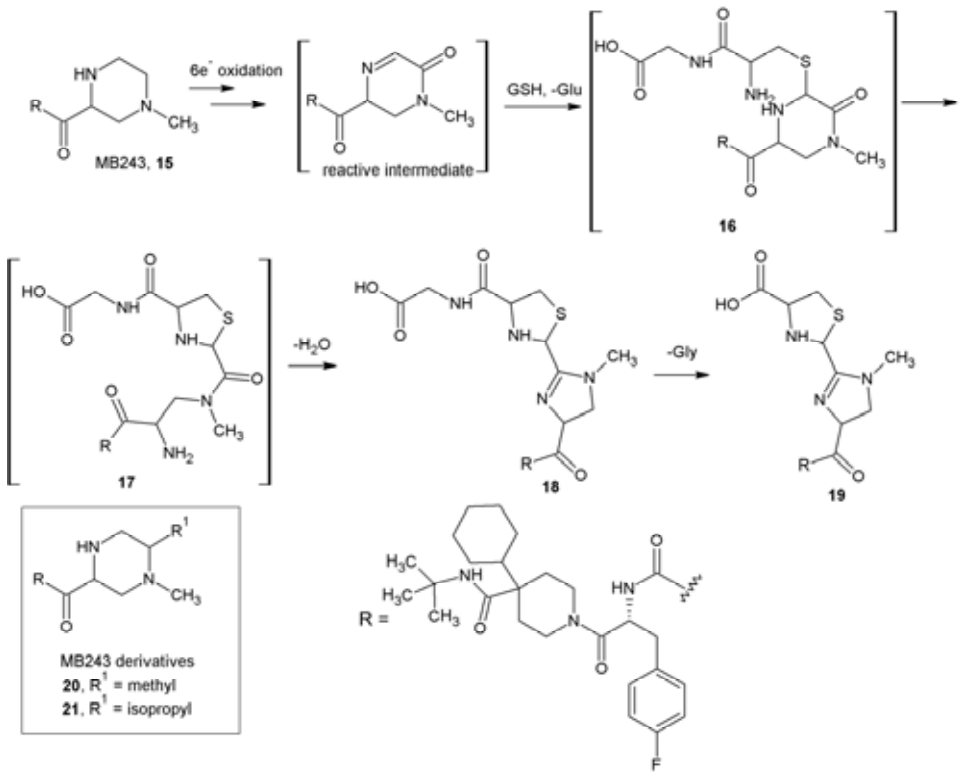

Fig. (4). Proposed bioactivation pathway of MB243 **15** involving a 1,3-disubstituted piperazine ring.

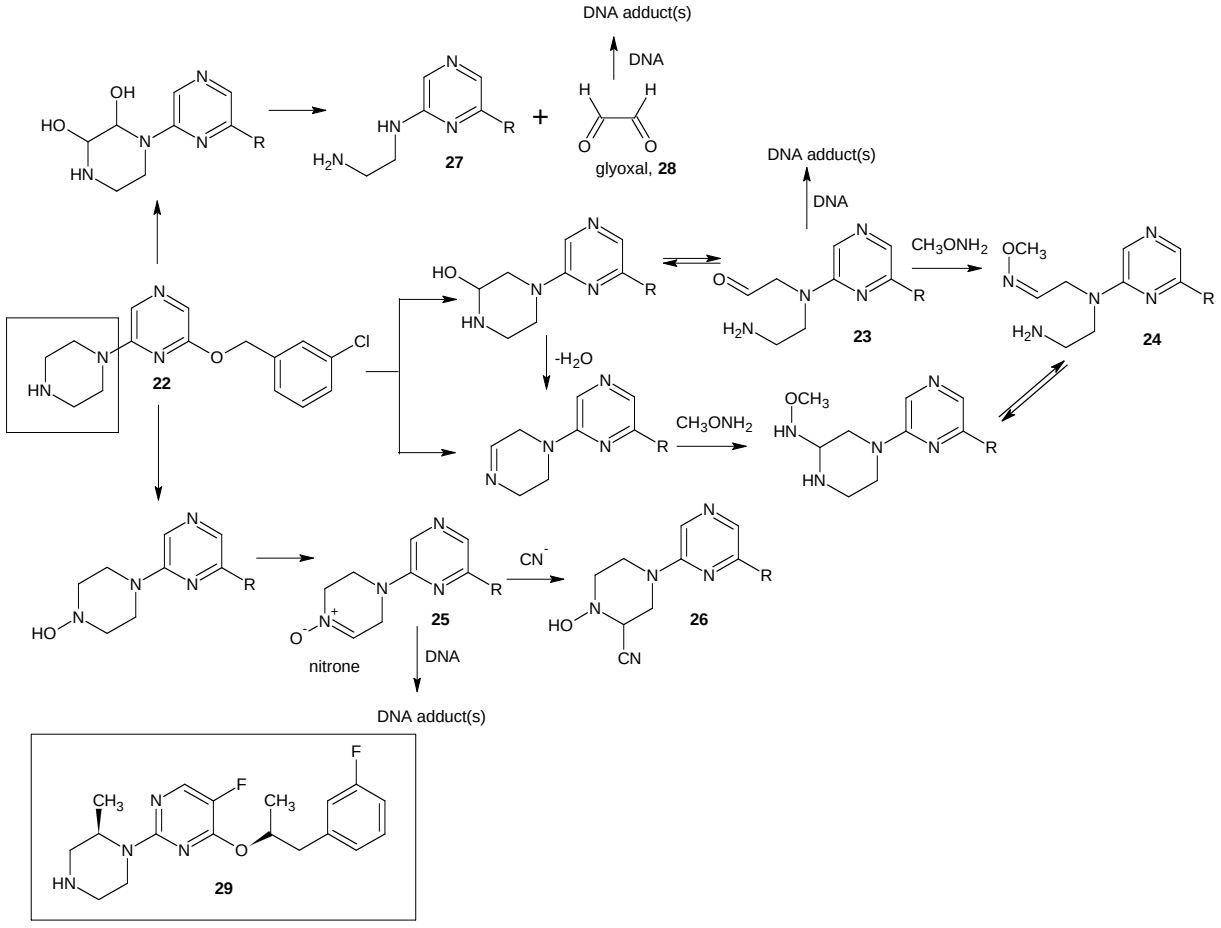

Fig. (5). Proposed bioactivation pathways of 5-HT_{2C} receptor agonist **22** involving 1-substituted piperazine ring and compound **29**, which devoids ames liability.

which reacted with cyanide. From a structure-mutagenicity relationship, the P450-catalyzed formation of the diamine metabolite **27**, the methoxyamine conjugate **24**, and the cyano conjugate **26** suggests that electrophilic carbonyl/iminium intermediates **23**, **25** and **28** (derived from the piperazine ring metabolism), as well as the quinine-methide (derived from the bioactivation of the 3-chlorobenzyl group), possesses the ability to react with DNA Fig. (**5**) [38]. Using this information, compound **29** was designed as a follow-on candidate by introducing a metabolic soft spot in the piperazine ring Fig. (**5**). Compound **29,** besides maintaining the primary pharmacology and attractive pharmacokinetic attributes of **22**, was devoid of the mutagenic response exhibited by **22**. Consistent with this finding, no amine and/or GSH conjugates of **29** were detected in rat S-9/NADPH incubations supplemented with trapping agents. It was speculated that the insertion of the α-methyl group in the piperazine ring serves as a metabolic soft spot and circumvents piperazine ring bioactivation. Similarly, the removal of the problematic 3-chlorobenzyloxy moiety eliminates the bioactivation pathway resulting in quinine-methide formation [5].

Another example of piperazine bioactivation is the tetracyclic antidepressant mianserin **30** Fig. (**6**) developed in the early 1970s. It has advantages over the related tricyclic antidepressants in terms of fewer cardiotoxic side-effects [39]. However, mianserine therapy is associated with a number of adverse reactions including skin rashes, hepatotoxicity, and blood dyscrasias [40]. The detection of specific mianserine antibodies against platelets in the serum of a patient with thrombocytopenia suggests that an immunologically mediated mechanism is involved in the development of mianserine-induced blood dyscrasias. In addition to CYP2D6 and CYP1A2 catalyzed formation of 8-hydroxymianserin, *N*-desmethylmianserin and mianserin-*N*-oxide as major urinary metabolites of mianserin **30** in humans [41], P450 is also known to bioactivate mianserin and its metabolites to the corresponding reactive iminium ions **31-33** [42, 43] Fig. (**6**). Studies with radiolabeled mianserin and its metabolites demonstrated the NADPH-dependent, irreversible incorporation of radiolabel into microsomal protein, and also demonstrated their cytotoxicity towards human mononuclear leukocytes included in the microsomal incubation. Structure-activity studies revealed that replacement of the nitrogen atom from the N5 position with a methine group (compound **34**) significantly reduces cytotoxicity, as does substitution of a methyl group for a hydrogen atom at position C14b (compound **35**) Fig. (**6**). These observations further confirm iminium ion formation as being on the pathway to toxicity. Apart from iminium intermediates discuses here recently quinone-imine intermediates of mianserin have been characterized, which were formed after initial 8-hydroxylation and subsequent two-electron oxidations. Both two types of reactive intermediates might be involved in the development of mianserin toxicity [8, 44].

Bauman *et al.* studied *in vitro* bioactivation of structurally related central nervous system agents nefazodone **36** (hepatotoxin) [45, 46] and aripiprazole **37** (nonhepatotoxin) in human liver microsomes, in an attempt to understand the differences in their toxicological profiles Fig. (**7**). NADPH supplemented microsomal incubations of nafazodone and glutathione generated conjugates derived from addition of thiol to quinonoid intermediates [47]. Inclusion of cyanide afforded cyano conjugates to iminium ions derived from α-carbon oxidation of the piperazine ring in nafazodone **36** and to downstream metabolites [28]. Although the arylpiperazine substructure in aripiprazole **37** did not succumb to bioactivation, the dihydroquinolinone group was bioactivated via an intermediate monohydroxy metabolite to a reactive species that was trapped by glutathione [48]. Moreover P4503A4 inactivation studies in microsomes indicated that, unlike nafazodone **36**, aripiprazole **37** was not a time-dependent inactivator of the enzyme. These studies thus reinforce the notion not all drugs that are bioactivated *in vitro* elicit a toxicological response *in vivo*. A likely explanation for the markedly improved safety profile of aripiprazole **37** compared with that of nafazodone **36**, despite the accompanying bioactivation liability, is the vast improvement of the pharmacokinetics due to reduced P4503A4-mediated metabolism/bioactivation, which results in a lower daily dose (5-20 mg/day) than needed with nefazodone **36** (200-400 mg/day). This attribute probably reduces the total body burden to reactive metabolite exposure, and may not exceed the toxicity threshold [49].

Prazosin **38** Fig. (**8**), an antihypertensive agent introduced to the market in 1976, is another example of a compound with bioactivation liability but, nevertheless, with an excellent safety record. The primary adverse reaction is postural hypotension and syncope, especially on initiation of medication, a result of its pharmacological activity [50]. There have been no reports of idiosyncratic toxicity to prazosine **38**. In *in vitro* studies, the presence of several metabolites was confirmed and several new ones were characterized, including a stable carbinolamine **39**, an iminium intermediate **40**, and an enamine **41** – all formed via oxidation of the piperazine ring. This work on prazosine demonstrated that, *in vitro*, bioactivation does not always lead to toxicity. The absence of idiosyncratic toxicity associated with prazosin is also probably due to its low maximal daily dose of 5 mg/day for hypertension [51].

Piperidines

A general reaction scheme for biotransformation of piperidine ring is presented in Fig. (**9**). Incubation of a piperidine-based compound, e.g. **42** with GSH and NADPH-supplemented P450 enzymes, can lead to the GSH conjugates **48** and **49** formed by the addition of the sulfhydryl nucleophile to a putative 2,3-dihydropyridinium intermediate **46**, presumably generated by a two

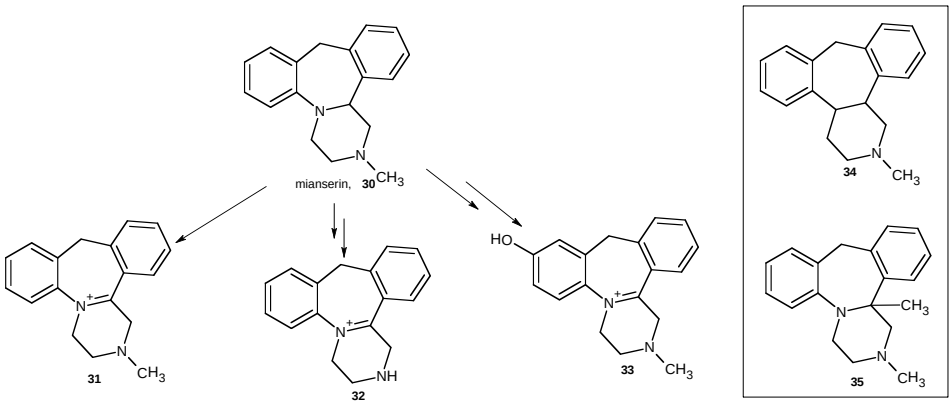

Fig. (6). P450 mediated bioactivation of the antidepressant mianserin to reactive iminium species.

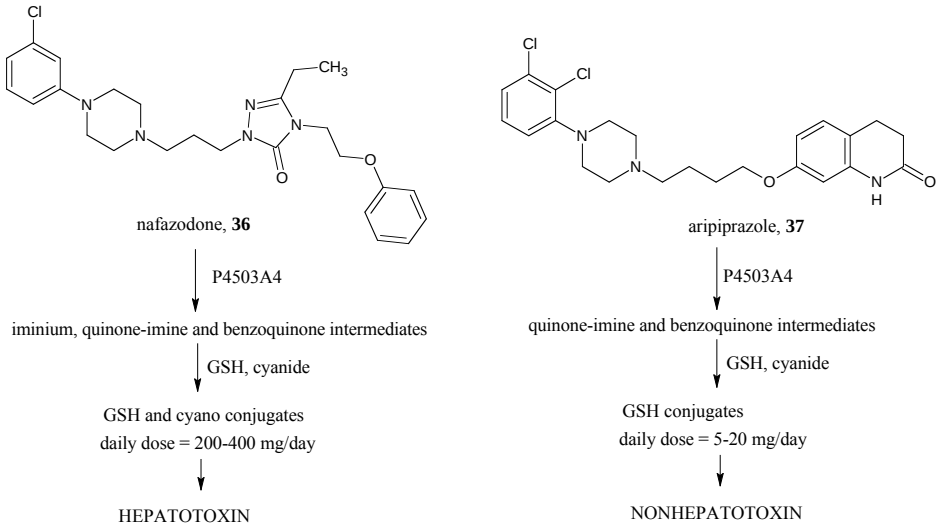

Fig. (7). Comparison of bioactivation potentials of the antidepressant and hepatotoxin nefazodone **36** and aripiprazole **37**.

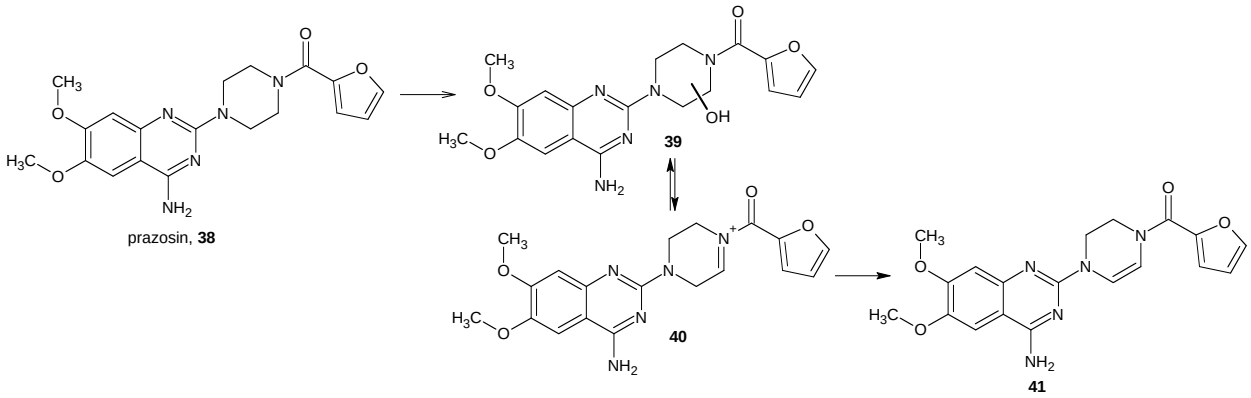

Fig. (8). Proposed *in vitro* bioactivation of antihypertensive prazosine **38** on the piperazine ring.

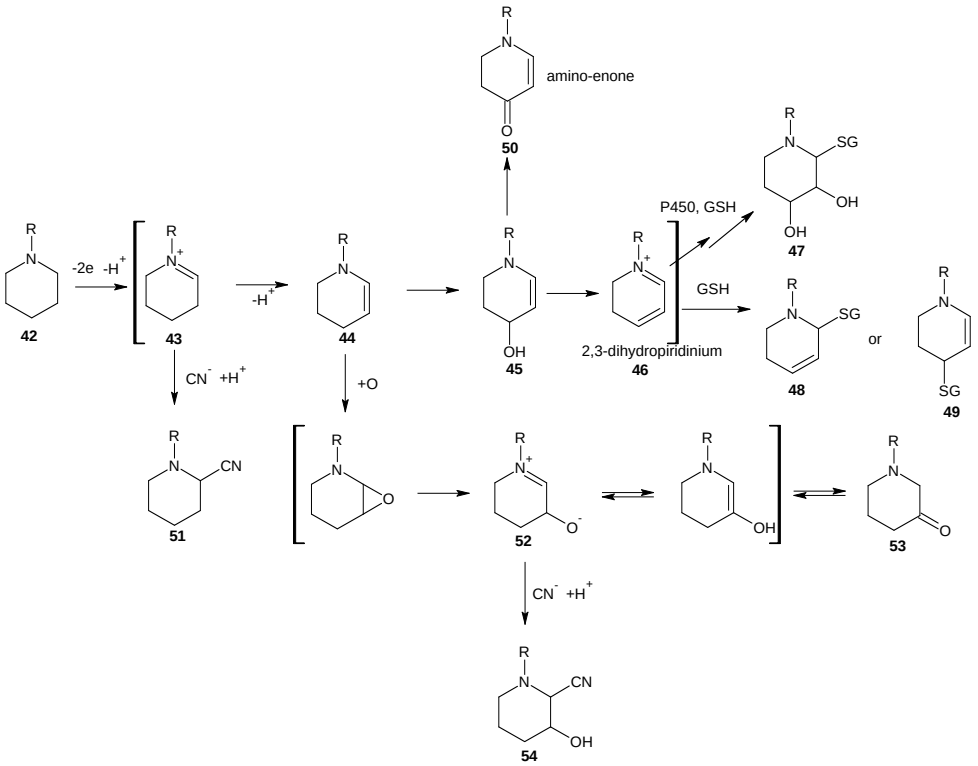

Fig. (9). General scheme of known bioactivation pathways of piperidine-based compounds in liver microsomes.

electron oxidation of iminium ion **43** Fig. (**9**). Under similar conditions, use of P450 enzymes can lead to the detection of a GSH conjugate [52] that had also undergone dihydroxylation on the piperidine ring Fig. (**9**). Co-incubation of piperidine-based compound with NaCN and NADPH-supplemented P450 enzymes can lead to CN conjugates formed by the addition of cyanide to a putative iminium ion **43**. Further, incubation of **43** with rat liver microsomes in the presence of an NADPH generating system led to the characterization of the aminoenone **50** [9,10]. Oxidation of endocyclic enamines like **44**, that are electron rich species and good candidates for metabolic one-electron oxidations mediated by cytochromes, can explain the formation of microsomal 3-one metabolite **53**. Addition of cyanide to the reactive species afforded the cyano adduct **54** [53, 54].

Phencyclidine (PCP, **55**) Fig. (**10**) was originally developed in the mid-1950s for use as an anesthetic agent, but its use in human medicine was soon discontinued because it produced serious adverse drug reactions that included a dose-dependent psychosis resembling schizophrenia. The etiology of these long-term and idiosyncratic side effects is not known; however, it has been proposed that they could result from the irreversible binding of **55** or its reactive metabolites to critical proteins in the central nervous system (CNS). More than two decades ago, the metabolism of **55** by cytochrome P450 enzymes was shown to result in the formation of covalent adducts with microsomal proteins and of reactive metabolites that lead to P450 inactivation [55, 56]. On the basis of these studies, it was thought that a PCP iminium ion (PCP-Im$^+$) metabolite **56**, obtained from the P450-mediated α-carbon oxidation of **55**, was responsible for both the covalent-binding and the enzyme inactivation events. This proposal was supported by the isolation of a PCP–cyano adduct **57** Fig. (**10**) and by the observation that inclusion of cyanide in incubations of liver microsomes protected against covalent binding of **56** to microsomal proteins. However, subsequent studies revealed that P450 inactivation by **56** requires the presence of NADPH, suggesting that **56** formation is an intermediary step in PCP bioactivation and that additional reactive intermediate(s) capable of inactivating P450 and/or binding to macromolecules may be formed from this iminium species [52, 56, 57].

Estrogens and selective estrogen modulators (SERMs) are prescribed widely in the clinic to alleviate symptoms in postmenopausal women, and they are metabolized to reactive intermediates that can elicit adverse effects [58, 59]. At Merck, in an attempt to develop safer SERM with an estrogen receptor α-subtype selectivity for the treatment of osteoporosis, compound **60** Fig. (**11**) was evaluated as a lead compound for covalent protein binding. It bound, both time- and NADPH-dependently, to proteins in human liver microsomes, reaching a value of 1106 pmol equiv/mg protein. At least three pathways are involved in the bioactivation of **60**, namely, oxidative cleavage of the dihydrobenzoxantin moiety to give a hydroquinone/para-benzoquinone redox couple, hydroxylation of the benzoxantiin moiety leading to an *o*-quinone intermediate, and metabolism of the piperidine ring to give iminium ion. The last reactive intermediate was identified as its bis-cyano adduct **61** [60]. Therefore structural modification of **60**, including a replacement of piperidine with a pyrrolidine ring, led to compound **62** Fig. (**11**) [61] which did not form a reactive iminium ion. Following the incubation of **62** with human liver microsomes, covalent binding to proteins was reduced (461 pmol equiv/mg protein), the residual level of binding being, apparently, due to the formation of a rearranged biphenyl quinine type metabolite. These studies demonstrate that gaining an understanding of bioactivation mechanisms can be exploited in terms of guiding structural modification of drug candidates to minimize covalent protein binding and, hopefully, to lower the potential for drug-mediated adverse effects [62].

Cyanide has also been incorporated into a series of *N*-alkyl substituted phenylpiperidines; while cyanide was incorporated into each substrate, no correlation was observed between the extent of incorporation and their pKa or logP of the substrates [8].

4-Hydroxypiperidines, 4-Fluoropiperidines and Related Compounds

4-Arylpiperidin-4-ol derivatives like haloperidol, **12**, Fig. (**12**) bearing a potential leaving group, such as a hydroxy group at C-4 position, are in the same oxidation state as the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP, **8**) Fig, (**2**). Consequently, ring α-carbon oxidation of **12** generates iminium products **63**, which, as the corresponding enamine free bases **64**, will undergo spontaneous elimination to form the unstable dihydropyridinium products **65** see Fig. (**12**). In the brain, which lacks aldehyde oxidase activity, these intermediates can oxidize spontaneously to the pyridinium products **66**. Various members of the cytochrome P450 family of enzymes and coenzymes are known to be present in rodent and human brain. Consequently, it is reasonable to speculate that appropriately functionalized piperidinyl derivatives can be converted to the corresponding pyridinium metabolites in the central nervous system. Haloperidol **12**, 4-phenyl-4-piperidinol derivative, resembles MPTP **8** in that it bears an aryl group at C-4 of the azacycle. Furthermore, simple dehydration of haloperidol, a reac-

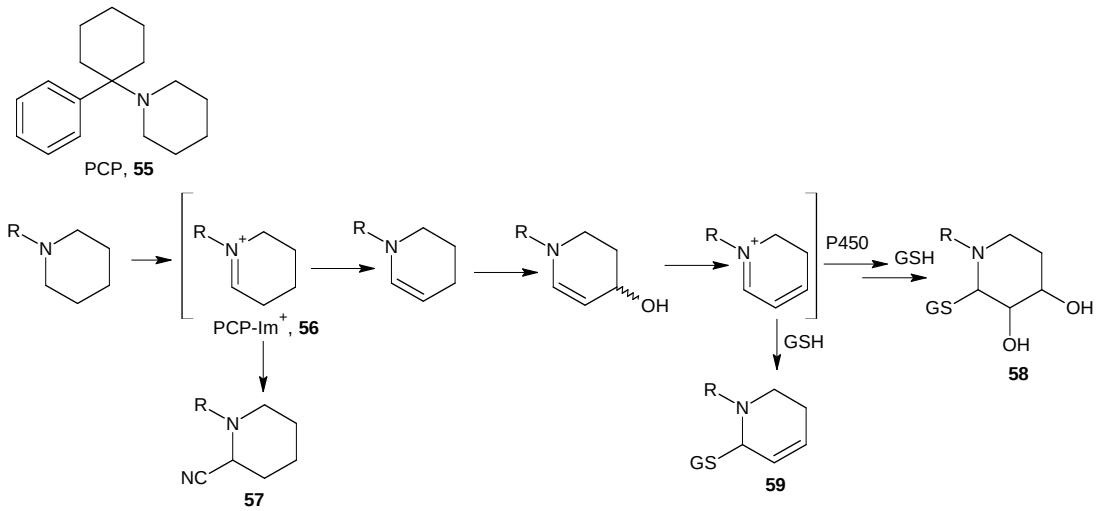

Fig. (10). Bioactivation of PCP in liver microsomes involving the piperidine ring.

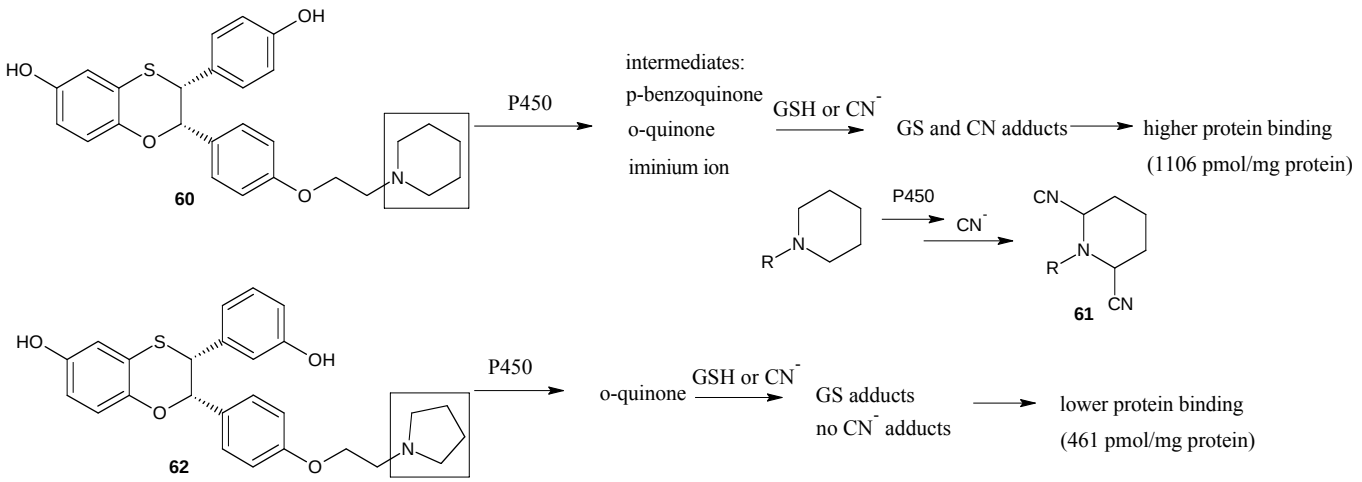

Fig. (11). Comparison of *in vitro* bioactivation of two dihydrobenzoxantiin selective estrogen receptor modulators by cytochrome P4503A4 in HLM.

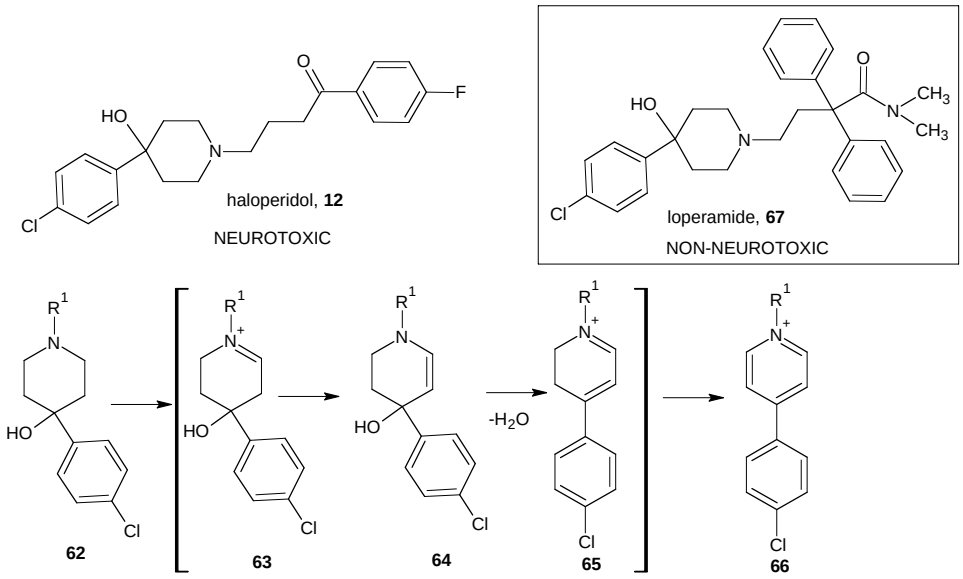

Fig. (12). Proposed bioactivation pathway of functionalized piperidinols on model compound haloperidol **12**.

tion which is reported to occur in microsomal incubations, gives the corresponding 1,2,3,6-tetrahydropyridine derivative **64**, an even closer analog of MPTP [9, 63]. However, the structurally related loperamide **67** Fig. (**12**), a μ-opioid receptor agonist in the myenteric plexus of the large intestine, is not neurotoxic despite a CYP450 catalyzed oxidation of the piperidinol ring, providing a further proof that not all compounds under bioactivation *in vitro* will necessarily elicit a toxic response *in vivo* [64].

The 4-hydroxy- and 4-fluoro-substituted piperidines are chemically similar cyclic tertiary amines that undergo cytochrome P450-catalayzed oxidative metabolism to form chemically reactive iminium species. Ring α-carbon oxidation of **68** and **69** Fig. (**13**) generates iminium products, which, as the corresponding enamine free bases, undergo spontaneous elimination to form the unstable dihydropyridinium product which, after addition of GSH, forms conjugates like **70**.

Pyrrolidines

MRL-A **71** and MRL-B **72** Fig. (**14**) are dipeptidylpeptidase-IV (DPP-IV) inhibitors with good pharmacokinetic properties and significant activity in the oral glucose tolerance test in lean mice. They contain a fluoropyrrolidine moiety in the structure, which undergoes metabolic bioactivation [65, 66]. The irreversible binding of these (tritium-labeled) compounds to rat liver microsomal proteins was time- and NADPH-dependent and was attenuated by the addition of GSH or *N*-acetylcysteine (NAC) to the incubation. Mass spectrometric analysis and further trapping experiments with semicarbazide (products **73** and **75**) indicated that the fluoropyrrolidine ring had undergone sequential oxidation and defluorination events, resulting in the formation of GSH or NAC conjugates **74** of the pyrrolidine moiety. The bioactivation of MRL-A **71** was catalyzed primarily by rat recombinant CYP3A1 and CYP3A2 [67].

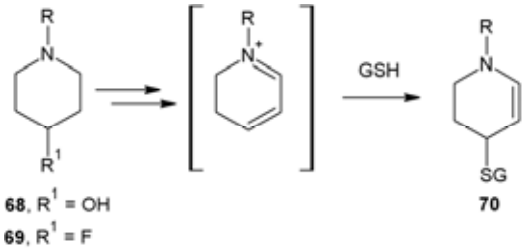

Fig. (13). Proposed mechanism for formation of glutathione adducts of 4-hydroxy- and 4-fluoropiperidines.

In the case of an unsymmetrical alicyclic tertiary amine like nicotine **76** Fig. (**15**), iminium ions, arising through oxidation at the

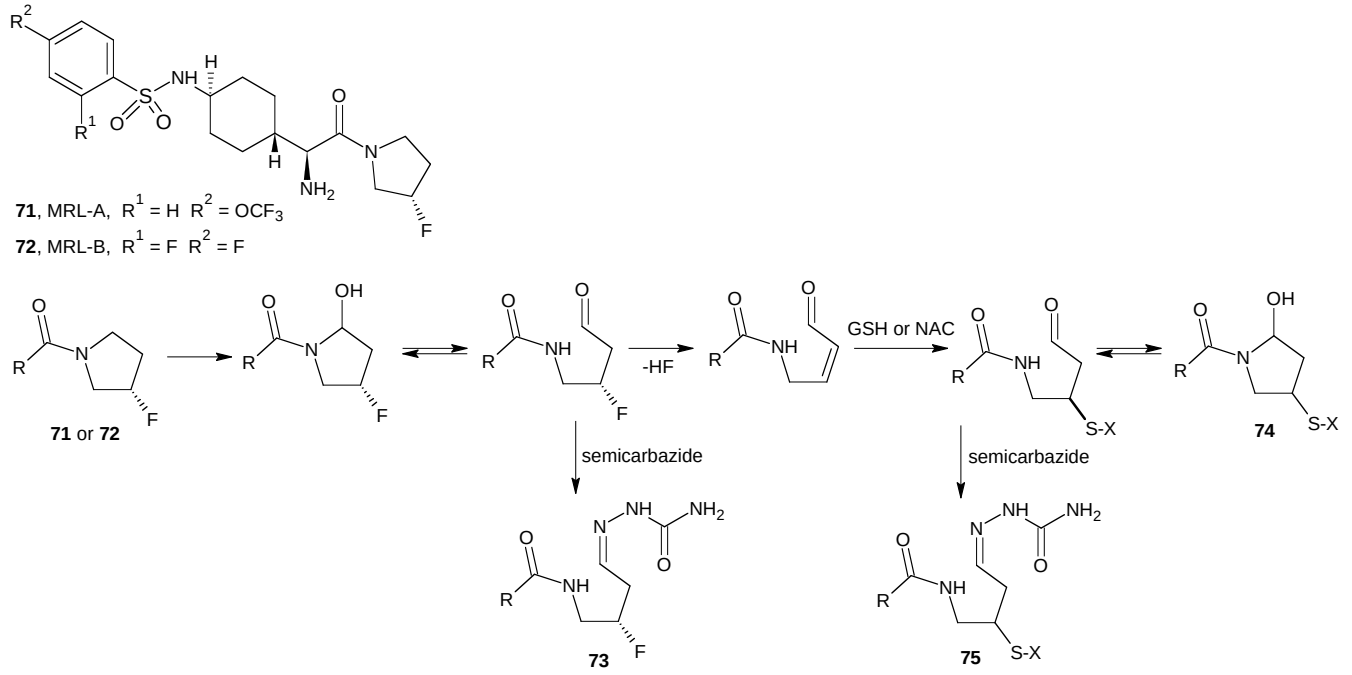

Fig. (14). Metabolic activation of the fluoropyrrolidine moiety of MRL-A **71** and MRL-B **72**.

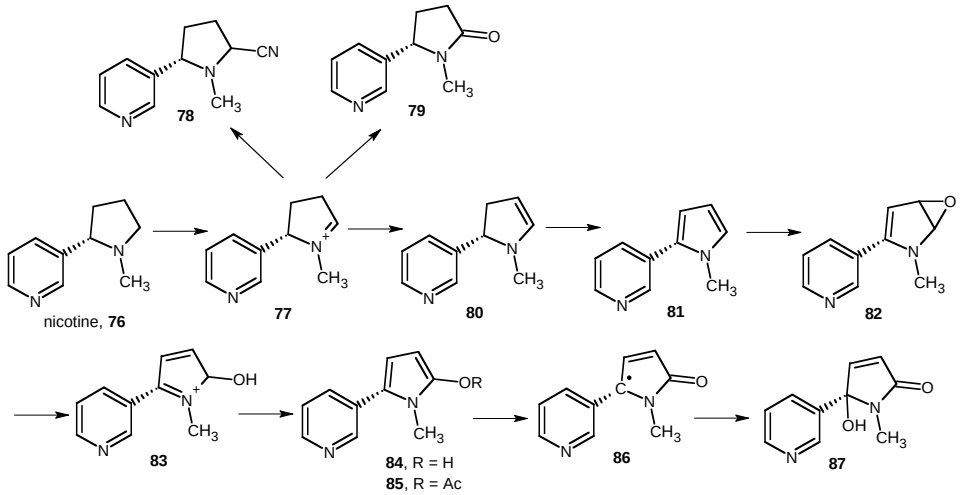

Fig. (15). Metabolic pathways of the alkaloid nicotine **76**.

unsubstituted α-carbon atoms, can occur and may also exist as aminocarbinol tautomers, leading to *N*-dealkylation or ring opening. [8] The bioactivation pathway is likely to proceed *via* the iminium intermediate **77**, since covalent binding is inhibited by cyanide ion, which traps **77** as the corresponding α-cyanoamine **78**, and by the addition of liver cytosolic fraction containing aldehyde oxidase, which catalyzes the conversion of **77** to the corresponding lactam (*S*)-cotinine (**79**). MAO-B has been shown to catalyze the oxidation of iminium intermediate to the corresponding pyrrolyl derivative **81**, presumably *via* the enamine free base **80**. These findings may be relevant to the report that brain MAO-B activity in smokers is almost 40% lower than that in nonsmokers and former smokers [68]. The metabolic conversion of (S)-nicotine to **82** led to metabolic studies on this electron rich pyrrolyl compound, and established conversion by rabbit liver microsomes to the hydroxypyrrolinone **87**. Although no direct evidence exists for the sequence **82** to **87**, incubation of **84**, generated *in situ* from **85**, results in the formation of a carbon-centered radical, most probably **86**, that can be trapped with a nitrone radical trapping compound [69, 70]. The possible toxicological significance of this metabolic pathway remains to be documented [9].

N-Alkyltetrahydroquinolines

Metabolic aromatization of xenobiotics is an unusual reaction. For instance, oxidation of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP, **8**) Fig. (**2**) to the neurotoxic pyridinium ion metabolite, 1-methyl-4-phenylpyridinium (MPP^+, **10**) Fig. (**2**), by monoamine oxidase (MAO) B in the brain has been of interest to a number of investigators. It has also been reported that, although the aromatization of *N*-methyl-tetrahydroisoquinoline **88**, Fig. (**16**) occurs with MAO-B to the corresponding *N*-methylisoquinolinium (**90**), the same enzyme does not result in its isomer, *N*-methyltetrahydroquinoline (**91**), by Fig. (**16**) [71].

The aromatization of an *N*-alkyl-tetrahydroquinoline substructure was identified during *in vitro* metabolite profiling of compound **92** Fig. (**17**), which was designed as a potent rennin inhibitor for the treatment of hypertension [72, 73]. The *N*-alkylquinolinium metabolite **93** was identified by LC-MS/MS of human liver microso-

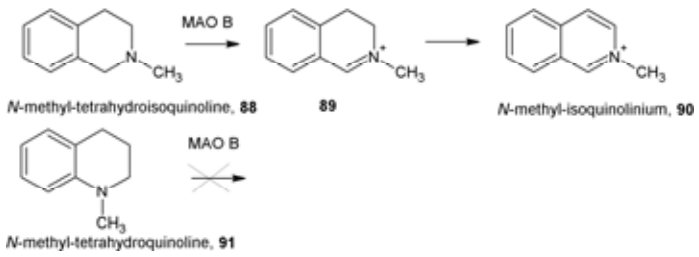

Fig. (16). Reactivities of *N*-Methyl-tetrahydroisoquinoline (**88**) and *N*-Methyl-tetrahydroquinoline (**91**).

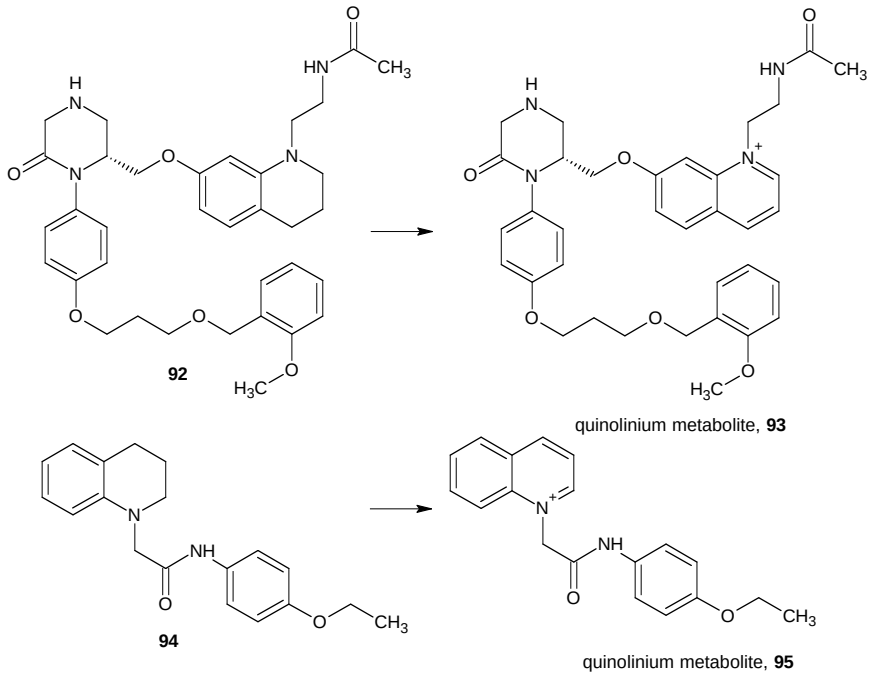

Fig. (17). Proposed quinolinium metabolites of compounds **92** and **94** generated in human liver microsomes.

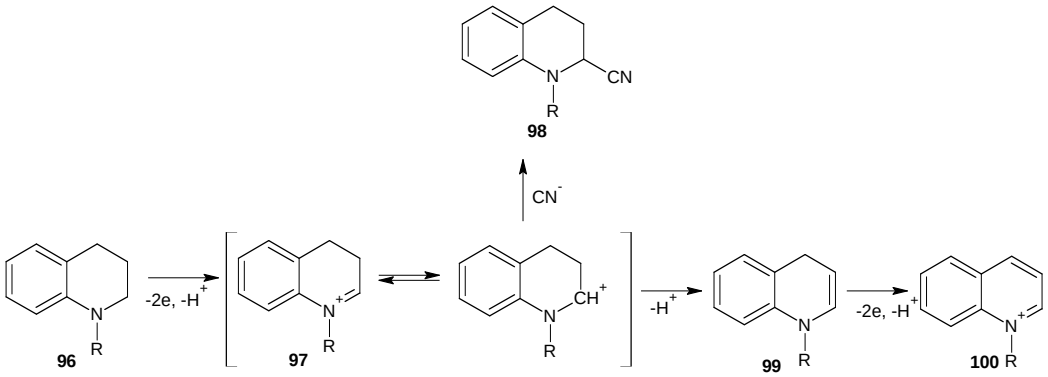

Fig. (18). Postulated mechanism of metabolic aromatization of *N*-Alkyl-1,2,3,4-tetrahydroquinoline (**96**).

mal incubates and proton NMR of the isolated metabolite. Further *in vitro* metabolism studies with the commercially available chemical (compound **94**) containing the same substructure, also generated an *N*-alkylquinolinium metabolite **95**. *In vitro* cytochrome P450 reaction phenotyping of compound **92** revealed that the metabolism was catalyzed exclusively by CYP3A4. Although compound **94** is a substrate for several P450 isoforms, its quinolinium metabolite **95** was also generated predominantly by CYP3A4. Neither **92** nor **94** was a substrate of MAOs. The quinolinium metabolites were readily produced by horseradish peroxidase, suggesting that aromatization of the *N*-alkyltetrahydroquinoline could occur via a mechanism involving single electron transfer from nitrogen Fig. (**18**). Although dihydro intermediates **97** and **99** from the tetrahydroquinoline substrates **96** were not observed in the formation of quinolinium metabolites **100**, cyanide trapping results (conjugate **98**) indicate the occurrence of iminium intermediates **97** [74].

Another example of compounds containing an *N*-methyltetrahydroisoquinoline ring and having the potential for being oxidized to quaternary dihydroisoquinolinium and isoquinolinium ions, is nomifensine **101** Fig. (**19**). It is an antidepressant agent that was removed from use because of high incidence of hemolytic anemia [75, 76]. Nomifensine possess an aniline group, which is a substitu-

ent in drugs frequently associated with blood and liver toxicities. Beside this structural element, nomifensin possess tetrahydroisoquinoline structural element that could be biotransformed to electrophilic nomifensine dihydroisoquinolinium metabolite. Human liver microsomes supplemented with NADPH generated the dihydroisoquinolinium ion metabolite **102**, along with other hydroxylated metabolites, whereas, when supplemented with *t*-butyl peroxide, only the dihydroisoquinolinium ion metabolite **102** was observed. MAO A, but not MAO B, catalyzed this reaction, as well as human hemoglobin supplemented with H_2O_2. Human myeloperoxidase catalyzed this reaction in the presence of H_2O_2, and activation of the reaction was observed when incubations were conducted in the presence of acetaminophen at concentrations relevant to those present in humans. The reaction was also observed in human whole blood. The dihydroisoquinolinium ion **102** was shown to react with cyanide, giving the corresponding cyanide adduct **104**, but not with glutathione. These findings suggest that the electrophilic nomifensine dihydroisoquinolinium metabolite **102**, which can be generated by several enzymes, could be responsible for the toxic responses to nomifensine **101**, such as hemolytic anemia and hepatotoxicity [77].

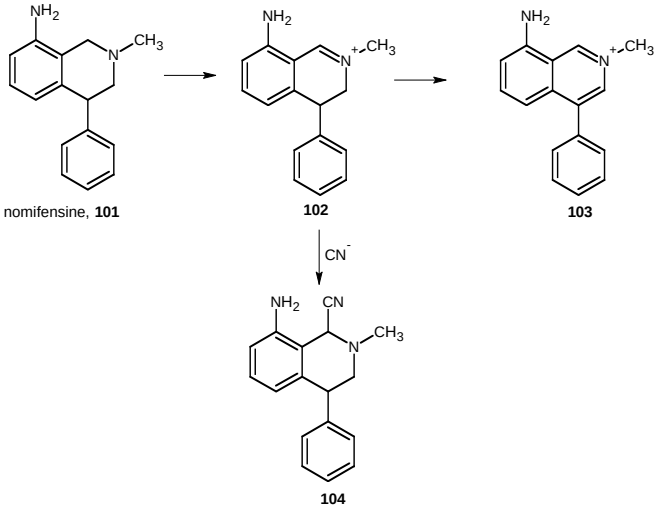

Fig. (19). Nomifensine **101** oxidation to nomifensine quinolinium through nomifensine dihydroisoquinolinium ion.

The anti-aggregatory agent ticlopidine **105** Fig. (**20**) [78], has some structural features that are similar to those of cyclic tertiary amines such as tetrahydroisoquinolines, which are converted to isoquinolinium metabolites via the iminium (dihydropyridinium) species. Metabolic studies with ticlopidine **105** have indicated that the principal routes of metabolism are *N*-dealkylation, *N*-oxidation, and oxidation of the thiophene ring [79]. The results from *in vitro* studies indicate the potential of ticlopidine to undergo a similar conversion, catalyzed by cytochrome P450, peroxidases, and monoamine oxidase (MAO). This suggested that, catalyzed by P450, horseradish peroxidase, and myeloperoxidase and, to a lesser extent, by MAO, ticlopidine undergoes an overall 4-electron oxidation to the novel thienopyridinium metabolite **105** via the thienodihydropyridinium metabolite **106**. In the peroxidase-mediated reactions **106** was the primary metabolite, quite stable to air oxidation or disproportionation. It was less electrophilic and did not form cyanide, glutathione, or *N*-acetylcysteine adducts. On the other hand, **108** was the major metabolite in P450-catalyzed oxidation of ticlopidine **105**. The results from this study have revealed that, in addition to metabolism of the thiophene ring of ticlopidine, the tetrahydropyridine moiety of the compound is susceptible to 2-electron and 4-electron oxidations, like other cyclic tertiary amines [80].

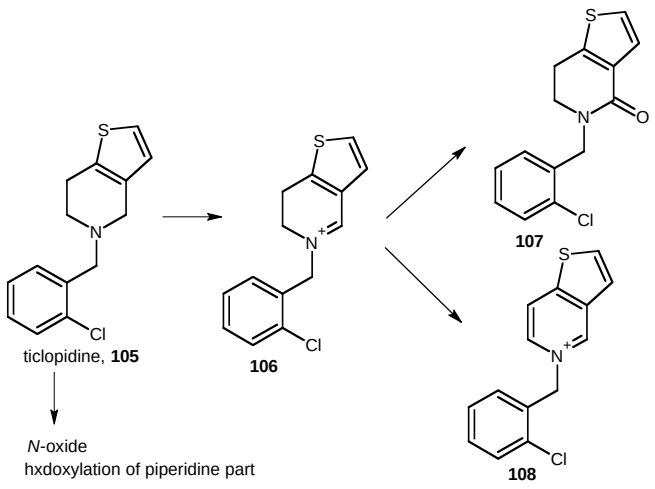

Fig. (20). Proposed bioactivation of ticlopidine **105** at the piperidine part of the molecule.

INFLUENCES ON THE TOXICITY OF IMINIUM REACTIVE INTERMEDIATES

Species Difference

At Merck they described a case of species-selective metabolic activation that correlated with species-selective hepatotoxicity. Preliminary *in vitro* metabolism studies with compound **109** Fig. (**21**) indicated that two major routes were followed in all species examined, namely, UGT-mediated conjugation of the carboxylic acid moiety to yield the corresponding acyl glucuronide, and CYP-mediated oxidation of the aryl and alkyl substituents denoted as R1 and R2. In order to examine the metabolic fate of **109** in more detail, a radiolabeled analogue was prepared for a series of *in vitro* and *in vivo* studies and for assessment of the propensity of the compound to undergo bioactivation to species that would bind covalently to protein. Interestingly, the results of the latter experiments revealed a striking species difference in the ability of [$^3H_2$] Compound **109** to alkylate liver microsomal proteins fortified with NADPH. Thus, while covalent binding was low (<15 pmol/mg protein) in preparations from rats, dogs, and humans, high levels of irreversibly bound radioactivity were noted in liver microsomal preparations from the rhesus monkey (686 pmol/mg protein). After comprehensive *in vivo* studies performed in rat and rhesus monkey, it appeared that **109** was subject to extensive metabolic activation in the rhesus monkey, leading to species-specific hepatotoxicity [5, 81].

A series of *in vitro* studies were conducted to compare the metabolite profiles and covalent binding properties of compound **109** in liver microsomal preparations from the rhesus monkey, in the absence and presence of nucleophilic trapping agents such as GSH and mercaptoethanol (thiol nucleophiles), cyanide ion (an effective trap for iminium ions), and methoxylamine (a trapping agent for reactive carbonyl species). These experiments revealed that, of the nucleophiles tested, only cyanide ion successfully reduced the covalent binding of **109** to protein. Compound **109** undergoes CYP-mediated two-electron oxidation, yielding an intermediate iminium species **110** which, upon deprotonation, affords a putative enamine species Fig. (**21**). A second cycle of CYP catalysis introduces an atom of oxygen, generating an unstable epoxide that undergoes spontaneous ring opening to the dipolar oxy-anion **111** that, in turn, is ultimately trapped by cyanide to give the stable end-product **112**. It is important to note that, in the specific case of compound **109**, the rhesus monkey was not representative of humans with respect to its metabolic properties, and therefore, this animal species would have been a poor choice in the preclinical toxicological evaluation of **110**, had it been selected for development [5, 81].

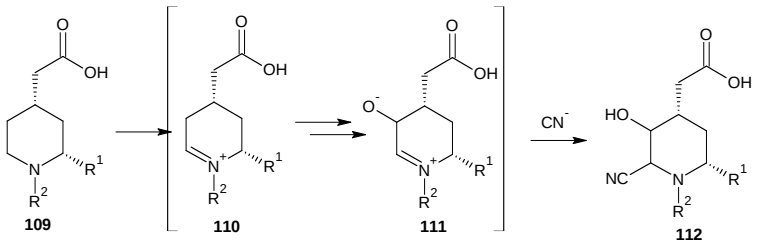

Fig. (21). Proposed bioactivation mechanism for compound **109**.

Removal of Structural Alert

The propensity of a compound to undergo bioactivation to reactive intermediates, usually electrophiles, is a function of the structure resulting from metabolism. Structural alerts that, upon bioactivation, afford reactive metabolites, along with numerous drugs that contain putative structural alerts associated with some form of toxicity are reviewed comprehensively by Kalgutkar *et al.* [82] We have presented nefazodone **36** Fig. (**7**) and nomifensine **101** Fig. (**19**) that possess the bioactivation labile cyclic tertiary amine moiety and have been withdrawn due to toxicity. In several cases, removal of bioactivation liability (structural alert) in prototype drugs has resulted in the elimination of toxicity in the successor drug. Structural alterations that successfully eliminate the propensity of new chemical leads to undergo bioactivation may also be detrimental to the desired pharmacological and pharmacokinetic properties. Therefore, strategies of chemical optimization, targeted to the elimination of bioactivation phenomenon, often entail an iterative process, dependent on close working between medicinal chemists, pharmacologists, and drug metabolism scientists.

Structural alerts of cyclic tertiary amines susceptible to bioactivation to reactive iminium intermediates are listed in Fig. (**22**). There are several strategies that medicinal chemists can utilize toward elimination of bioactivation potential of compounds. One involves direct replacement of the potential structural alert with substituents resistant to metabolism. Cases where reactive iminium metabolite formation can be eliminated by direct replacement in the offending motifs are compound **22** Fig. (**5**) and mianserin **30** Fig. (**6**). Removal of structural alerts in prototype compounds translates into improved safety profiles.

Blocking the Site of Bioactivation

An alternate approach involves blocking the site of metabolic activation to preclude bioactivation. An example of successful implementation of this approach, that prevents formation of reactive iminium intermediates, is evident in the structure-bioactivation studies conducted on compound MB243 **15** Fig. (**4**). Compound **6** is a 1,3-disubstituted piperazine derivative that is a selective melanocortin receptor subtype-4 agonist with potential application in the treatment of obesity and/or erectile dysfunction. Introducing simple alkyl substitutions (methyl, isopropyl) α to the N^1-methyl functionality afforded compounds **20** and **21** Fig. (**4**) that did not undergo metabolic bioactivation to iminium intermediates and were therefore more attractive as drug candidates.

Introduction of Metabolic Soft Spots

Apart from strategies that focus on replacement of structural alerts or blocking metabolic sites susceptible to bioactivation, medicinal chemists can also introduce metabolic soft spots elsewhere on the structure to divert the biotransformation reactions. An example in the case of cyclic tertiary amines is agonist compound **22** Fig. (**5**). Information on the structure of reactive intermediates leading to mutagenic response and, hence, a rationale on which base subsequent chemical optimization. Using this information, compound **29** was designed as a follow-on candidate by introduction of a metabolic soft spot in the piperazine ring Fig. (**5**). Compound **29**, besides maintaining the primary pharmacology and attractive pharmacokinetic attributes of **22**, was devoid of the mutagenic response observed with **22**. Consistent with this finding, no amine and/or GSH conjugates of compound **29** were detected in rat S-9/NADPH incubations supplemented with trapping agents. The insertion of the α-methyl group in the piperazine ring serves as a metabolic soft spot and circumvents piperazine ring bioactivation. Similarly, removal of the problematic 3-chlorobenzyloxy moiety eliminates the bioactivation pathway, resulting in quinone-methide formation.

Other Factors that Mitigate the Toxicity Risk Associated with Compounds Containing Structural Alert

There are several other factors that can determine whether the presence of a putative structural alert in the compound will eventually be manifested in unanticipated toxicity. Susceptibility of a structural alert to metabolic activation needs to be clearly understood, since there are several examples of successful drugs which do not exhibit toxicity despite containing structural alerts. In many such cases structural alert is not involved in metabolism and primary route of metabolism proceed normally at an alternate site or by non-metabolic processes. In other instances, bioactivation may be discernible in a standard *in vitro* system, such as liver microsomes, but the principal clearance mechanism *in vivo* may involve a different and perhaps more facile metabolic pathway yielding non-reactive metabolites, such in the case of raloxifene [83-85]. Drugs used in a chronic setting appear to be more prone to adverse drug reactions than those used in an acute setting. Because toxicity is evident only after a few weeks of administration, agents that are administered for two weeks or less are rarely associated with bioactivation-related toxicity. This is illustrated with the widely used antidiarrheal agent loperamide **67** Fig. (**12**) that is rarely associated with especially tardive dyskinesia and Parkinsonism, despite its structural similarity to haloperidol and despite its P4503A4-mediated metabolism to a potentially neurotoxic pyridinium intermediate Fig. (**12**). Plausible reasons for the lack of neurotoxic complications associated with loperamide, relative to haloperidol, include opiate activity that is restricted to the gastrointestinal tract and therapy that usually last for a few days, in contrast to haloperidol use in a chronic setting. In addition, loperamide and its positively charged pyridinium metabolite, but not haloperidol, are p-glycoprotein substrates and are denied access to the central nervous system, where they could potentially damage critical neurons.

Finally, the efficacious dose of the drug or drug candidate may be pivotal as a factor mitigating the risk of toxicity. There are actually no examples of drugs that are dosed below 10 mg/day that cause idiosyncratic adverse drug reactions. There are many examples of two structurally related drugs that possess a common structural alert prone to bioactivation, but the one administered at the lower dose is much safer than the one given at a higher dose. The two examples noted previously in this review are the antihypertensive agent prazosin **38** Fig. (**8**), and the nefazodone **36**/aripiprazole **37** comparison Fig. (**7**).

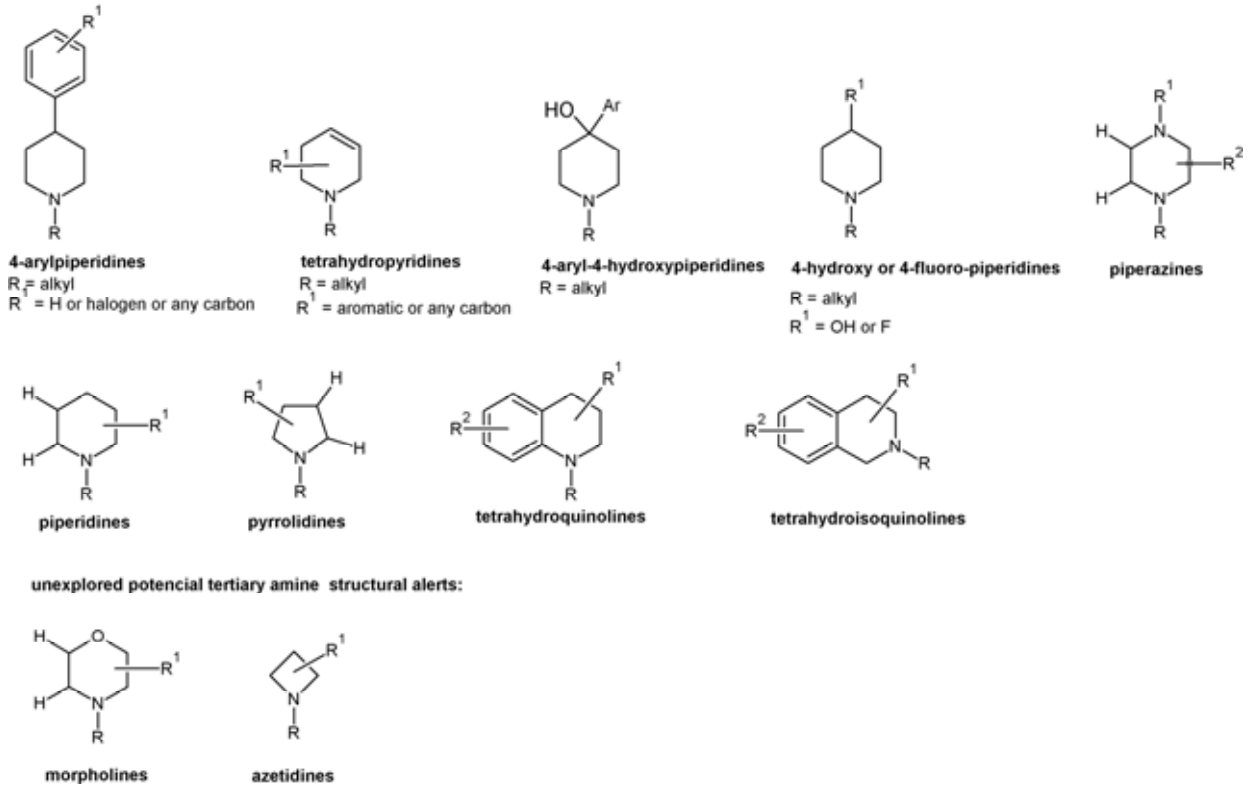

Fig. (22). A list of tertiary amine structural alerts susceptible to bioactivation to the reactive iminium intermediates.

SUMMARY

The metabolism of several tertiary amines of toxicologic significance, e.g. MPTP and phencyclidine, has been shown to be associated with low levels of covalent binding and/or suicide enzyme inactivation, although the physiologic significance of such covalent binding is uncertain. The possibility that the iminium species formed upon two-electron oxidation by cytochrome P-450 (or in the case of MPTP, by mitochondrial MAO), might act as electrophilic intermediates capable of binding covalently to cellular nucleophiles was initially believed. It is still not established that simple cyclic iminium species form stable covalent adducts with cellular nucleophiles and no evidence has been obtained for persistent binding of iminium species directly under physiological conditions. More recent studies and presented examples in the article have demonstrated that most, if not all, covalent binding activity arises from metabolism beyond the iminium stage of oxidation. While the detection of a bioactivation process *in vitro* is relatively straightforward, the downstream consequence of this process as it relates to toxicity remains poorly understood. Given the inability to predict whether bioactivation phenomenon detected *in vitro* will ultimately lead to toxicity in the clinic, a general strategy adopted by many within pharmaceutical industry involves the assessment of reactive metabolite formation as early as possible in the selection of drug candidates, with the goal of eliminating or minimizing the formation of reactive species by rational structural modification of compound. The complex nature of adverse drug reactions still limits our ability to predict whether *in vitro* bioactivation and accompanying covalent binding of a potential drug candidate to hepatic tissue(s) will or will not ultimately translate in some form of toxicity in animals or for that matter in humans. Even if the drug candidate fails to cause organ toxicity in preclinical species, there is always some concern that bioactivation observed in human hepatic tissue may have potential to elicit idiosyncratic immune-mediated adverse drug reactions in the susceptible population.

The potential for toxicity of a new drug candidate depends on a variety of factors including the extend of metabolism via bioactivation pathway, daily dose, acute or chronic therapeutic regimen, and the intended target population; factors that need to be taken into consideration when making a final decision to develop a drug candidate that is biotransformed to reactive metabolites. The detection of reactive metabolites *in vitro* is not a prerequisite for toxicity. The efficacious dose of the drug or drug candidate may be pivotal as a factor mitigating the risk of toxicity. Many drugs possess dose dependent toxicity like acetaminophen and hydralazine. There are actually no examples of drugs that are dosed below 10 mg/day that cause idiosyncratic adverse drug reactions. Based on our increased knowledge of the field of bioactivation reactions and mechanisms, drug metabolism scientists in the industry/academia have in their arsenal some tools to qualitatively probe reactive metabolite formation in a drug discovery/early development setting. Future application of new techniques may be able to improve our understanding of the specificity and selectivity of the binding electrophilic species to endogenous molecules. A low risk strategy in drug discovery could potentially preclude the use of structural alerts susceptible to bioactivation altogether, but this is likely to limit a detailed exploration of SAR around a chemical series of interest. Thus a proactive approach between medicinal chemists, pharmacologists and metabolism scientists is establishing detailed pharmacological SAR alongside screening for bioactivation potential is more appropriate and balanced especially in the lead optimization stage. If the chemical matter is susceptible to bioactivation, than the efforts to minimize the metabolic activation by iterative chemical intervention should be considered. If an alternate structural series that does not form reactive metabolites is available, than this series could be progressed as a suitable replacement for further optimization of pharmacology and/or absorption, distribution, metabolism, and excretion (ADME) properties. Despite decades of research in the area of bioactivation and toxicity, accurate prediction of toxicity

potential of a drug candidate susceptible to bioactivation remains elusive.

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