Comparison of the Bioactivation Potential of the Antidepressant and Hepatotoxic Nefazodone with Aripiprazole, a Structural Analog and Marketed Drug

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Received January 22, 2008; accepted March 5, 2008

ABSTRACT:

In vitro metabolism/bioactivation of structurally related central nervous system agents nefazodone (hepatotoxic) and aripiprazole (nonhepatotoxic) were undertaken in human liver microsomes in an attempt to understand the differences in toxicological profile. NADPH-supplemented microsomal incubations of nefazodone and glutathione generated conjugates derived from addition of thiol to quinonoid intermediates. Inclusion of cyanide afforded cyano conjugates to iminium ions derived from α-carbon oxidation of the piperazine ring in nefazodone and downstream metabolites. Although the arylpiperazine motif in aripiprazole did not succumb to bioactivation, the dihydroquinolinone group was bioactivated via an intermediate monohydroxy metabolite to a reactive synthetic, which was trapped by glutathione. Studies with synthetic dehydroaripiprazole metabolite revealed an analogous glutathione conjugate with molecular weight 2 Da lower. Based on the proposed structure of the glutathione conjugate(s), a bioactivation sequence involving aromatic ortho- or para-hydroxylation on the quinoline followed by oxidation to a quinone-imine was proposed. P4503A4 inactivation studies in microsomes indicated that, unlike nefazodone, aripiprazole was not a time- and concentration-dependent inactivator of the enzyme. Overall, these studies reinforce the notion that 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 (versus nefazodone) despite the accompanying bioactivation liability is the vastly improved pharmacokinetics (enhanced oral bioavailability, longer elimination half-life) due to reduced P4503A4-mediated metabolism/bioactivation, which result in a lower daily dose (5–20 mg/day) compared with nefazodone (200–400 mg/day). This attribute probably reduces the total body burden to reactive metabolite exposure and may not exceed a threshold needed for toxicity.

In an increasing number of cases, a broader understanding of the biochemical basis for adverse drug reactions (ADRs) has aided to replace a vague perception of a chemical class effect with a sharper picture of an individual molecular peculiarity. Evidence from such investigations suggests that bioactivation of drugs to reactive intermediates constitutes a rate-limiting step in the etiology of some ADRs (Nelson, 2001; Evans et al., 2004; Park et al., 2005; Kalgutkar et al., 2005a; Kalgutkar and Soglia, 2005). Information to qualify certain functional groups as “structural alerts” or “toxicophores” also has been inferred from such studies based on myriad examples of protoxins containing these motifs, which are bioactivated to reactive metabolites (Kalgutkar et al., 2005a). Consequently, there often exists a notion among medicinal chemists that structural alerts should generally be avoided in drug design efforts. Such a strategy has also come under scrutiny given the plethora of examples of drugs devoid of ADRs despite containing toxicophores susceptible to bioactivation. An interesting example of this dilemma is evident with the central nervous system agents 2-[3-[4-(3-chlorophenyl)-1-piperazinyl]propyl]-5-ethyl-2,4-dihydro-4-(2-phenoxethyl)-3H-1,2,4-triazol-3-one (nefazodone) and 7-[4-[2-(3-dichlorophenyl)-1-piperazinyl]butoxy]-3,4-dihydrocarbostyril (aripiprazole), which, despite containing common toxicophores(s), display markedly different safety profiles.

Antidepressant therapy with nefazodone has been associated with idiosyncratic hepatic ADRs including necrosis and failure, often resulting in liver transplantation or death; liver biopsy results have been consistent with toxic injury (García-Pando et al., 2002; Stewart, 2002; Choi, 2003; Andrade and Lucena, 2003). Circumstantial evidence that reactive intermediates may be ultimately responsible for liver injury has been presented. In humans, the oxidative pathway of nefazodone metabolism to para-hydroxynefazodone (1), a major circulating metabolite (Mayol et al., 1994), results in a para-hydroxyaniline analog and well established toxicophore, which undergoes P4503A4-mediated bioactivation to electrophilic quinonoid intermediates in human liver microsomes. The structure of these reactive metabolites was inferred through the characterization of the corresponding glutathione (GSH) conjugates (Fig. 1) (Kalgutkar et al., 2005b). Additionally, reactive iminium ion intermediates arising from α-carbon oxidation

REFERENCES:

ADRs, adverse drug reaction; P450, cytochrome P450; 1, para-hydroxynefazodone; GSH, reduced glutathione; LC-MS/MS, liquid chromatography tandem mass spectrometry; CID, collision-induced dissociation; MRM-EPI, multiple reaction monitoring-enhanced product ion; Rt, retention time.
The lack of reports on nefazodone-like hepatotoxicity with the atypical antipsychotic aripiprazole is intriguing. Since its introduction in 2003, aripiprazole has enjoyed great commercial success in the treatment of schizophrenia with 2006 revenues exceeding 1 billion U.S. dollars. Like nefazodone, P4503A4-mediated aromatic hydroxylation on the arylpiperazine motif in aripiprazole results in the formation of the para-hydroxyaripiprazole metabolite [aripiprazole metabolism data submitted by the manufacturer of the drug to the FDA; for a review, see Caccia (2007)], which is capable of undergoing bioactivation on the double bond forming an electrophilic epoxide that can potentially react directly with GSH (Fig. 2, pathway E) or undergo oxidation to the quinone-imine/quinone intermediates (Fig. 2, pathway A). In addition, the piperazine ring in aripiprazole can succumb to α-carbon oxidation resulting in the formation of reactive iminium ion intermediates (Fig. 2, pathway B). Furthermore, unlike nefazodone, aripiprazole contains an acetanilide motif, capable of undergoing a bioactivation sequence involving ortho- or para-aromatic hydroxylation followed by oxidation to the quinone-imine intermediate (Fig. 2, pathway C) in a manner similar to that observed with acetaminophen (Hinson, 1983). Finally, dehydroaripiprazole (Fig. 2, pathway D), the active metabolite of aripiprazole (Molden et al., 2006), is a α,β-unsaturated carbonyl compound and can potentially react directly with GSH (Fig. 2, pathway E) or undergo oxidation on the double bond forming an electrophilic epoxide that can react with GSH (Fig. 2, pathway F). Pathway F depicted in Fig. 2 is known to occur on the quinolinine ring in the inotropic agent toborizone (Kitani et al., 1997).

Since detailed information on the overall biotransformation pathways of aripiprazole in humans including the potential for bioactivation is currently unavailable in the primary literature, we decided to characterize the in vitro metabolic/bioactivation pathways of aripiprazole in human liver microsomes. Nefazodone was included as a positive control in these studies. Special emphasis was placed on the characterization of reactive iminium species of nefazodone in human liver microsomes, since previous information on this bioactivation pathway was limited to the rat (Argoti et al., 2005). Studies comparing the inhibition (reversible and time-dependent) of P450 enzymes involved in aripiprazole and nefazodone metabolism were also undertaken, since time-dependent, irreversible inactivation can also be caused by covalent modification of P450 enzyme(s) that catalyze bioactivation.

**Materials and Methods**

**Chemicals.** All chemicals and solvents were purchased from Aldrich Chemical Co. (Milwaukee, WI). Nefazodone hydrochloride, NADPH, GSH, and potassium cyanide were purchased from Sigma-Aldrich (St. Louis, MO). Aripiprazole and dehydroaripiprazole were synthesized at Pfizer Global Research and Development (New York, NY). Pooled human liver microsomes and recombinant human P4503A4 and P4502D6 isozymes, coexpressed with NADPH-P450 oxidoreductase in baculovirus-insect cells, were purchased from BD Gentest (Woburn, MA).

**Metabolism Studies.** Stock solutions of nefazodone, aripiprazole, and dehydroaripiprazole were prepared in methanol or dimethyl sulfoxide/acetonitrile [25%/75%, (v/v)], respectively. The final concentration of organic solvent in the incubation medium was 0.2% (v/v). Incubations were carried out at 37°C for 60 min in a shaking water bath. The incubation volume was 1 ml and consisted of the following: 0.1 M potassium phosphate buffer (pH 7.4) containing MgCl2 (10 mM), human liver microsomes (P450 concentration = 0.5 μM) or recombinant enzymes (25 pmols of P4503A4 or P4502D6), NADPH (1.2 mM), substrate (20 μM for metabolite identification and 100 μM for reactive metabolite trapping), and GSH (5 mM) or potassium cyanide (5 mM). Incubations that lacked either NADPH or trapping agents served as negative controls, and reactions were terminated by the addition of ice-cold acetonitrile (1 ml). The solutions were centrifuged (3,000g, 15 min), and the supernatants were dried under a steady nitrogen stream. The residue was
reconstituted with mobile phase and analyzed for metabolite formation by liquid chromatography tandem mass spectrometry (LC-MS/MS).

**Metabolite Identification Using Triple Quadrupole Linear Ion Trap LC-MS/MS.** A bioanalytical methodology similar to the one described by Zheng et al. (2007) was used for characterization of metabolites including GSH and/or cyano conjugates. The LC system consisted of Shimadzu LC20AD pumps, DGU20A5 degasser, and VP Option box (Columbia, MD), an HTC PAL autosampler (Leap Technologies, Cary, NC), and a Phenomenex Synergi 150 mm, 4.6 µm column (Torrance, CA). LC mobile phase A was 10 mM ammonium formate/isopropyl alcohol/formic acid in water [98.9%-1.0%-0.1%, (v/v)], and mobile phase B was formic acid in acetonitrile (0.1%). The flow rate was 0.7 ml/min. The LC gradient started at 5% B for 5 min, ramped linearly to 50% B over 30 min, increased to 90% B over 5 min, and then returned to the initial condition over 1.0 min. Post-column flow was split such that mobile phase was introduced into the mass spectrometer via an electrospray interface at a rate of 175 µl/min. The remaining flow was diverted to the photodiode array detector to provide simultaneous UV detection (254 nm) and total ion chromatogram. The LC system was interfaced to an API 4000 Q-trap mass spectrometer (Sciex, Toronto, ON, Canada) equipped with the TurboIonSpray source. Metabolites including GSH and cyano conjugates were initially characterized in the full scan mode (from m/z 100 to 850) by comparing t = 0 samples to t = 60 min samples (with or without cofactor or trapping agent), and structural information was generated from the collision-induced dissociation (CID) spectra of the corresponding protonated molecular ions. To improve sensitivity, metabolites, GSH, and cyano adducts were also characterized using the multiple reaction monitoring-enhanced product ion (MRM-EPI) mode following preset MRM transitions derived from the CID information. In the case of GSH and cyano adducts, these MRM transitions corresponded to the loss of the pyroglutamic acid moiety (129 Da) and cyanide (27 Da), respectively, from the molecular weight (MH+) of the conjugate(s). In the MRM analysis, the source temperature was set at 350°C, and the ion spray voltage was set to 5.0 kV. Nitrogen was used as the nebulizer and auxiliary gas. The dwell times for MRM analysis (up to 12 transitions) were 150 ms, and the interscan pause time for all MRM analysis was 5 ms. The same declustering potential (30 V), collision energy (50 eV), and a collision energy spread (±15 eV) were applied for all potential GSH and cyano adducts in the MRM-EPI mode.

**P450 Inhibition Studies in Human Liver Microsomes.** For nonpreincubation (time-independent) inhibition of P4503A4 activity, a pooled human liver microsomal preparation (0.01 mg/ml) was incubated with midazolam (3 µM) and test compound (nefazodone or aripiprazole) at concentrations ranging from 0.5 to 50 µM at 37°C for 20 min in the presence of NADPH (1.3 mM). For preincubation-dependent (time-dependent) P4503A4 inhibition, microsomes (0.01 mg/ml), NADPH (1.3 mM), and test compound (nefazodone or aripiprazole) at concentrations ranging from 0.5 to 50 µM were incubated for 30 min at 37°C, after which further incubation with midazolam (3 µM) and a second aliquot of NADPH (1.3 mM) was carried out for an additional 20 min. In separate studies, reversible and time-dependent inhibition of P4502D6 activity in human liver microsomes by aripiprazole (0.5–50 µM) was also examined in an identical fashion using the selective probe P4502D6 substrate dextromethorphan (25 µM). All inhibition studies were conducted in triplicate. Incubations were quenched by the addition of 2 volumes of acetonitrile containing levallophan (100 ng/ml) as internal standard. Remaining P4503A4 and P4502D6 activity as measured by the formation of 1′-hydroxymidazolam and dextromethorphan, respectively, was determined by LC-MS/MS using previously described bioanalytical conditions (Bertelsen et al., 2003; Kalugutkar et al., 2005b). For all high-performance liquid chromatography analysis, peak areas of the metabolite (1′-hydroxymidazolam or dextrophan) were expressed as a ratio to the internal standard (levallophan) peak area for each concentration of the inhibitor. These peak area ratios represent the remaining midazo-
lam-1'-hydroxylate and dextromethorphan demethylase activity in the microsomes and were expressed as a percentage of the time-matched control samples without inhibitor.

Results

Metabolic Profile of Nefazodone and Aripiprazole in Human Liver Microsomes. The metabolism of nefazodone has been studied extensively in vitro (Kalugtark et al., 2005b; Peterman et al., 2006; Li et al., 2007) and in vivo (Mayol et al., 1994). In the current investigation, we reassessed nefazodone metabolism in human liver microsomes for comparative purposes only. Figure 3 indicates the metabolic profile of nefazodone in NADPH-supplemented human liver microsomes. The molecular ions (MH\(^+\)), MS/MS fragmentation pattern, and plausible structures of the various nefazodone metabolites (M1–M11) are depicted in Table 1. The formation of metabolites M1–M7 and M9 is consistent with previously published findings and requires no further discussion (Mayol et al., 1994; Kalugtark et al., 2005b; Peterman et al., 2006; Li et al., 2007). Three new nefazodone metabolites, M8, M10, and M11, were also observed in this study. The presence of fragment ions at \(m/z\) 274 and 246 in the mass spectra of dihydroxylated metabolite M8 (MH\(^+\) = 502) and monohydroxylated metabolite M11 (MH\(^+\) = 486), respectively, suggested that the N-propyl-5-ethyl-2,4-dihydro-4-(2-phenoxyethyl)-triazolone substituent was unmodified. In contrast, the fragment ions at \(m/z\) 274 and 246 in the dihydroxylated metabolite M10 shifted by 16 Da to \(m/z\) 290 and 262, respectively, suggesting that one site of hydroxylation was on the N-propyl-5-ethyl-2,4-dihydro-4(2-phenoxyethyl)-triazolone motif. In M8, the fragment ion at \(m/z\) 241 (Table 1) indicated that both hydroxylations had occurred on the 3-chlorophenylpiperazine ring. The fragment ion at \(m/z\) 223 (loss of 18 mass units from \(m/z\) 241) suggests that one site of hydroxylation is the phenyl ring and the other site is on the piperazine ring. Likewise, with M10 and M11, the 18-Da shift in the fragment ion at \(m/z\) 225 to \(m/z\) 207 was also consistent with piperazine ring oxidation. Treatment of the incubation mixture with the reducing agent TiCl\(_3\) led to the disappearance of the peaks, suggesting that they were N-oxides. Finally, the assigned regiochemistry for hydroxylations on the phenyl ring in M8 and the ethyl group in M10 is based on the information available from previously characterized nefazodone metabolites (Peterman et al., 2006).

Figure 4 shows chromatograms of the MS total ion current and UV absorption (\(\lambda = 254\) nm) for extracts from NADPH-supplemented human liver microsomal incubations with aripiprazole (20 \(\mu M\)). The CID spectrum of aripiprazole (MH\(^+\) = 448) is revealed in Fig. 5. The molecular ions (MH\(^+\)), MS/MS fragmentation pattern and plausible structures of the various aripiprazole metabolites (M12–M19) are depicted in Table 2. The molecular weights of M14 and M12 correspond to those of the 2,3-dichlorophenylpiperazine and the para-hydroxy-2,3-dichlorophenylpiperazine metabolites, which have been characterized previously (Caccia, 2007). The molecular weight of M13 was consistent with a dihydroxylated aripiprazole metabolite. The fragment ions at \(m/z\) 301 and 259 in the CID spectrum of M13 were derived from addition of 16 Da to the fragment ions at \(m/z\) 285 and \(m/z\) 243 in the CID spectrum of aripiprazole. This observation suggested the 2,3-dichlorophenylpiperazine and the dihydroquinolinone ring systems as the sites of hydroxylation. The molecular weight and CID spectrum of metabolite M15 reflected O-dealkylation of the ether linkage in aripiprazole. The molecular weights of metabolites M16 and M17 were consistent with aripiprazole monohydroxylation. Fragment ions at \(m/z\) 301 and \(m/z\) 259 in the CID spectrum of M16 were consistent with monohydroxylation on the 2,3-dichlorophenylpiperazine ring system in the parent compound. In contrast, the observation that the fragment ion at \(m/z\) 285 was retained in the CID spectrum of M17 indicated monohydroxylation on the dihydroquinolinone core. The molecular weight and CID spectrum of M19 were consistent with that previously reported for dehydroaripiprazole (Molden et al., 2006). Finally, metabolite M18 was derived from the monohydroxylation of dehydroaripiprazole M19, and CID data suggested the site of monohydroxylation to be on the dehydroquinolinone motif.

Reactive Metabolite Formation: Nefazodone. Glutathione conjugates. LC-MS/MS analysis of NADPH-supplemented human liver microsomal incubations containing nefazodone and GSH led to the detection of two conjugates, GS1 and GS2, with molecular ions (MH\(^+\)) at 807 [retention time (\(R_t\)) = 25.89 min] and 791 (\(R_t = 28.66\) min), respectively (Fig. 6A). These conjugates were not observed when NADPH and/or GSH were omitted from the incubation mixture. The molecular weights of GS1 and GS2 were consistent with the addition of one molecule of GSH to dihydroxylated and monohydroxylated nefazodone metabolites, respectively. The CID spectrum
### TABLE 1
Molecular ions (MH$^+$) and MS$^2$ fragmentation pathways of nefazodone metabolites in NADPH-supplemented human liver microsomes

<table>
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<tr>
<th>Metabolite</th>
<th>Proposed Structure</th>
<th>MH$^+$ (m/z)</th>
<th>MS$^2$</th>
</tr>
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<tr>
<td>M1</td>
<td><img src="image1.png" alt="M1 Structure" /></td>
<td>213</td>
<td>170 (100%), 134</td>
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<tr>
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<td>290 (100%), 272</td>
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<td>M4</td>
<td><img src="image4.png" alt="M4 Structure" /></td>
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<td>154 (100%), 118</td>
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<tr>
<td>M5</td>
<td><img src="image5.png" alt="M5 Structure" /></td>
<td>374</td>
<td>288 (100%), 246, 168, 140</td>
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<tr>
<td>M6</td>
<td><img src="image6.png" alt="M6 Structure" /></td>
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<td>290 (100%), 262, 253, 225</td>
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<tr>
<td>M7</td>
<td><img src="image7.png" alt="M7 Structure" /></td>
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<tr>
<td>M8</td>
<td><img src="image8.png" alt="M8 Structure" /></td>
<td>502</td>
<td>484, 274 (100%), 246, 241, 223</td>
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</table>
of GS1 (Fig. 6B) displayed diagnostic fragment ions at \( m/z \) 678, 534, and 290. The fragment ion at \( m/z \) 678 corresponded with the loss of the pyroglutamate moiety, which represents a characteristic fragment ion derived from GSH conjugates (Baillie and Davis, 1993). Likewise, the fragment ion at \( m/z \) 534 was assigned as cleavage of the cysteiny1 thioether moiety with the retention of sulfur on the drug molecule. Finally, the presence of the fragment ion at \( m/z \) 290 (addition of 16 Da to the fragment ion at \( m/z \) 274 present in parent nefazodone) indicated monohydroxylation on the \( N^\prime \)-propyl-5-ethyl-2,4-dihydro-4-(2-phenoxymethyl)-triazolone substituent in nefazodone. This observation also

<table>
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<th>Metabolite</th>
<th>Proposed Structure</th>
<th>( MH^+ (m/z) )</th>
<th>( MS^2 )</th>
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<td>M9</td>
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<tr>
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<td><img src="image" alt="M11 Structure" /></td>
<td>486 468, 274 (100%), 246, 225, 207</td>
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</table>

**TABLE 1 (continued)**

**FIG. 4.** Total ion chromatogram (A) and UV spectrum (\( \lambda = 254 \text{ nm} \)) (B) of an incubation mixture containing aripiprazole (10 \( \mu M \)) and NADPH-supplemented human liver microsomes.
suggests that the second hydroxylation site is on the 3-chlorophenylpiperazine ring. Based on this information, the proposed structure for GS1 that fits the CID data is shown in Fig. 6B. The regiochemical assignments for the aromatic and aliphatic hydroxylations in GS1 are based on the previously characterized dihydroxylated metabolite of nefazodone in human liver microsomes (Peterman et al., 2006; Li et al., 2007). Finally, the CID spectrum of GS2, which displayed fragment ions at \( m/z \) 662, 518, 274, and 140, was identical to the CID spectrum of the GSH conjugate of para-hydroxynefazodone (structure 1 in Fig. 1) (Kalugutkar et al., 2005b). As shown in Fig. 1, GS2 is formed via addition of GSH to the reactive quinone-imine metabolite of para-hydroxynefazodone.

**Cyanide conjugates.** Incubations of nefazodone in NADPH-supplemented human liver microsomes in the presence of potassium cyanide led to the detection of several cyano conjugates, CN1–CN6, which were not observed when NADPH and/or cyanide were omitted from the incubation mixture (Fig. 7). These conjugates were also observed in nefazodone incubations with NADPH-supplemented recombinant P4503A4 (data not shown). The molecular ions (\( MH^+ \)) for GS1 are listed in Table 3. Indicated regiochemistry of aromatic and aliphatic hydroxylation is assumed based on the previous structural characterization of the dihydroxynefazodone metabolite (Peterman et al., 2006; Li et al., 2007). The \( MH^+ \) at \( m/z \) 511 for CN5 and CN6 (see Table 3 for structures) was consistent with the addition of one molecule of cyanide (27 Da) to a monohydroxylated nefazodone metabolite. With CN5, the fragment ions at \( m/z \) 274 and 246, which correspond to the phenoxyethyl-5-ethyltriazolone-ethyl moiety, remained intact but the fragment ion at \( m/z \) 194 shifted by 16 Da to \( m/z \) 210, consistent with oxygenation on the 3-chlorophenylpiperazine ring. In contrast, with CN6, the fragment ions at \( m/z \) 274 and 246 shifted by 16 Da to \( m/z \) 290 and 262, respectively, which suggested monoxygenation of the 5-ethyltriazolone substituent.

**Aripiprazole.** GS3 was detected in aripiprazole incubations with recombinant P4503A4 and P4502D6 enzymes, which are involved in aripiprazole metabolism. GS3 was detected in aripiprazole conjugations with recombinant P4503A4 (Fig. 8B) but not P4502D6. The molecular weight of GS3 was consistent with the addition of one molecule of GSH to a monohydroxylated aripiprazole metabolite. The CID spectrum of GS3 (Fig. 8C) displayed diagnostic fragment ions at \( m/z \) 640, 496, 463, 285, and 243. The fragment ion at \( m/z \) 640 and 496 were assigned to the characteristic losses of pyroglutamate and C-S bond cleavage with retention of sulfur on the aripiprazole molecule. The fragment ions at \( m/z \) 285 and 243 suggest that monohydroxylation and GSH conjugation had occurred on the dihydroquinolinone core. Two plausible structures (A and B) of GS3, which are consistent with the CID spectrum, are shown in Fig. 8C. As depicted in Fig. 2, structure A is derived from the aripiprazole bioactivation pathways D and F, whereas structure B is derived from bioactivation pathway C.

The availability of the synthetic standard of dehydroaripiprazole (metabolite M19) allowed additional studies to be conducted, which provided further insight into the structure of GS3. As shown in Fig.
<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Proposed Structure</th>
<th>MH&lt;sup&gt;+&lt;/sup&gt; (m/z)</th>
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<td><img src="image" alt="Structure M12" /></td>
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<td>204, 168 (100%), 133</td>
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<td>M13</td>
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<td>480</td>
<td>462, 301 (100%), 259</td>
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<td><img src="image" alt="Structure M16" /></td>
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<td>301 (100%), 259, 218, 176</td>
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<td>M19</td>
<td><img src="image" alt="Structure M19" /></td>
<td>446</td>
<td>285 (100%), 243, 216</td>
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</table>
9B, LC-MS/MS analysis of an incubation mixture of dehydroaripiprazole in NADPH-supplemented human liver microsomes in the presence of GSH indicated the formation of a GSH conjugate GS4. The observed molecular ion (MH$^+$/H$_1$ at 767 Da and $R_t$ of GS4 were different from that for GS3 (Fig. 9A). The molecular weight of GS4 was 2 Da lower than that of GS3. The CID spectrum of GS4, which is shown in Fig. 9C, displayed diagnostic fragment ions at $m/z$ 638, 494, and 285. The fragment ion at $m/z$ 285 indicated that monohydroxylation and GSH conjugation had occurred on the dehydroquinoline core. The fragment ions at $m/z$ 638 and 494 were assigned to the characteristic losses of pyroglutamate and C-S bond cleavage with retention of sulfur on the aripiprazole molecule. Based on these observations, a structure for GS4, which is consistent with the mass spectrum, is shown in Fig. 9C. Thus, GS4 is a structural analog of GS3 and the formation of these conjugates appears to be derived from hydroxylation para (or ortho) to the quinoline nitrogen atom in aripiprazole (or dehydroaripiprazole) followed by 1) two-electron oxidation to the electrophilic quinone-imine derivatives and 2) Michael addition of GSH to these quinone-imine intermediates. Finally, it is noteworthy to point out that, although NADPH- and GSH-supplemented incubations of dehydroaripiprazole did not give rise to GS3, we did observe a metabolite M20 (MH$^+$ = 480, $R_t$ = 34.78 min) derived from the dihydroxylation of dehydroaripiprazole. The CID of M20 (Fig. 10B) was consistent with dihydroxylation of the olefinic bond within the dehydroquinolinolone moiety.

Cyanide conjugates. Incubations of aripiprazole in NADPH-supplemented human liver microsomes in the presence of potassium cyanide did not reveal the formation of cyanide adducts.

**Inhibitory Effect on P450 Activity in Human Liver Microsomes.** Inhibition studies were repeated with nefazodone, since the present set of experiments used a much lower human liver microsomal concentration of 0.01 mg/ml to minimize nonspecific binding of inhibitor to microsomes (Obach et al., 2007). Our previous studies with nefazodone were conducted with a microsomal concentration of 0.8 mg/ml (Kalgutkar et al., 2005b). Consistent with our previous results, nefazodone was a potent concentration- and time-dependent inhibitor of P4503A4-mediated midazolam-1'-hydroxylase activity in human liver microsomes. The magnitude of enzyme inhibition was higher when the experiment was carried out with preincubation in the presence of NADPH. The IC$_{50}$ value for the time-independent and time-dependent inhibition of P4503A4 activity by nefazodone was 1.5 $\mu$M and 0.015 $\mu$M, respectively. Under the present assay conditions, the IC$_{50}$ value for the time-independent and time-dependent inhibition of P4503A4 activity by nefazodone was 1.5 $\mu$M and 0.015 $\mu$M, respectively. Under the present assay conditions, the IC$_{50}$ value for the time-independent and time-dependent inhibition of P4503A4 activity by nefazodone was 1.5 $\mu$M and 0.015 $\mu$M, respectively. Under the present assay conditions, the IC$_{50}$ value for the time-independent and time-dependent inhibition of P4503A4 activity by nefazodone was 1.5 $\mu$M and 0.015 $\mu$M, respectively. Under the present assay conditions, the IC$_{50}$ value for the time-independent and time-dependent inhibition of P4503A4 activity by nefazodone was 1.5 $\mu$M and 0.015 $\mu$M, respectively. Under the present assay conditions, the IC$_{50}$ value for the time-independent and time-dependent inhibition of P4503A4 activity by nefazodone was 1.5 $\mu$M and 0.015 $\mu$M, respectively.
Discussion

In vitro characterization of reactive quinonoid species derived from the bioactivation of para-hydroxynefazodone, a circulating metabolite of nefazodone in humans, suggests a causative role for reactive metabolites in nefazodone hepatotoxicity (Kalgutkar et al., 2005b). Our present studies on nefazodone metabolism in human liver microsomes and recombinant P4503A4 revealed additional bioactivation pathways involving α-carbon oxidation on the piperazine ring in nefazodone and several of its downstream metabolites, yielding reactive iminium ion intermediates. Argoti et al. (2005) have also reported a similar finding following nefazodone incubations with rat liver microsomes. Overall, the observations that nefazodone bioactivation by P4503A4 results in the mechanism-based inactivation of the isozyme presumably via covalent modification of an active site nucleophile(s) by reactive quinonoid and/or iminium intermediates (Kalgutkar et al., 2005b) suggests a possibility that these metabolites also can covalently modify macromolecules other than P450, some of which may be essential for critical pathophysiology in the liver.

Like nefazodone, P450-mediated metabolism on the 2,3-dichlorophenyl ring in aripiprazole resulted in the formation of the corresponding para-hydroxyaniline toxicophores as metabolites. However, the lack of formation of GSH conjugates of these metabolites suggests
that they do not undergo further two-electron oxidation to electrophilic quinone-imine intermediates in an analogous manner as nefazodone. The absence of N-dearylated aripiprazole metabolites in the microsomal incubations further supports this hypothesis, since, the mechanism of N-dearylation observed with nefazodone is thought to involve hydrolytic cleavage of the reactive quinone-imine species (Fig. 1). Furthermore, unlike the case with nefazodone, we did not detect cyanide conjugates of aripiprazole and its downstream metabolites, which would be consistent with α-carbon oxidation on the piperazine ring to yield reactive iminium ions. Overall, the biochemical basis for the marked differences in the bioactivation profile involving the 3-chlorophenyl- and the 2,3-dichlorophenyl-piperazine ring systems in nefazodone and aripiprazole, respectively, remains unclear at the present time. Whether the presence of the extra chlorine atom in aripiprazole alters the electronic environment (reduces the propensity toward two-electron oxidation) of the hydroxyaniline metabolite(s) needs to tested, experimentally. Toward achieving this goal, we have undertaken the synthesis of a nefazodone analog, which contains the additional chlorine atom on the C-2 position, in order to evaluate the propensity of this analog to form quinone-imine and/or iminium ion intermediates.

Although the 2,3-dichlorophenylpiperazine ring appeared to be latent toward bioactivation, a GSH conjugate (GS3) derived from bioactivation of the dihydroquinolinone motif in aripiprazole was detected. The molecular weight of GS3 suggested the addition of GSH to a monohydroxylated aripiprazole metabolite, and the CID data indicated that this modification had occurred on the dihydroquinolinone nucleus. The formation of GS3 can arise via the two pathways C and D/F as depicted in Fig. 2. The bioactivation pathway C can arise via hydroxylation para (or ortho) to the quinolinone nitrogen atom followed by 1) two-electron oxidation to the electrophilic quinone-imine and 2) Michael addition of GSH to the quinone-imine intermediate. The bioactivation pathway D/F is analogous to the one reported for the dihydroquinolinone-containing inotropic agent toborinone (Kitani et al., 1997) and involves olefin epoxidation of the initially formed dehydroaripiprazole metabolite. Epoxide ring opening by GSH then affords GS3. The finding that NADPH- and GSH-supplemented human liver microsomal incubations of synthetic dehydroaripiprazole metabolite did not yield GS3 and argues against the pathways D/F. Instead, the formation of a new GSH conjugate GS4 with molecular weight 2 Da lower than that of GS3 suggests that both aripiprazole and dehydroaripiprazole succumb to an identical bioactivation pathway. Consequently, on the basis of these findings, we speculate that the formation of GS3 from aripiprazole and GS4 from dehydroaripiprazole arises via the bioactivation pathway shown in Fig. 2, pathway C. Within this context, it is interesting that P450-mediated oxidation of dehydroaripiprazole in human liver microsomes did result in the formation of a dihydroxylated metabolite M20 with mass spectral properties indicative of olefin dihydroxylation.

![Figure 8](https://example.com/figure8.png)

**Fig. 8.** Extracted ion chromatogram of an incubation mixture containing aripiprazole (100 μM) and NADPH-supplemented human liver microsomes (A) or recombinant P4503A4 (B) in the presence of GSH (5 mM). C, CID spectrum of the MH+ ion (m/z 769) of GS3 (Rt = 28.07 min). The structures depicted represent one possible regiosomer and is for illustrative purposes only.
tion. If so, this finding is consistent with olefin epoxidation in dehydroaripiprazole followed by hydrolysis to yield the diol M20. Why the epoxide was not trapped by GSH remains unclear at the present time. Overall, these results constitute the first report on the P450-catalyzed bioactivation of aripiprazole. Besides literature reports on the involvement of P4503A4 in the metabolism of aripiprazole in humans, our studies also demonstrated a key role for the enzyme in the bioactivation of this drug. Although both P4503A4 and P4502D6 are thought to be involved in aripiprazole metabolism (Caccia, 2007), we did not observe the formation of GS3 in aripiprazole incubations with recombinant P4502D6. A plausible explanation for this finding may be that only P4503A4 is capable of catalyzing aripiprazole bioactivation involving aromatic hydroxylation on the quinolinone moiety. Given that a bioactivation pathway was detected with aripiprazole (albeit different from the one in nefazodone), we decided to compare the time-dependent P450 inhibitory potencies of nefazodone and aripiprazole. Consistent with our previous findings, the potency of P4503A4 inhibition by nefazodone significantly increased with time (~1000-fold increase based on the IC50 values of 15 and 0.015 μM for time-independent and time-dependent inhibition). The potent time- and concentration-dependent inactivation of P4503A4 activity by nefazodone is consistent with the numerous examples of pharmacokinetic interactions of nefazodone with P4503A4 substrates and with the nonstationary pharmacokinetics of this drug due to autoinactivation of its elimination mechanism (Greene and Barbhaiya, 1997). In contrast, aripiprazole did not display the marked time-dependent differences in P4503A4 and P4502D6 inhibitory potencies. The finding that aripiprazole is not a P4503A4 inactivator despite undergoing bioactivation suggests that the reactive metabolite escapes the P4503A4 active site prior to the inactivation step. Alternately, it is likely that the area where the dihydroquinolinone ring resides in the P4503A4 active site lacks a nucleophilic amino acid residue(s). Despite some reversible inhibition of P4503A4 and P4502D6 activities observed in our studies, there are no reports on clinical drug-drug interactions between aripiprazole and P4503A4/P4502D6 substrates (aripiprazole package insert). The most likely explanation for this discrepancy is that the IC50 values for P450 isozyme inhibition by aripiprazole are significantly greater than the free maximal plasma concentrations of 2.2 to 8.8 nM in humans after daily oral administration of the drug for 14 days at a dose range of 5 to 20 mg (Mallikaarjun et al., 2004). Our findings on the P4503A4-mediated bioactivation of nefazodone and aripiprazole in human liver microsomal incubations represent a classical assessment of bioactivation potential of xenobiotics and drugs. Often, such a finding can be interpreted as being a harbinger of a potential toxicological response in the clinic. Clearly, the hepatotoxic ADRs associated with nefazodone support this notion. But this is not the case with aripiprazole since, despite the bioactivation liability and the fact that both drugs are intended for chronic use, there have been no reports of idiosyncratic hepatotoxicity associated with this drug. Plausible explanations for this discrepancy may be accounted for by the differences in dose levels and the pharmacokinetics of the two drugs. The absolute oral bioavailability of nefazodone (Greene and Barbhaiya, 1997) and aripiprazole (aripiprazole metabolism data submitted by the manufacturer of the drug to the FDA) in humans has been estimated to be ~20 and 87%, respectively.
The low oral bioavailability of nefazodone stems from extensive first pass metabolism mediated by P4503A4 in the small intestine and liver. The total clearance and volume of distribution at steady state of nefazodone following intravenous administration to humans is 7.5 ml/min/kg (hepatic extraction of 40% based on a human hepatic blood flow of 21 ml/min/kg) and 0.51 l/kg, which translates into a short half-life of ~1 h (Barbhaiya et al., 1996). In contrast, the terminal half-life of aripiprazole is very long, averaging 75 h, and is a consequence of a significantly lower total clearance (0.8 ml/min/kg, hepatic extraction of < 5%) and increased volume of distribution at steady state (5 l/kg) (aripiprazole metabolism data submitted by the manufacturer of the drug to the FDA).

Given the longer half-life of aripiprazole compared with nefazodone, the daily dose is ~10- to 30-fold lower (5-20 mg once per day) than the daily dose of nefazodone (200-400 mg once per day). The lower daily dose and the lesser affinity toward metabolism/bioactivation likely reduce the total body burden to reactive metabolite exposure upon aripiprazole administration as compared with that following nefazodone administration. There are several examples (clozapine versus olanzapine and troglitazone versus rosiglitazone/pioglitazone) of structurally analogous drugs that display differential safety profiles despite forming reactive metabolites. In all of these cases, the drugs that are associated with idiosyncratic ADRs are dosed at considerably greater levels. For example, clozapine is dosed at 200 mg/day compared with the 10 mg/day dose of olanzapine. Overall, the studies described herein provide yet another example that in vitro bioactivation of a drug (or drug candidate) cannot necessarily be assumed to be predictive of toxicity. It is important to place results from in vitro bioactivation studies within the proper context of overall factors (e.g., indication, acute versus chronic therapy, dose size, and regimen) when making a final decision on whether to proceed with the development of the drug.

**References**


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