BIOACTIVATION OF THE NONTRICYCLIC ANTIDEPRESSANT NEFAZODONE TO A REACTIVE QUINONE-IMINE SPECIES IN HUMAN LIVER MICROSOMES AND RECOMBINANT CYTOCHROME P450 3A4

Amit S. Kalgutkar, Alfin D. N. Vaz, Mary E. Lame, Kirk R. Henne, John Soglia, Sabrina X. Zhao, Yuri A. Abramov, Franco Lombardo, Claire Collin, Zachary S. Hendsch, and Cornelis E. C. A. Hop


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ABSTRACT:
The therapeutic benefits of the antidepressant nefazodone have been hampered by several cases of acute hepatotoxicity/liver failure. Although the mechanism of hepatotoxicity remains unknown, it is possible that reactive metabolites of nefazodone play a causative role. Studies were initiated to determine whether nefazodone undergoes bioactivation in human liver microsomes to electrophilic intermediates. Following incubation of nefazodone with microsomes or recombinant P450 3A4 in the presence of sulfhydryl nucleophiles, conjugates derived from the addition of thiol to a monohydroxylated nefazodone metabolite were observed. Product ion spectra suggested that hydroxylation and sulfhydryl conjugation occurred on the 3-chlorophenylpiperazine-ring, consistent with a bioactivation pathway involving initial formation of p-hydroxynefazodone, followed by its two-electron oxidation to the reactive quinone-imine intermediate. The formation of novel N-dearylated nefazodone metabolites was also discernible in these incubations, and 2-chloro-1,4-benzoquinone, a by-product of N-dearylation, was trapped with glutathione to afford the corresponding hydroquinone-sulfhydryl adduct. Nefazodone also displayed NADPH-, time-, and concentration-dependent inactivation of P450 3A4 activity, suggesting that reactive metabolites derived from nefazodone bioactivation are capable of covalently modifying P450 3A4. A causative role for 2-chloro-1,4-benzoquinone and/or the quinone-imine intermediate(s) in nefazodone hepatotoxicity is speculated. Although the antianxiety agent buspirone, which contains a pyrimidine ring in place of the 3-chlorophenyl-ring, also generated p-hydroxybuspirone in liver microsomes, no sulfhydryl conjugates of this metabolite were observed. This finding is consistent with the proposal that two-electron oxidation of p-hydroxybuspirone to the corresponding quinone-imine is less favorable due to differences in the protonation state at physiological pH and due to weaker resonance stabilization of the oxidation products as predicted from ab initio measurements.

2-[3-[4-(3-Chlorophenyl)-1-piperazinyl]propyl-5-ethyl-2,4-dihydro-4-[2-phenoxyethyl]-3H-1,2,4-triazol-3-one (nefazodone) (Fig. 1) was approved as an antidepressant in the United States in late 1994. Nefazodone is structurally distinct from selective serotonin reuptake inhibitors, tri- and tetracyclics, and monoamine oxidase inhibitors. Nefazodone derives its clinical efficacy by inhibiting serotonin and norepinephrine reuptake and by antagonizing the postsynaptic 5-hydroxytryptamine 2-receptor and α1-adrenergic receptors (Eison et al., 1990). However, despite its therapeutic benefits, there are several reported cases of idiosyncratic adverse reactions including hepatobiliary dysfunction and cholestasis that are associated with the use of this drug (Aranda-Michel et al., 1999; Lucena et al., 1999; García-Pando et al., 2002; Stewart, 2002; Andrade and Lucena, 2003; Choi, 2003). The U.S. Food and Drug Administration has received reports of at least 55 cases of liver failure, including 20 deaths, and another 39 cases of less severe liver injury since nefazodone sales commenced in 1994. Although the exact cause of hepatotoxicity by nefazodone is currently unknown, a probable causal link between nefazodone use and hepatic injury has been established based on the temporal relationship between nefazodone administration and the onset of hepatotoxicity in most cases. Furthermore, liver biopsy specimens from a patient who succumbed to the hepatic injury revealed marked centrozonal necrosis, which is consistent with a toxic etiology (García-Pando et al., 2002).

The principal clearance mechanism of nefazodone in humans involves intestinal and hepatic metabolism catalyzed by cytochrome P450 3A4 (Mayol et al., 1994; Greene and Barbhaiya, 1997; von Moltke et al., 1999; Rotzinger and Baker, 2002). Of particular interest...
in the many biotransformation pathways of nefazodone is the aromatic hydroxylation, which occurs para to the piperazinyl nitrogen affording p-hydroxynefazodone (metabolite 1) (Fig. 1), which has been detected as a circulating metabolite in plasma following oral administration to humans and in vitro microsomal incubations (Kaul et al., 1995; von Moltke et al., 1999). In addition, 1-(m-chlorophenyl)piperazine (m-CPP), which results from the N-dealkylation of nefazodone (Mayol et al., 1994; von Moltke et al., 1999; Rotzinger and Baker, 2002), undergoes further P450 2D6-mediated oxidation, yielding p-hydroxy-m-CPP (metabolite 2) (see Fig. 1) (Rotzinger et al., 1998). We postulated that metabolites 1 and 2 are oxidatively bioactivated to the corresponding quinone-imine species 3 and 4, respectively (Fig. 1), and that these reactive species may play a role in nefazodone-induced hepatic necrosis. It is noteworthy to point out that, like nefazodone, the antianxiety drug 8-[4-[4-(2-pyrimidinyl)piperazinyl]butyl]-8-azaspiro[4,5]-decane-7,9-dione (buspirone) also undergoes a P450 3A4-catalyzed aromatic hydroxylation that occurs para to the piperazinyl nitrogen leading to p-hydroxybuspirone (metabolite 5) (Fig. 1) (Gammans et al., 1986; Jajoo et al., 1989; Kivisto et al., 1997). Furthermore, buspirone-N-dealkylation generates the corresponding 1-(2-pyrimidinyl)piperazine (1-PP) metabolite (Mayol et al., 1985; Caccia et al., 1986), which upon further oxidation furnishes p-hydroxy-1-PP (metabolite 6) (Fig. 1) (Jajoo et al., 1989; von Moltke et al., 1998). These hydroxylated metabolites also can undergo further two-electron oxidation to generate an electrophilic quinone-imine intermediate (e.g., intermediate 7). However, despite the similarity in oxidative biotransformation pathways of nefazodone and buspirone, there are no documented reports on idiosyncratic hepatotoxicity associated with buspirone use in the clinic.

Consequently, we examined the ability of nefazodone and buspirone and their respective metabolites m-CPP and 1-PP to undergo oxidative bioactivation in human liver microsomes and recombinant P450 enzymes. The studies reported here provide evidence that the p-hydroxylated metabolite of nefazodone, but not of buspirone, is indeed metabolically activated in vitro and that this activation is catalyzed by P450 3A4.

**Materials and Methods**

**Chemicals.** Nefazodone hydrochloride, buspirone hydrochloride, 1-PP, 2-chloro-1,4-benzoquinone, NADPH, reduced glutathione (GSH), and reduced glutathione ethyl ester (GSH-EE) were purchased from Sigma-Aldrich (St. Louis, MO). m-CPP hydrochloride was purchased from Avocado Research Chemicals Ltd. (Lancashire, UK). Recombinant human P450 3A4 and 2D6 isoforms, coexpressed with NADPH-P450 oxidoreductase in baculovirus-insect cells, were purchased from BD Gentest (Woburn, MA).

**Human Liver Microsomal Preparations.** Microsomal fractions were prepared from human livers (International Institute for the Advancement of Medicine, Jessup, PA) using standard protocols. Protein concentrations were determined using the bicinchoninic acid assay method (Pierce Chemical, Rockford, IL). Total P450 content was measured according to published protocols (Omura and Sato, 1964), and human liver microsomes were characterized using P450-specific marker substrate activities. Human liver microsomes were isolated from 56 individual livers, and aliquots from the individual preparations were pooled on the basis of equivalent protein concentrations to yield a representative microsomal pool.

**Microsomal Incubations.** Stock solutions of the test compounds were prepared in methanol. The final concentration of methanol in the incubation media 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), human liver microsomes (P450 concentration = 0.5 μM) or recombinant enzymes (25 pmol of P450 1A2, P450 2D6, P450 2C9, P450 3A4, or P450 2C19), NADPH (1.2 mM), and substrate (20 μM). The reaction mixture was prewarmed at 37°C for 2 min before adding NADPH. GSH (5 mM) or GSH-EE (5 mM) was added 3 min after initiation of the reaction with NADPH. Incubations that lacked either NADPH or GSH/GSH-EE served as negative controls, and reactions were terminated by the addition of ice-cold acetonitrile (1 ml). The solutions were centrifuged (3000g, 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).

**Characterization of the GSH-EE Conjugate(s) of 2-Chloro-1,4-benzoquinone.** Human liver microsomes (P450 concentration = 0.3 μM) containing nefazodone (100 μM), GSH-EE (1 mM), and NADPH (1.2 mM) in 100 mM potassium phosphate buffer (pH 7.4) were incubated for 30 min at 37°C. The final incubation volume was 250 μl. Samples without either NADPH or nefazodone were used as negative controls. Incubations of synthetic 2-chloro-
1.4-benzoquinone (100 μM) and GSH-EE (1 mM) in 100 mM potassium phosphate buffer (pH 7.4) were also conducted for 30 min at 37°C in the absence or presence of microsomes containing NADPH. The reactions were quenched by the addition of acetoneitrile (375 μL) and after centrifugation (3000g, 15 min), the supernatant was concentrated under a steady nitrogen stream. Sulfydryl conjugates were extracted from each sample using a Waters Oasis HLB 96-well microextraction solid phase extraction plate (Waters, Milford, MA). A 384-channel manual-150 pipettor fitted with a 96-channel head (Apricot Designs Inc., Monrovia, CA) was used during solid phase extraction (SPE) to facilitate solvent transfer. The SPE plate was conditioned by passing methanol (200 μL), followed by water (200 μL), through each of the SPE cartridges. To initiate solvent flow through the absorbent, vacuum was applied to the receiving side of the SPE plate using a 96-well extraction manifold (Tomtec, Orange, CT). Samples were added to the 96-well plate and washed with water (200 μL). Analyte was desorbed using 40:60 (v/v%) acetoneitrile/isopropanol (100 μL). The desorption solvent was collected in a 96-well polypropylene plate. Solvent was evaporated using a 96-channel Evaporlex EVX-192 evaporator (Apricot Designs Inc.) utilizing nitrogen as a drying gas. Samples were reconstituted with 5:95 (v/v%) acetoneitrile/water/formic acid, 5 mM ammonium formate (50 μL) before analysis.

P450 3A4 Inhibition Studies in Human Liver Microsomes. For non-preincubation (time-independent) P450 3A4 inhibition, a pooled human liver microsomal preparation (P450 concentration = 0.25 μM) was incubated with midazolam (3 μM) and nefazodone at concentrations ranging from 0.5 to 50 μM at 37°C for 5 min in the presence of NADPH (1.2 mM). For preincubation-dependent (time-dependent) inhibition, microsomes (P450 concentration = 0.25 μM) were incubated with nefazodone at concentrations ranging from 0.5 to 50 μM in the presence of NADPH (1.2 μM) for 30 min at 37°C, after which incubation with midazolam (3 μM) was carried out for 5 min. To determine whether the preincubation-dependent inhibition also was NADPH-dependent, a third incubation in which NADPH was absent during the preincubation period was carried out. Incubations were quenched by the addition of acetoneitrile containing a proprietary internal standard (0.2 μL) before analysis. The formation of 1'-hydroxymidazolam was determined by LC/MS/MS. Inhibition studies were conducted in triplicate.

Computational Methods. Ab initio calculations were performed using the Gaussian 03 quantum chemistry package (Gaussian Inc., Wallingford, CT). The geometry of N-methyl-1-(3-chloro-4-hydroxyphenyl)piperazine and N-methyl-1-(4-hydroxypropyrimidyl)piperazine motifs was optimized at the B3LYP/6-31G* level of theory in vacuum. Single-point energy calculations were performed using the Gaussian 03 quantum chemistry package (Gaussian Inc., Wallingford, CT). Samples were reconstituted with 5:95 (v/v%) acetoneitrile/water/formic acid, 5 mM ammonium formate (50 μL) before analysis.

LC/MS/MS Assay for 1'-Hydroxymidazolam Analysis. 1'-Hydroxymidazolam formation was assessed on a Micromass Quattro Ultima (Waters) triple quadrupole mass spectrometer in line with a Hewlett Packard Series 1100 HPLC system (Hewlett Packard, Palo Alto, CA). Analytes were chromatographed on a Phenomenex Primesep 5-μm C18-HC 30 × 2.0 mm column (Phenomenex, Torrance, CA) using a 2-min gradient consisting of a mixture of 95% water/5% acetonitrile with 0.1% formic acid (solvent A) and acetonitrile/ammonium formate (5 mM)/formic acid (80:20:0.05, v/v) (solvent B) at a flow rate of 50 μL/min. The LC gradient was programmed as follows. Solvent A to solvent B ratio was held at 100:0 (v/v) for 3 min and then adjusted to 90:10 (v/v) from 3 min and from 10:90 (v/v) to 100:0 (v/v) from 35 to 40 min. The column was re-equilibrated for 6 min before the next analytical run. Postcolumn flow was split such that mobile phase was introduced into the mass spectrometer via an electrospray interface at a rate of 50 μL/min. The remaining flow was diverted to the PDA detector to provide simultaneous UV detection (λ = 240–260 nm) and total ion chromatogram. The capillary temperature was 250°C, and CID analyses were performed using a collision energy of 30 eV. Sulfydryl conjugates of nefazodone were identified in the full scan mode (from m/z 100 to 850) by comparing t = 0 samples with t = 60-min samples (with or without cofactor), and structural information was generated from the CID spectra of the corresponding molecular ions (MH+ and/or comparison with synthetic standards).

LC/MS/MS Assay for GSH and GSH-EE Conjugate Formation. Metabolites were identified by electrospray LC/MS/MS using a Finnigan TSQ Quantum Ultra mass spectrometer (Thermo Finnigan) in conjunction with a Finnigan Surveyor LC system comprising a high-performance liquid chromatograph with a built-in degasser, autosampler, UV detector, and a Finnigan LCQ-Classic ion trap mass spectrometer (Thermo Finnigan, San Jose, CA). The UV detector was set to scan between 240 and 260 nm, the mass spectrometer was auto-tuned with nefazodone in the positive ion mode, and data-dependent scans were acquired up to MS2 with dynamic exclusion parameters set to 5 scans and an exclusion time limit of 0.5 min. A constant flow rate of 1.0 mL/min was used. Samples were injected on the column equilibrated in 10% acetonitrile/90% 5 mM ammonium formate (pH 3.0) and held for 5 min under these conditions. The acetonitrile concentration was then ramped linearly at 1.33%/min up to 90% and held at this condition for 5 min, and then returned to the original conditions. Nefazodone and its identifiable metabolites eluted in the first 30 min. Metabolites were identified 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), and structural information was generated from the CID spectra of the corresponding molecular ions (MH+ and/or comparison with synthetic standards).

Characterization of the GSH-EE Conjugate of 2-Chloro-1,4-benzoquinone by MicroESI. A capillary liquid chromatography system (Dionex Corp., Sunnyvale, CA) was used for the analysis. The autosampler module was configured to inject samples from 96-well plates. Chromatography was performed in a Vydac 300 μm i.d. × 5 cm C8 column that contained 5-μm particles with a pore size of 300 Å (Grace Vydac, Hesperia, CA). GSH-EE conjugates were chromatographed using a binary mobile phase consisting of acetoneitrile/ammonium formate (5 mM)/formic acid (10:90:0.05, v/v) (solvent A) and acetoneitrile/ammonium formate (5 mM)/formic acid (80:20:0.05, v/v) (solvent B) at a flow rate of 5 μL/min. Following injection of 0.6 μL of sample, a starting mobile phase composition of 90% solvent system A/10% solvent system B was increased linearly to 40% solvent system A/60% solvent system B from 0 to 5 min. From 5 to 10 min, the percentage of solvent system B was held isocratic. At 10 min, the solvent system was returned to 90% solvent
Results

Metabolite Identification Studies. Nefazodone. Figure 2 shows chromatograms of the MS total ion current and UV absorption (λ = 240–260 nm) for extracts from NADPH-supplemented human liver microsomal incubations with nefazodone (20 μM). Consistent with previous reports on nefazodone metabolism in human liver microsomes (von Moltke et al., 1999) and in vivo (Mayol et al., 1994), product ion spectra of the monohydroxylated metabolites established the 3-chlorophenylpiperazine motif or the ethyl group on the 5-ethyltriazol-3-one moiety (Fig. 2). The origin of the characteristic ions is as indicated.

Additional metabolites derived from nefazodone N-dealkylation, including 5-ethyl-2-(3-hydroxypropyl)-4-(2-phenoxyethyl)-2,4-dihydrotriazol-3-one (Rt = 21.50 min; Fig. 2) and its oxidation product 3-(3-ethyl-5-oxo-4-(2-phenoxyethyl)-4,5-dihydrotriazol-1-propionic acid (Rt = 18.57 min; Fig. 2), were also detected in the incubates as previously reported (Mayol et al., 1994). Of interest to the present studies was the detection of I (MH+ = 486, R = 24.84 min), 3-CPP (MH+ = 197, R = 9.35 min), and 2 (MH+ = 213, R = 2.85 min). The product ion spectrum obtained by CID of the molecular ion (MH+) of I is displayed in Fig. 3. The R and product ion spectrum of m-CPP was identical to that observed with the synthetic standard, whereas the product ion spectrum of 2 was consistent with that reported in the literature (Mayol et al., 1994).

Of considerable interest were our current observations on the formation of three novel nefazodone metabolites 8, 9, and 10 that eluted at R = 8.87 min, 10.63 min, and 4.03 min, respectively (see Fig. 2). The characteristic chlorine isotope pattern observed with the parent compound was missing in the three metabolites. Metabolite 8 displayed a protonated molecular ion (MH+) at 360, 110 mass units lower than the MH+ for nefazodone. The formation of 8 was not observed in control incubations lacking NADPH, implicating the involvement of P450 in its formation. The product ion spectrum (Fig. 4) obtained by CID of the molecular ion (MH+) for 8 indicated fragment ions at m/z 274, 246, and 140 that corresponded with the 5-ethyltriazol-3-one and the 5-ethyltriazolone propyl moiety. Based on the CID data, the metabolite 8 was tentatively identified as the product of N-dearylation of the 3-chlorophenyl-ring in nefazodone (see Fig. 4). Metabolite 9 displayed a protonated molecular ion (MH+) at 374, 96 mass units lower than the MH+ for nefazodone and 14 mass units higher than the MH+ for 8. This observation suggested that 9 was derived from the further oxidation of 8. The product ion spectrum obtained by CID of the molecular ion (MH+) for 9 indicated fragment ions at m/z 274, 246, and 140.
ions at \( m/z \) 288, 260, 246, 168, and 140 (Fig. 5). The fragment ion at \( m/z \) 288 was consistent with the addition of 14 mass units to the fragment ion at \( m/z \) 274 observed in the product ion spectra of 8. This fragment ion indicated that the piperazine ring in 9 was unaltered. The fragment ions 246 and 140 corresponded with the phenoxethyl-5-ethyltriazolone-ethyl moiety, implying that this region of the molecule remained unchanged. Based on these observations, metabolite 9 was tentatively identified as the amide metabolite obtained by the further oxidation of 8 (see Fig. 5). Metabolite 10 displayed a protonated molecular ion \((\text{MH}^+)\) at 376, 94 mass units lower than the \( \text{MH}^+ \) for nefazodone and 16 mass units higher than the \( \text{MH}^+ \) for 8. This observation suggested that 10 was a monohydroxylated derivative of 8. The presence of the fragment ion at \( m/z \) 290 in the product ion spectrum of 10 suggested that the piperazine ring was not the site of monohydroxylation (Fig. 6). The detection of fragment ions at \( m/z \) 262 and 170 was consistent with monohydroxylation on the 5-ethyltriazolone substituent. Addition of \( \text{TiCl}_3 \) to the microsomal incubation did not lead to the disappearance of the metabolite signal, suggesting that 10 was not derived from \( N \)-oxidation of the triazolone nitrogen. Therefore, we proposed hydroxylation of the ethyl group in 8 as the probable structure for 10 (see Fig. 6).

Separate incubations of nefazodone in recombinant P450 isozymes including P450 1A2, 2C9, 2C19, 2D6, and 3A4 revealed that only P450 3A4 catalyzed the oxidative metabolism of buspirone. Consistent with previous reports on buspirone metabolism in humans (Gammans et al., 1986; Jajoo et al., 1989), metabolite identification studies in NADPH-supplemented human liver microsomes indicated the presence of \( N \)-dealkylated and several mono- and dihydroxylated metabolites of the parent compound. A full scan total ion chromatogram and UV \((\lambda = 240–260 \text{ nm})\) of an NADPH-supplemented human liver microsomal incubation with buspirone (20 \( \mu \text{M} \)) is depicted in Fig. 7. The \( R_t \) and product ion spectrum of I-PP \((\text{MH}^+ = 165, R_t = 10.04 \text{ min})\) and 6 \((\text{MH}^+ = 181, R_t = 8.43 \text{ min})\) were identical to those of their respective synthetic standards. The metabolite at \( R_t = 17.89 \text{ min} \) \((\text{MH}^+ = 402)\) was tentatively identified as 5 on the basis of the following observations. First, the diagnostic fragment ions in the mass spectrum at \( m/z \) 265 and 222 that corresponded with the butylazaspirodecaneidene substituent implied that this region of the molecule was unaltered. Furthermore, the fragment ion at \( m/z \) 138, which was indicative of the addition of 16 mass units to the fragment ion at \( m/z \) 122 (corresponding with the pyrimidinyl piperazine ring cleavage fragment) observed in the mass spectrum of buspirone and 6, was consistent with hydroxylation on the pyrimidinyl ring system. Finally, the assignment of the \( \text{para} \) regiochemistry for pyrimidine ring hydroxylation was assumed based on the unambiguous identification of 5 and several of its downstream metabolites in humans (Gammans et al., 1986; Jajoo et al., 1989). Individual incubations of buspirone in several recombinant P450 isozymes indicated that only P4503A4 catalyzed the oxidative metabolism of buspirone (data not shown). The role of P4503A4 in buspirone metabolism is
consistent with the in vivo observations on the marked increases in buspirone plasma concentrations in humans pretreated with selective P4503A4 inhibitors including grapefruit juice, erythromycin, and itraconazole (Kivisto et al., 1997; Lilja et al., 1998).

**Formation of GSH and GSH-EE Conjugates.** Reduced GSH and a close analog of GSH, reduced GSH-EE, were used as exogenous nucleophiles in human liver microsomal incubations to trap reactive metabolites of the test compounds. Reduced GSH-EE was also chosen based on the recent report (Soglia et al., 2004) on the improved sensitivity in conjugate detection with the ethyl ester derivative of GSH relative to the free carboxylic acid analog.

**Nefazodone.** LC/MS/MS analysis of NADPH-supplemented human liver microsomal incubations containing nefazodone and GSH led to the detection of a single conjugate, 11 (tR = 16.9 min), with a molecular ion [(M+H)+] at 791 (Fig. 8A). The molecular weight of this conjugate was consistent with the addition of GSH to a monohydroxylated nefazodone metabolite. The product ion spectrum obtained by CID of the [M+H]+ ion at m/z 791 produced fragment ions at m/z 662, 518,
274, and 140 (Fig. 8B). The presence of the fragment ions at m/z 274 and 140 established that the N-propyl-5-ethyl-2,4-dihydro-4-(2-phenoxethyl)-triazolone substituent in nefazodone was unaltered (see Fig. 8B), indicating that hydroxylation and subsequent GSH conjugation had occurred on the 3-chlorophenylpiperazine-ring system. The fragment ion at m/z 662 corresponded with the loss of the pyroglutamate moiety, which represents a characteristic fragment ion derived from GSH conjugates (Baillie and Davis, 1993). The fragment ion at m/z 518 was assigned as a cleavage adjacent to the cysteinyl thioether moiety with charge retention on the hydroxynefazodone residue. The occurrence of the fragment ion at m/z 518 is consistent with the presence of an aromatic thioether motif in 11 (Baillie and Davis, 1993). A proposed structure for 11 that is consistent with the observed mass spectrum is shown in Fig. 8. The assignment of the para regiochemistry for aromatic hydroxylation in 11 is based upon the report by von Moltkc et al. (1999), which demonstrated the exclusive formation of 1 from nefazodone in human liver microsomes. The corresponding ortho- and meta-isomers were not detected.

When GSH was replaced with GSH-EE in the microsomal incubations, conjugate 12 eluting at R_t = 18.1 min (MH^+ = 819) was observed (Fig. 9A). The molecular weight of 12 was once again consistent with the addition of GSH-EE to a monohydroxylated nefazodone metabolite. The product ion spectrum obtained by CID of the MH^+ for 12 produced fragment ions at m/z 690, 518, 274, and 140 (Fig. 9B). The fragment ions at m/z 274 and 140 established that hydroxylation and subsequent GSH conjugation had occurred on the 3-chlorophenylpiperazine-ring system (Fig. 9B). Based on this information and the characteristic fragment ion at m/z 690 (loss of pyroglutamate) and at m/z 518 (aromatic thioether bond cleavage), the proposed structure for 12, analogous to 11, is depicted in Fig. 9. Furthermore, GSH and GSH-EE conjugates 11 and 12 of 1 were only formed in incubation mixtures containing recombinant P450 3A4. The formation of 11 and 12 was not observed in incubations containing recombinant P450 1A2, 2C9, 2C19, and 2D6.

Buspirone. LC/MS/MS analysis of NADPH-supplemented human liver microsomal or recombinant P450 3A4 incubations of buspirone and GSH or GSH-EE in the full scan or in the MRM mode (5-GSH conjugate, m/z 707→578; 5-GSH-EE conjugate, m/z 735→606; loss of the pyroglutamate moiety) did not indicate the formation of GSH and/or GSH-EE conjugates of 5.

m-CCP and 1-PP. LC/MS/MS analysis of NADPH-supplemented human liver microsomal incubations of m-CCP or 1-PP and GSH or GSH-EE in the full scan or MRM mode (2-GSH conjugate: m/z 518→389; 2-GSH-EE conjugate, m/z 546→417; 6-GSH conjugate, m/z 486→357; 6-GSH-EE conjugate, m/z 514→385; loss of the pyroglutamate moiety) did not indicate the formation of GSH and/or GSH-EE conjugates of 2 and 6, respectively. Likewise, no conjugates were detected when m-CP and 1-PP were incubated with recombinant P450 2D6, the enzyme primarily responsible for the para aromatic hydroxylation of m-CP and 1-PP. Evidence for the Formation of 2-Chloro-1,4-benzoquinone in Liver Microsomal Incubations of Nefazodone: Characterization of the Sulfydryl Conjugate(s) of 2-Chloro-1,4-benzoquinone. The

FIG. 8. Representative LC/MS/MS chromatogram (A) and product ion spectrum (B) obtained by CID of the MH^+ ion (m/z 791) of 11 (R_t = 16.9 min) in NADPH-supplemented human liver microsomal incubations. The origins of the characteristic ions are as indicated.

FIG. 9. Representative LC/MS/MS chromatogram (A) and product ion spectrum (B) obtained by CID of the MH^+ ion (m/z 819) of 12 (R_t = 18.1 min) in NADPH-supplemented human liver microsomal incubations. The origins of the characteristic ions are as indicated.

FIG. 10. Representative LC/MS/MS analysis of a solid phase-extracted sample following incubation of nefazodone (100 μM) in NADPH-supplemented human liver microsomes in the presence of GSH-EE (1 mM) (A), incubation of 2-chloro-1,4-benzoquinone (100 μM) with GSH-EE in phosphate buffer (B), and incubation of 2-chloro-1,4-benzoquinone (100 μM) in NADPH-supplemented human liver microsomes in the presence of GSH-EE (1 mM) (C).
the possibility that N-dearylation of nefazodone results in the liberation of the electrophilic 2-chloro-1,4-benzoquinone was also examined in NADPH-supplemented human liver microsomes containing nefazodone (100 μM) and GSH-EE (1 mM). LC/MS/MS analysis using the anticipated mass transition (m/z 478→m/z 349) that corresponds to the loss of the pyroglutamate moiety from the GSH-EE conjugate of 2-chloro-1,4-benzoquinone (compound 15) led to the detection of a prominent peak at \( R_t = 7.21 \) min (Fig. 10A). This peak was also observed upon nonenzymatic reaction of commercially available 2-chloro-1,4-benzoquinone (100 μM) with GSH-EE (1 mM) in phosphate buffer (Fig. 10B) and upon incubating 2-chloro-1,4-benzoquinone (100 μM) and GSH-EE (1 mM) in the presence of NADPH-supplemented human liver microsomes (Fig. 10C). The product ion spectrum obtained by CID of the MH\(^+\) ion at 478 in each of these cases produced identical fragment ions at m/z 375, 349, 218, and 158 (Fig. 11). The proposed structure of 15, consistent with the observed fragment ions, is also depicted in Fig. 11. It is noteworthy to point out that the product ion spectra of the additional peaks at 7.21 min (Fig. 10A) from NADPH-supplemented human liver microsomal incubations of 2-chloro-1,4-benzoquinone (100 μM) and GSH-EE (1 mM) (A), and incubation of 2-chloro-1,4-benzoquinone (100 μM) in NADPH-supplemented human liver microsomes in the presence of GSH-EE (1 mM) (C). The origins of the characteristic ions are as indicated.

**Inhibitory Effect of Nefazodone on Human Liver Microsomal Midazolam-1'-Hydroxylase Activity.** Figure 12 shows the effect of varying concentrations of nefazodone on P450 3A4-mediated midazolam-1’-hydroxylation in human liver microsomes both with and without preincubation with microsomes with the antidepressant. 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 inhibition of P450 3A4 activity by nefazodone was 45.5 ± 12.8 μM, whereas the IC\(_{50}\) value for the time-dependent inhibition of P450 3A4 activity by nefazodone was 3.21 ± 0.90 μM. When NADPH was omitted from the preincubation inhibition studies, the IC\(_{50}\) value for the time-independent inhibition of P450 3A4 activity by nefazodone was 42.4 ± 19.6 μM. These data suggested that nefazodone is an NADPH- and time-dependent inhibitor of P450 3A4 activity in human liver microsomes.

**Ab Initio Predictions of the Relative Oxidative Stability of N-Methyl-1-(3-chloro-4-hydroxy)phenylpiperazine and N-Meth-yl-1-(4-hydroxy pyrimidinyl)piperazine.** Ab initio quantum chemical calculations were performed to estimate the relative oxidative stability of the (3-chloro-4-hydroxy)phenylpiperazine and the 1-(4-hydroxy-2-pyrimidinyl)piperazine ring system in nefazodone and buspirone, respectively. For the purpose of simplicity, the analysis was performed on the corresponding N-methyl derivatives of p-hydroxy-m-CPP and p-hydroxy-1-PP. Furthermore, considering that the reported pK\(_{a}\) values of trazodone [a nefazodone analog that also contains the 1-(3-chlorophenyl)piperazine ring] and buspirone are 6.13 ± 0.04 (Ruiz et al., 2003) and 7.60 ± 0.01 (Takacs-Novak and Avdeef, 1996), respectively, it was assumed for the calculations that at physiological pH (7.4), N-methyl-1-(3-chloro-4-hydroxy)phenylpiperazine (A) would exist primarily in the protonated state, whereas N-methyl-1-(4-hydroxy-4-pyrimidinyl)piperazine (B) would exist primarily in the protonated state as shown in Table 1. The energetics of the sequential thermodynamic steps involving the two-electron oxidation of A and B to the corresponding quinone-imines was studied (Table 1). Step I depicts the first single-electron transfer step in the oxidation of A and B in aqueous medium, and step II illustrates subsequent hydrogen atom abstraction from the phenolic hydroxyl group to generate the corresponding quinone imines. The relative oxidative stability of A and B was evaluated by calculating the free energy difference for A and B for each thermodynamic step, so that for reaction N, ∆ΔG(N) = ∆G(N = B) − ∆G(N = A). Calculation of the relative free energies ∆ΔG(N) (Table 1) of the reactions allowed counterbalancing contributions and corrections such as zero-point and thermal corrections to the molecular free energies, which were not considered in the current theoretical study.

Our experimental observation that p-hydroxybuspirone (5) does not undergo further two-electron oxidation to the corresponding quinone-imine 7 in a manner similar to that observed with p-hydroxynefazodone (1) is consistent with the ab initio calculations on the simple N-methyl derivatives, which suggest both oxidation steps to be significantly less favorable for buspirone. A plausible explanation for the oxidative stability differences is that the stronger aromatic nature of the 3-chloro-4-hydroxyphenyl ring in 1, relative to the 4-hydroxy pyrimidine group in 5, leads to oxidation product(s) of greater stability. Another explanation is the pK\(_{a}\) differences between the two compounds; buspirone is protonated at physiological pH, whereas nefazodone is charge-neutral. Therefore, electron transfer in the case of 5 (or molecule B) is less favored relative to 1 (or molecule A). To test this hypothesis, calculations were also performed with the neutral form of molecule B. Although this process dramatically lowers ∆ΔG for the initial electron transfer step, the overall calculated free energy difference for both steps together is only reduced by about half (data not shown). These observations suggest that the differences in the protonation states of the p-hydroxylated metabolites of nefazodone...
TABLE 1

<table>
<thead>
<tr>
<th>Thermodynamic step (N)</th>
<th>ΔΔG(N)*, kCal/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>13.4</td>
</tr>
<tr>
<td>II</td>
<td>7.1</td>
</tr>
<tr>
<td>I + II</td>
<td>20.5</td>
</tr>
</tbody>
</table>

Thermodynamic step I for molecules A and B are as follows:

A → A^+ e^- + B^+ e^- → B^{++}

Thermodynamic step II for molecules A and B are as follows:

A^+ H^- → A'^+ H^+ (I-A)

B^{++} H^- → B'^++ H^+ (I-B)

*ΔΔG(N) = ΔG(N-B) - ΔG(N-A), where ΔG(N-X) is the calculated free energy difference for the reaction N-X.

The results from our current investigation clearly demonstrate the bioactivation of the 3-chlorophenylpiperazine motif in nefazodone to the reactive quinone-imine species 3, which can be trapped by GSH or the corresponding GSH-EE derivative. This investigation is the first to report on the bioactivation of nefazodone. Apart from the literature reports on the exclusive involvement of P450 3A4 in the metabolism of nefazodone in humans, our studies also demonstrated a key role for the enzyme in the metabolic activation of this antidepressant. Also of considerable interest was our result on the characterization of the enzyme in the metabolic activation of this antidepressant. Also of interest was our result on the characterization of the enzyme in the metabolic activation of this antidepressant. Also of interest was our result on the characterization of the enzyme in the metabolic activation of this antidepressant.

And buspirone at physiological pH and the difference in the resonance stabilization of the subsequent two-electron oxidation products could contribute about equally to the observed experimental difference in reactivity.

**Discussion**

The results from our current investigation clearly demonstrate the bioactivation of the 3-chlorophenylpiperazine motif in nefazodone to the reactive quinone-imine species 3, which can be trapped by GSH or the corresponding GSH-EE derivative. This investigation is the first to report on the bioactivation of nefazodone. Apart from the literature reports on the exclusive involvement of P450 3A4 in the metabolism of nefazodone in humans, our studies also demonstrated a key role for the enzyme in the metabolic activation of this antidepressant. Also of considerable interest was our result on the characterization of the N-dearylated metabolites of nefazodone, 8 to 10. A similar P450-catalyzed N-dearylation sequence involving the phenylpiperazine ring system has been reported with the phenylpiperazine-containing anti-emetic agent mosapirone and several of its analogs (Enreclle et al., 1991; Galmier et al., 1991). Apart from P450, the role of myeloperoxidase in N-dearylation has also been established as exemplified by the phosphodiesterase inhibitor vesarimide (Etuct et al., 1994) and the anti-inflammatory agent prinomide (Parrish et al., 1997). Potential mechanisms of nefazodone N-dearylation to afford 8 and 2-chloro-1,4-benzoquinone are shown in Fig. 13. Pathway A depicts the initial two-electron oxidation of 1 to the corresponding quinone-imine intermediate 3 bearing a permanently positive charge on the piperazine nitrogen. This quinone-imine intermediate can participate in a 1,4-Michael addition with GSH or GSH-EE, resulting in the formation of the aromatic thioether conjugates 11 and 12, respectively, or undergo hydrolysis across the iminium bond, resulting in the formation of the unstable carbinol amine intermediate 14, which would spontaneously decompose to 2-chloro-1,4-benzoquinone and 8. N-Dearylation via pathway B involves epoxidation across the phenyl ring carbons meta and para to the hydroxy group in 1, resulting in the formation of 13. Epoxide ring opening in 13 would then lead to 14. In pathway C, the hydroxy group in 1 participates in a nucleophilic reaction with the hemeperoxo intermediate, resulting in the direct formation of 14. This pathway was considered on the basis of previous observations on the involvement of the heme hydroperoxy intermediate as the oxidant with aromatic rings containing electron-donating groups (A. D. N. Vaz, unpublished results). Evidence for the formation of the electrophilic 2-chloro-1,4-benzoquinone was also obtained via characterization of the corresponding GSH-EE conjugate 15 (see Fig. 13). Additional studies designed to probe the mechanism of N-dearylation in greater detail are currently in progress. In the light of literature reports on the cytotoxicity (Moore et al., 1987) and genotoxicity (Westmoreland et al., 1992) observed with structurally diverse 1,4-benzoquinone derivatives, we speculate that 2-chloro-1,4-benzoquinone and quinone-imine 3 may contribute to the etiology of nefazodone hepatotoxicity. The reason(s) for the lack of metabolic activation of the para-hydroxy-m-CPP metabolite 2 of nefazodone in human liver microsomes remains unclear. One possibility is that 2 escapes from the active site of the P450 prior to the bioactivation step.

Although not investigated in this preliminary analysis, it is reasonable to expect that the reactive quinone-imine or p-benzoquinone intermediates will form covalent adducts to hepatic proteins, with potentially deleterious consequences. That the potency of P450 3A4 inhibition by nefazodone significantly increases with time certainly provides preliminary evidence for covalent adduction formation with the enzyme that results in its inactivation. On the basis of the time-dependent P450 3A4 inhibition data, it is tempting to speculate that the nonlinear pharmacokinetics of nefazodone in humans after q.i.d. and b.i.d. dose escalation (Barbhaya et al., 1995a, 1996; Kaul et al., 1995) occur due to autoinactivation of P450 3A4, the primary enzyme responsible for nefazodone metabolism in humans. The P450 3A4 inactivation data in our studies are consistent with the clinical data that suggest that nefazodone may be an inhibitor of P450 3A4 in vivo to a significant extent. A plethora of examples on the pharmacokinetic interactions of nefazodone with diverse P450 3A4 substrates such as triazolam (Barbhaya et al., 1995b), alprazolam (Greene et al., 1995), carbamazepine (Ashton and Wolin, 1996), cyclosporine (Helms-Smith et al., 1996; Wright et al., 1999), terfenadine (Abernethy et al., 2001), astemizole (Dresser et al., 2000), and tacrolimus (Olyaei et al., 1998) has appeared in the literature.

The lack of bioactivation of buspirone despite heteroaromatic hydroxylation para to the piperazinyl nitrogen is consistent with our ab initio calculations that suggest that a weaker resonance stabilization of the oxidation products and the greater acidity make p-hydroxybuspirone less favorable for the two-electron oxidation. Overall, these observations appear to correlate with the lack of idiosyncratic hepatotoxicity with buspirone in the clinic. Finally, the observed differences in the bioactivation of the phenyl- and the pyrimidinylpipera- zine ring systems is consistent with the notion that the 2-aminopyridine or 2-aminopyrimidine derivatives present a “safer” alternative to aniline-based compounds, which are prone to bioactivation. However, additional examples may be required to further validate this hypothesis.
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Address correspondence to: Amit S. Kalgutkar, Pharmacokinetics, Dynamics, and Metabolism Department, Pfizer Global Research and Development, Groton, CT 06340. E-mail: amit_kalgutkar@groton.pfizer.com