SHORT COMMUNICATION

CHARACTERIZATION OF A NOVEL METABOLITE INTERMEDIATE OF ZIPRASIDONE IN HEPATIC CYTOSOLIC FRACTIONS OF RAT, DOG AND HUMAN BY ESI-MS/MS, H/D EXCHANGE AND CHEMICAL DERIVATIZATION

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REDUCTIVE CLEAVAGE OF A BENZISOTHIAZOLE ANALOG

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1 Abbreviations used are: ZIP, ziprasidone (5-[2-{4-(1,2-benzisothiazol-3-yl)piperazin-1-yl}ethyl]-6-chloro-1,3-dihydro-indol-2-one) hydrochloride hydrate; 5-HT, 5-hydroxytryptamine; BITP, 3-(piperazin-1-yl)-1,2-benzisothiazole; OX-acetic acid, 6-chloro-2-oxo-2,3-dihydro-1H-indol-5-yl)acetic acid; dihydro-ZIP, 6-chloro-5-(2-{4-[imino-(2-mercapto-phenyl)-methyl]-piperazin-1-yl}-ethyl)-1,3-dihydro-indol-2-one; S-methyl-dihydro-ZIP, (6-chloro-5-(2-{4-[imino-(2-methylsulfanyl-phenyl)-methyl]-piperazin-1-yl}-ethyl)-1,3-dihydro-indol-2-one; CYP, cytochrome P450; radio-HPLC, HPLC with on-line radioactivity detection; β-RAM, radioactivity monitor; CID, collision induced dissociation; XO, xanthine oxidase; AO, aldehyde oxidase; TMT, thiol methyltransferase.
Abstract

Ziprasidone (geodone), a novel atypical antipsychotic agent, is recently approved for the treatment of schizophrenia. It undergoes extensive metabolism in preclinical species and humans after oral administration and only a very small amount of administered dose is excreted as unchanged drug. In vitro studies using human liver microsomes have shown that the oxidative metabolism of ziprasidone is mediated primarily by CYP3A4. However, co-administration of ziprasidone with ketoconazole, a CYP3A4 inhibitor, showed only a modest increase in its exposure. Therefore, in vitro metabolism of ziprasidone was investigated in hepatic cytosolic fractions to further understand its clearance mechanisms in preclinical species and humans. The major metabolite from incubation of ziprasidone in cytosolic fractions of rat, dog and human was characterized by LC-MS/MS and found to be the product of reductive cleavage. Derivatization and H/D exchange were used to deduce that the addition of 2 hydrogen atoms had occurred at the benzisothiazole moiety. Further studies to determine the enzyme involved in the formation of this metabolite are currently in progress. The identification of this novel metabolite in cytosol has clarified the clearance mechanism of ziprasidone in humans and preclinical species.
Ziprasidone (ZIP1), a substituted benzisothiazolyl-piperazine (fig. 1), is a recently approved novel antipsychotic agent effective in the treatment of schizophrenia. It exhibits potent and highly selective dopamine D2 and serotonin 5HT2 receptor antagonistic activities. It also has a high affinity for the 5HT1A, 5HT1D and 5HT2C receptor subtypes that could contribute to the overall therapeutic effect (Seeger et al., 1995). The metabolism of ZIP has been studied both in vitro and in vivo in preclinical species and humans (Prakash et al., 1997a, 1997b and 2000). In vitro studies using human liver microsomes suggested the formation of four oxidative metabolites, ZIP-sulfoxide, ZIP-sulfone, BITP and Ox-AA (fig.1). Further in vitro studies using CYP isoform-selective chemical inhibitors, correlation studies and metabolism by recombinant human CYP isoforms suggested that the formation of these oxidative metabolites is mediated mainly by human liver CYP3A4 (Prakash et al., 2000). However, Phase II clinical studies showed only modest increases in both the AUC and Cmax of ZIP (<40% increase with single and multiple dose evaluations) with concomitant administration of ketoconazole, a potent inhibitor of CYP3A4 (Miceli et al., 2000a). It is known that substrates that are primarily dependent on CYP3A4 for metabolism demonstrate inter-subject variability in exposure on the order of 50-fold and have significant increases in exposure with concomitant administration of ketoconazole (Lin and Lu, 1998; Thummel and Wilkinson, 1998). On the other hand, co-administration with carbamazepine (200 mg BID), an inducer of CYP3A4, resulted only in a small decrease (<40%) in exposure of ZIP (Miceli et al., 2000b). In addition, concomitant administration of cimetidine (400 mg/day), a general inhibitor of CYP isoforms, showed only modest increase in both the AUC and Cmax of ZIP (<40% increase with single and multiple dose; Wilner et al., 2000). Therefore, it could be hypothesized that the major clearance of ZIP in preclinical species and humans is due to metabolism by non-CYP enzymes.
In vivo metabolism studies suggested that ZIP is extensively metabolized both in animals and humans and only a small amount (<1%) of drug is excreted unchanged in urine (Prakash et al., 1997a, 1997b). In addition to metabolites found in vitro, a major metabolite, S-methyl-dihydro-ZIP of ZIP was identified in rats, dogs and humans. This metabolite accounted for >60% of the administered dose in feces of humans following a single 20 mg oral dose of radiolabelled ZIP (Prakash et al., 1997b). Therefore, it could be envisioned that enzymes other than CYP isoforms form the S-methyl-dihydro-ZIP metabolite. The formation of S-methyl-dihydro-ZIP metabolite was speculated to involve an initial reductive cleavage of the benzisothiazole moiety to give an intermediate that can be further converted to a methyl thioether by methylation with S-thiomethyl transferases (Prakash et al., 1997c). However, the intermediate metabolite was not detected either in vivo or in vitro possibly due to its instability or its rapid metabolism to S-methyl metabolite. The present work reports the vitro metabolism of ZIP in hepatic cytosolic fractions from preclinical species and humans to further understand the clearance mechanisms of ZIP in these species. The major metabolite of ZIP in these incubations was characterized by LC-MS/MS, H/D exchange and chemical derivatization with N-dansylaziridine and found to be the product of reductive cleavage of the benzisothiazole ring. Taking all these data together, the new metabolite was identified as dihydroziprasidone (6-chloro-5-(2-4-[imino-(2-mercapto-phenyl)-methyl]-piperazin-1-yl}-ethyl)-1,3-dihydro-indol-2-one). The identification of this novel metabolite in cytosol has provided the understanding of the clearance mechanism of ziprasidone in humans and preclinical species.

Materials and Methods

Chemicals and Reagents. Commercially obtained chemicals and solvents were of HPLC or analytical grade. [14C]ZIP (Fig. 1), specific activity 8.2 mCi/mmol and dihydro-ZIP were
synthesized at Pfizer Global Research and Development (Groton, CT). Dansylaziridine, menadione, allopurinol, CD$_3$ COOD and D$_2$O were purchased from Sigma-Aldrich Chemical Co (St Louis, MO).

**Incubations.** The cytosolic fractions of rat, dog and human liver were prepared using standard centrifugal methods (Prakash et al., 2000). The incubations of ZIP with cytosolic fractions of rat, dog and human liver were conducted using standard procedures. A typical incubation mixture (1 ml) contained $[^{14}\text{C}]$ZIP (10 µM in 5 µl methanol), 10 mg/ml cytosolic protein, 2-hydroxypyrimidine (40 µM) and 100 mM potassium phosphate buffer, pH 7.4. The reaction was initiated by the addition of the cytosolic fraction and the incubation was carried out for 20 min. The reaction was stopped by the addition of methanol (5 ml). Control incubations using boiled cytosols were also carried out under the same conditions. The reaction mixtures were centrifuged (1900 x g) and the supernatants were transferred to clean tubes to be analyzed by LC-MS/MS. Incubations were also conducted with allopurinol (a mechanism-based inhibitor of XO) and menadione (a specific inhibitor of AO) at a concentration of 100 µM each as described above.

**HPLC-MS.** The mixture of metabolites was subjected to chromatography on an HPLC system that consisted of a HP-1050 solvent delivery system, a HP-1050 auto injector, a radioactivity monitor ($\beta$-RAM, Tampa, FL) and a SP 4200 computing integrator (Riviera Beach, FL). Chromatography was conducted on an YMC basic HPLC column (4.6 x 150 mm, 3µm) with a binary mixture of 10 mM ammonium acetate (pH 5.0, solvent A) and methanol (solvent B) and the flow rate was established at 1 ml/min. Analysis of metabolites was performed on a PE-SCIEX API III HPLC/MS/MS system (Perkin Elmer-Sciex, Boston, MA) using ion spray. The effluent from the HPLC column was split and about 50 µl/min was introduced into the atmospheric ionization source via an ion spray interface. The remaining effluent was directed
into the flow cell of β-RAM (Tampa, FL). The β-RAM response was recorded in real time by the mass spectrometer that provided simultaneous detection of radioactivity and mass spectrometry data. The ion spray interface was operated at 5000 V, and the mass spectrometer was operated in the positive ion mode. CID studies were performed using argon gas at collision energy of 25-30 eV and a collision gas thickness of $2 \times 10^{15}$ molecules/cm$^2$.

**Derivatization with Dansylaziridine.** The supernatant from the incubation was dried under nitrogen in a Turbo Vap LV evaporator (Zymark, Hopkinton, MA). The dried incubation mixture was dissolved in 2M KOH (100 µl). 500 µl of 3 mM N-dansylaziridine in methanol was added and the reaction mixture was stirred at room temperature for 1 h (Orford et. al., 1989). Following evaporation of the solvents, the residue was dissolved in mobile phase and an aliquot was injected to HPLC-MS/MS system without further purification.

**Results and Discussion**

**Metabolite Profiles of ZIP in Cytosolic Fractions.** Representative HPLC-radiochromatograms of metabolites from incubations of ZIP with cytosolic fractions of rat, dog and human liver are shown in fig. 2. A major metabolite designated M11, along with few additional minor metabolites (M4, M4A, M7 and M8) were detected in the chromatograms. These metabolites were not detected with boiled liver cytosols, suggesting the formation of these metabolites by enzymatic reactions. We have earlier reported the structural characterization of metabolites (M4, M4A, M7 and M8 in humans but M11 was not detected in vivo in humans (Prakash et al., 1997b). Full scan mass spectrum of metabolite M11 revealed a protonated molecular ion $[M+H]^+$ at $m/z$ 415, 2 Da higher than the drug. The product ion mass spectrum of $m/z$ 415 produced fragment ions at $m/z$ 280, 263, 237, 194 and 159 (fig. 3a). The ion at $m/z$ 280 corresponds to a charge initiated fragmentation of the piprazinyl nitrogen-benzisothiazole carbon.
bond with the expulsion of the benzisothiazole as a neutral molecule (Prakash et. al. 1997a). The ions at \( m/z \) 263 resulted by the loss of ammonia from fragment ion at \( m/z \) 280. The presence of other characteristic fragment ions at \( m/z \) 194 and 263 in its CID spectrum further suggested that the addition of 2 Da had occurred remote from the oxindole part of the molecule.

Hydrogen/deuterium (H-D) exchange followed by mass spectrometry has long been recognized as a valuable means to study the mechanism of ion formation, metabolic pathways of xenobiotics, and to differentiate the isomeric structures of metabolites (Kamel et al., 2002, 2004). H-D exchange techniques are useful for determination of the presence, number, and position of H/D exchangeable functional groups on the metabolite structures and serve as an aid for structure elucidation of metabolites (Nassar, 2003). Solution phase H/D exchange of M11 using D\(_2\)O, showed a shift of the protonated molecular ion from \( m/z \) 415 to \( m/z \) 419 for the full exchanged species \([M(d_3)+D]^+\). On the other hand, the full scan MS of ZIP after D\(_2\)O treatment showed the deuterated molecular ion \((M(d)+D)^+\) at \( m/z \) 415 (2 mass units higher than the corresponding \([M+H]^+\)). The increase of 2 mass units for the deuterated molecular ion of M11 compared to the deuterated molecular ion of the parent is in agreement with the presence of two exchangeable hydrogen atoms. The product ion MS spectrum of \( m/z \) 419 showed fragment ions at \( m/z \) 283, 265, 195 and 160 (fig. 3b). These data indicated that the addition of two hydrogen atoms had occurred at the benzisothiazole moiety by its reductive cleavage. Based on these data the structure of the major metabolite M11 was proposed as dihydroziprasidone.

The proposed structure of M11 was further corroborated by its derivatization with dansylaziridine, a specific derivatizing reagent for the thiol group (Orford et. al., 1989). Treatment of M11 with dansylaziridine formed a new product at a HPLC retention time of 42.2 min. The full scan MS of derivatized product revealed a protonated molecular ion at \( m/z \) 691,
176 Da higher than the (M+H)$^+$ of M11, suggesting the addition of a dansylaziridine moiety. The product ion MS spectrum of $m/z$ 691 showed fragment ions at $m/z$ 455, 412, 309, 280, 263, 194 and 170, consistent with the proposed structure (fig. 4a). The assignment of these ions was confirmed by parallel CID spectrum of $m/z$ 693 (MH$^+$,$^{37}$Cl), which gave the fragment ions at $m/z$ 455, 412, 309, 282, 265, 196 and 194 (not shown). The structure of M11 was confirmed unambiguously by comparison of its retention time and MS spectral data with a synthetic standard.

This is the first report on the identification of such an intermediate metabolite of ZIP. There are several marketed drugs including the anticonvulsant zonisamide and the antipsychotic risperidone and iloperidone, which contain the 1, 2-isoaxazole ring structure. It has been reported that these drugs undergo reductive metabolism resulting in the cleavage of N-O bond to form the intermediate imines (Dalvie et al., 2002, Mutlib et al., 1995, Mannens et al, 1993; Stiff et al., 1992). Although the intermediate imines themselves have never been isolated, due to their rapid hydrolysis in aqueous media to ketones, Sugihara et al. (1996) provided the evidence for this mechanism when they demonstrated stoichiometric production of ammonia and 2-sulfamoylacetophenol from zonisamide. We report here that the 1,2 benzisothiazole ring structure of ZIP also undergoes similar reductive metabolism resulting in the cleavage of N-S bond to form an intermediate amidine. The structure of this intermediate amidine was characterized by LC-MS/MS, H/D exchange and chemical derivatization with a thiol specific derivatization reagent (Orford et. al., 1989). The intermediate amidine was not detected in vivo in preclinical species either due to its instability or its rapid metabolism to a methyl thioether by methylation with S-thi methyl transferases (Prakash et. al., 1997c). Hillenweck et al. (1997)
have shown the formation of methylthio metabolites of chlorothanil, a fungicide used in agriculture, in digestive contents of rat, dog and human.

It is well established that the reductive metabolism of zonisamide is catalyzed by several enzymes, liver microsomal CYP450, especially CYP3A4 (Nakasa et al 1993), AO (Sugihara et al., 1996), as well as, mammalian intestinal bacteria (Kitamura and Tatsumi, 1984; Kitamura et al., 1997). However, the reduction of the isoxazole ring in risperidone was attributed mainly to gut microflora (Mannens et al, 1993). Recently, Tschirret-Guth and Wood (2003) have reported that 3-(indole-1-yl)-1,2 benzisoxazoles are reductively N-dearylated by rat liver microsomes under anaerobic conditions. One of the proposed mechanisms for the reductive N-dearylation involved the cleavage of the N-O bond followed by hydrolysis.

It is speculated that the cleavage of the benzisothiazole ring of ZIP could also be mediated by any of the above enzyme systems. M11 or its S-methyl metabolite were not detected in the incubation of ZIP with human fecal samples under anaerobic conditions suggesting that the gut microflora may not be playing a role in the formation of these metabolites (data not shown). Further, the ring cleaved S-methyl metabolites of ZIP were earlier found in both urine and serum of humans and preclinical species, suggesting that the liver enzymes mediate the opening of the benzisothiazole ring. Our previous in vitro studies ruled out the possibility of involvement of microsomal enzyme in the formation of M11 (Prakash et al., 2000). However, the present study has demonstrated that the cytosolic fractions from rat, dog and human can catalyze the reduction of the benzisothiazole ring. Our preliminary studies in human liver cytosolic fractions showed that the formation of M11 was inhibited slightly by a XO inhibitor, allopurinol (22%). However, 100 μM menadione (a specific AO inhibitor) largely abolished the formation of dihydroziprasidone (>70% inhibition) suggesting that the formation
of dihydroziprasidone metabolite may be mainly mediated by AO (Kamel et al., unpublished work). These results indicated that the opening of the benzisothiazole ring might be mediated by AO. As reported previously, AO, a liver cytosolic metalloflavoprotein, in the presence of its electron donor, catalyzes the reduction of a variety of compounds such as N-oxides, hydroxamic acids, and oximes (Kitamura and Tatsumi, 1984; Tatsumi and Ishigai, 1987). The reduction of oximes formed the intermediate ketimines that subsequently can undergo nonenzymatic hydrolysis to ketones (Tatsumi and Ishigai, 1987). The benzisothiazole derivatives may also undergo reductive metabolism by AO to the corresponding amidine. The details of the mechanism for the formation of M11 are currently under investigation in this laboratory.

In summary, ZIP is eliminated by two distinct pathways in humans and preclinical species. In humans, cytosolic enzyme(s) mediates approximately two thirds of the ZIP metabolism and therefore, reduces the potential of CYP-450 based drug-drug interaction with co-administered drugs. Finally, the identification of this novel metabolite of ZIP allows us to understand the clearance mechanism of ZIP and the results from clinical studies.

**Acknowledgments.** We would like to thank Drs. Keith McCarthy and Stan Walinsky for synthesizing radiolabelled ziprasidone and synthetic standard, Mr. Larry Cohen for providing cytosol and Drs. Hassan Fouda, Scott Obach, Robert Ronfeld and Larry Tremaine for helpful suggestions and advice.
References


Footnotes:

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This work was presented in part at the 4th American Society of Mass Spectrometry and Allied Topics. Chicago, IL 2001.
Figure Legends

FIG. 1. Structures of ziprasidone and its major metabolites

FIG. 2. HPLC-radiochromatograms of ZIP metabolites in cytosolic fraction from rat, dog and human liver

FIG. 3. CID product ion spectra of metabolite M11: (a) before and (b) after H-D exchange

FIG. 4. CID product ion spectrum of metabolite M11 after derivatization with dansylaziridine

Number in the parentheses refer to $^{37}$Cl fragment ions
Fig. 1

* Denotes the position of $^{14}$C label
Fig. 2