An Evaluation of the Cytochrome P450 Inhibition Potential of Lisdexamfetamine in Human Liver Microsomes

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ABSTRACT:

The human cytochrome P450 (P450) system is implicated in many drug interactions. Lisdexamfetamine dimesylate (NRP104), the proposed generic name for a new agent under investigation for treatment of attention deficit hyperactivity disorder, was recently analyzed for inhibitory drug-drug interactions with seven major P450 isoforms using pooled human liver microsomes. Probe substrates were used near the $K_m$ concentration values reported in the literature for CYP1A2 (phenacetin), CYP2A6 (coumarin), CYP2B6 (bupropion), CYP2C9 (tolbutamide), CYP2C19 (S)-mephentoin, CYP2D6 (dextromethorphan), and CYP3A4 (midazolam and testosterone), and lisdexamfetamine was evaluated at concentrations ranging from 0.01 to 100 μM for its ability to inhibit the activity of these seven P450 isoforms. NADPH was added to one set of samples to initiate metabolic reactions, which were then terminated by adding organic solvent, vortexing the samples, and placing them on ice. The relevant substrates were then introduced to both sets of samples so that the percentage of remaining activity could be measured and compared. In addition, these samples were compared with other samples with the same concentrations of lisdexamfetamine but without preincubation. None of the seven P450 isoforms showed any concentration-dependent inhibition. Comparison of results from microsomes preincubated with and without NADPH showed no mechanism-based inhibition. Neither concentration-dependent nor mechanism-based inhibition caused by time-dependent inactivation of human P450 isoforms was shown for lisdexamfetamine during in vitro testing. The evidence suggests that lisdexamfetamine has a low potential for drug-drug interactions or initiation of drug-drug interactions.

Approximately 3.9 million children have been diagnosed with attention deficit hyperactivity disorder (ADHD), making it one of the most common behavioral disorders of childhood (Centers for Disease Control and Prevention, 2005). Epidemiologic studies have estimated the prevalence of ADHD in school-aged children to be 8 to 10% (Dulcan, 1997; American Academy of Pediatrics, 2000). Using efficacious, well tolerated drug treatments with a decreased likelihood of drug interactions in this vulnerable population is of paramount importance.

Food and Drug Administration approval is being sought for lisdexamfetamine dimesylate (proposed generic name), a medication developed for treatment of ADHD in children (Mickle et al., 2006). Lisdexamfetamine is an inactive prodrug in which the pharmacologically active d-amphetamine is covalently bonded to l-lysine, an essential amino acid. After ingestion, the pharmacologically active d-amphetamine is released when the covalent bond is cleaved during metabolism. The covalent bond linking l-lysine to d-amphetamine is an amide bond, and the structure of lisdexamfetamine resembles a dipeptide. Therefore, a proteolytic enzyme or enzymes are likely responsible for the biotransformation of the prodrug to release d-amphetamine. Lisdexamfetamine is stable to hydrolysis in serum, and minimal amounts of d-amphetamine are released when the drug is administered by parenteral routes (Mickle et al., 2006). When administered at therapeutic doses by the intended p.o. route, lisdexamfetamine releases d-amphetamine bioequivalent to equimolar doses of d-amphetamine sulfate. Thus, the specific enzyme or enzymes that metabolize lisdexamfetamine appear to be digestive enzymes that are more abundant in the gastrointestinal tract (Mickle et al., 2006). Lisdexamfetamine was designed to have efficacy and tolerability comparable with that of current extended-release stimulant formulations used to treat ADHD, but with reduced potential for abuse, diversion, and overdose toxicity (Jasinski and Krishnan, 2006).

Animal studies have indicated that in rats, the LD50 of the lisdexamfetamine is 5 times larger than that reported for amphetamine sulfate (S. Krishnan, unpublished observations). Compared with d-amphetamine sulfate, exposure to d-amphetamine is decreased and delayed after i.v. and intranasal administration and at high p.o. doses above the therapeutic level of lisdexamfetamine (Mickle et al., 2006).

Although the toxicity and tolerability of lisdexamfetamine appear to be favorable, if drug-drug interactions result in serious adverse events (AE), it could limit the usefulness of the compound. One of the

ABBREVIATIONS: ADHD, attention deficit hyperactivity disorder; P450, cytochrome P450; HPLC, high-performance liquid chromatography; APAP, acetaminophen; 7OHCMN, 7-hydroxycoumarin; OHBP, hydroxybupropion; HTB, hydroxytolbutamide; 4HMPN, 4'-hydroxymephenytoin; DRR, dextrorphan; 1OHMDZ, 1'-hydroxymidazolam; 6βT, 6β-hydroxytestosterone; QC, quality control; LC/MS/MS, liquid chromatography/tandem mass spectrometry.
most important determinants of potential drug interactions is the human cytochrome P450 (P450) system. The P450 system is a family of heme protein isozymes responsible for the oxidative metabolism of drugs, environmental contaminants, and xenobiotics (Prough and Spearman, 1987; Yang and Lu, 1987; Peter et al., 1990; Wrighton et al., 1993). P450 drug interactions usually result from either enzyme inhibition or enzyme induction (Cupp and Tracy, 1998). Inhibition may be competitive or noncompetitive, as well as reversible or irreversible as observed through damage to the heme or apoprotein, such as with cimetidine and chloramphenicol (Nedelcheva and Gut, 1994). Interactions may also be potentiated by genetic polymorphisms because some individuals are poor metabolizers through certain P450 isozymes, whereas others are extensive metabolizers (Goldstein et al., 1994; Nedelcheva and Gut, 1994; Cupp and Tracy, 1998). Adverse reactions that may not occur when a drug is administered alone may emerge when the compound is taken in conjunction with other medications that either induce or inhibit one or more P450 isozymes (Cupp and Tracy, 1998). Inhibition may result in elevated serum levels of the unmetabolized agent, which can cause increases in both primary and adverse effects. Conversely, if the primary compound is a prodrug, inhibition may result in delayed or inhibited onset of action and reduced efficacy.

Although P450 isozymes can be found in many body tissues, they are most prevalent in the liver (Cupp and Tracy, 1998). The isozymes of the CYP3A subfamily are the most abundant cytochrome enzymes in humans, constituting 30% of liver P450 enzymes, and are involved in many clinically important drug interactions (Shimada et al., 1994; Slaughter and Edwards, 1995; Cupp and Tracy, 1998). Because of interspecies differences in substrate specificities for some P450 isozymes (Waxman et al., 1988), in vitro human liver microsomes were used in this study to evaluate the likelihood of drug-drug interactions for lisdamphetamine. The isozymes CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 were included in the investigation.

### Materials and Methods

**Chemicals.** The test compound, lisdamphetamine (NRP104), was supplied by Organichem Corp. (Rensselaer, NY), with a certificate of analysis reporting 99.4% purity. A purity of 100% was assumed for preparation of spiking solutions, which was completed daily so that a 2-μl spike of the appropriate solution into 500 μl of total volume would achieve the desired concentration. Potassium phosphate monobasic, potassium phosphate dibasic, sodium carbonate, NADPH tetrasodium salts, and other reagents were purchased from Sigma Chemical Co. (St. Louis, MO) or equivalent vendors. Methanol, acetonitrile, and water [all high-performance liquid chromatography (HPLC) grade] and ethyl acetate, acetone, and other solvents were purchased from Fisher Scientific International, Inc. (Hampton, NH), Honeywell Burdick and Jackson (Muskegon, MI), Mallinckrodt Baker Inc. (Phillipsburg, NJ), or equivalent vendors. The following compounds were supplied by CellzDirect Inc (Pittsboro, NC): phenacetin, acetaminophen (APAP), d₄-acetaminophen, α-naphthoflavone, coumarin, 7-hydroxycoumarin (7OHCMN), 4-methyl-7-

### Table 1: Human Liver Microsomes

<table>
<thead>
<tr>
<th>Isoform Monitored</th>
<th>Marker Substrate</th>
<th>Substrate Concentration</th>
<th>Incubation Time</th>
<th>Protein Concentration</th>
<th>Metabolite Formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>Phenacetin</td>
<td>50</td>
<td>30</td>
<td>0.1</td>
<td>Acetaminophen</td>
</tr>
<tr>
<td>CYP2A6</td>
<td>Coumarin</td>
<td>1</td>
<td>5</td>
<td>0.025</td>
<td>7-Hydroxycoumarin</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>Bupropion</td>
<td>125</td>
<td>20</td>
<td>0.25</td>
<td>7-Hydroxybupropip</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>Tolbutamide</td>
<td>140</td>
<td>20</td>
<td>0.1</td>
<td>Hydroxytolbutamide</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>(S)-Mephenytoin</td>
<td>50</td>
<td>30</td>
<td>0.1</td>
<td>4'-Hydroxymephyton</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>Dextromethorphan</td>
<td>7.5</td>
<td>30</td>
<td>0.1</td>
<td>Dextromphan</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>Midazolam</td>
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<td>4</td>
<td>0.025</td>
<td>1'-Hydroxymidazol</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>Testosterone</td>
<td>50</td>
<td>7</td>
<td>0.05</td>
<td>6β-Hydroxytestosterone</td>
</tr>
</tbody>
</table>

**Human Liver Microsomes.** Human liver microsomes pooled from males and females (n = 15) were provided by CellzDirect for use in this study. The microsomes previously characterized for major P450 activity by the In Vitro Drug Development Services Division of CellzDirect were CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4.

**Analytical Methods and Incubation Procedure.** Human liver microsomal samples were preincubated with lisdamphetamine, both with and without NADPH, at 37°C for 15 min. An incubation buffer of 0.5 ml of 100 mM potassium phosphate buffer, pH 7.4, was used in all the reaction mixtures, all of which met calibration and quality control (QC) standards. All the samples were injected onto a Micromass Quattro II liquid chromatography/tandem mass spectrometry (LC/MS/MS) equipped with an appropriate HPLC column. Quantitation for all the following was performed with a weighted (1/x) linear least-squares regression analysis generated from fortified matrix calibration standards, except for hydroxybupropip (CYP2B6) and 6β-hydroxytestoster- one (CYP3A4), for which a weighted (1/x²) linear least-squares regression analysis was used.

**Microsomal Incubations for IC₅₀ Estimation.** The ability of the test compound to inhibit the activity of each of the seven P450 isoforms was evaluated in pooled human liver microsomes. The tested isoforms, their respective marker substrates, and incubation conditions are summarized in Table 1. Human liver microsomes diluted in 100 mM potassium phosphate buffer at pH 7.4 were spiked with the appropriate marker substrate and lisdamphetamine (0.01, 0.1, 1, 10, and 100 μM), and the triplicate suspensions were allowed to equilibrate. Total incubation volumes were approximately 0.5 ml. The final concentration of each substrate was near the Km value reported in the literature (Waxman et al., 1988; Miles et al., 1990; Peter et al., 1990; Veronese et al., 1991; Wrighton et al., 1993; Goldstein et al., 1994; Gorski et al., 1994; Kerry et al., 1994; Kobayashi et al., 1999; Faucette et al., 2000; Hesse et al., 2000). Metabolic reactions were initiated by adding NADPH (1 mM final concentration). Reactions were terminated by adding organic solvent, vortexing, and placing on ice. Samples were then extracted and analyzed as described previously.

**Determination of APAP (CYP1A2).** The marker metabolite APAP was quantitated from 1 to 1000 ng/ml. Freshly prepared samples containing APAP in a reaction mixture containing 0.1 mg/ml final human liver microsomal protein were treated with ethyl acetate and the internal standard d₄-acetaminophen. All the samples were then basified and extracted in ethyl acetate. The organic layer was transferred to a new tube, evaporated to dryness, reconstituted in a mobile phase, and injected onto a Micromass Quattro II LC/MS/MS equipped with an appropriate HPLC column. Peak areas of the m/z 152→110 product ion of APAP were measured against peak areas of the m/z 156→114 product ion of the deuterated internal standard.
human liver microsomal protein were treated with ethyl acetate and the internal standard 4-methyl-7-hydroxyxocoumarin. All the samples were then acidified. The organic layer was transferred to a new tube, evaporated to dryness, reconstituted in a mobile phase, and injected onto a Micromass Quattro II LC/MS/MS equipped with an appropriate HPLC column. Peak areas of the m/z 161→133 product ion of 7OHCMN were measured against peak areas of the m/z 175→133 product ion of the internal standard.

**Determination of OHBP (CYP2B6).** The marker metabolite OHBP was quantitated from 1 to 1000 ng/ml. Freshly prepared samples containing OHBP in a reaction mixture containing 0.25 mg/ml final human liver microsomal protein were treated with isooamyl alcohol/ethyl acetate (1:100, v/v) and the internal standard trazodone. A saturated sodium chloride solution was added to all the samples. The organic layer was transferred to a new tube, evaporated to dryness, reconstituted in a mobile phase, and injected onto a Micromass Quattro II LC/MS/MS equipped with an appropriate HPLC column. Peak areas of the m/z 257→239 product ion of OHBP were measured against peak areas of the m/z 372→176 product ion of the internal standard.

**Determination of HTB (CYP2C9).** The marker metabolite HTB was quantitated from 10 to 2000 ng/ml. Freshly prepared samples containing HTB in a reaction mixture containing 0.1 mg/ml final human liver microsomal protein were treated with acetone and the internal standard d₄-hydroxytolbutamide. The samples were acidified and extracted into diethyl ether/ethyl acetate (1:1, v/v). The organic layer was transferred to a new tube, evaporated to dryness, reconstituted in a methanol/ammonium acetate buffer (40:60, v/v), and injected onto a Micromass Quatro II LC/MS/MS equipped with an appropriate HPLC column. Peak areas of the m/z 285→186 product ion of HTB were measured against peak areas of the m/z 294→186 product ion of the internal standard.

**Determination of 4HMPN (CYP2C19).** The marker metabolite 4HMPN was quantitated from 5 to 1000 ng/ml. Freshly prepared samples containing 4HMPN in a reaction mixture containing 0.1 mg/ml final human liver microsomal protein were treated with acetone. The samples were acidified and extracted into diethyl ether/ethyl acetate (1:1, v/v). The organic layer was transferred to a new tube, evaporated to dryness, reconstituted in a mobile phase, and injected onto a Micromass Quatro II LC/MS/MS equipped with an appropriate HPLC column. Peak areas of the m/z 233→161 product ion of 4HMPN were measured.

**Determination of DRR (CYP2D6).** The marker metabolite DRR was quantitated from 5 to 1000 ng/ml. Freshly prepared samples containing DRR in a reaction mixture containing 0.1 mg/ml final human liver microsomal protein were treated with acetone. The samples were basified with NaOH and extracted into ethyl acetate. The organic layer was transferred to a new tube, evaporated to dryness, reconstituted in a mobile phase, and injected onto a Micromass Quatro II LC/MS/MS equipped with an appropriate HPLC column. Peak areas of the m/z 325→157 product ion of DRR were measured.

**Determination of 1OHMDZ (CYP3A4).** The marker metabolite 1OHMDZ was quantitated from 0.5 to 200 ng/ml. Freshly prepared samples containing 1OHMDZ in a reaction mixture containing 0.025 mg/ml final human liver microsomal protein were treated with ethyl acetate and the internal standard α-hydroxytriazolam. The organic layer was transferred to a new tube, evaporated to dryness, reconstituted in a mobile phase, and injected onto a Micromass Quatro II LC/MS/MS equipped with an appropriate HPLC column. Peak areas of the m/z 342→168 product ion of 1OHMDZ were measured against peak areas of the m/z 359→331 product ion of the internal standard.

**Determination of 6βT (CYP3A4).** The marker metabolite 6βT was quantitated from 5 to 5000 ng/ml. Freshly prepared samples containing 6βT in a reaction mixture containing 0.05 mg/ml final human liver microsomal protein were treated with ethyl acetate and the internal standard d₄ 6β-hydroxytestosterone. The organic layer was transferred to a new tube, evaporated to dryness, reconstituted in 1 mM ammonium acetate buffer, pH 2.5/ethanol (1:1, v/v), and injected onto a Micromass Quatro II LC/MS/MS equipped with an appropriate HPLC column. Peak areas of the m/z 305→269 product ion of 6βT were measured against peak areas of the m/z 308→272 product ion of the internal standard.

**QC Samples and Incubations.** Analytical QC samples containing high, medium, or low concentrations of the appropriate metabolite were examined within each group of incubation samples. Analytical runs were accepted only if two-thirds of all the QC samples and at least 50% of the QC samples at each level were ±25% theoretical value. To ensure that the enzymatic reaction was operating within the criteria established during the CellzDirect validation of each individual assay, a series of duplicate QC incubations at either five or six different substrate concentrations was conducted parallel to each study incubation. The QC incubations were used to estimate the Kᵦ value for the substrate/enzyme. Results from the study incubation were considered acceptable if the Kᵦ value estimated from the samples decreased between one-third and 3 times the mean Kᵦ value established during the CellzDirect validation exercise. In addition, the specificity of experimental conditions was evaluated with specific inhibitors to verify that the experimental conditions for IC₅₀ values were susceptible to inhibition. The specific inhibitors used during these incubations were furafylline (CYP1A2), tranylcypromine (CYP2A6), triethylenetriaminophosphoramide (CYP2B6), sulfaphenazole (CYP2C9), ticlopidine (CYP2C19), quinidine (CYP2D6), and ketoconazole (CYP3A4). Results were considered satisfactory if significant inhibition (>50%) was observed.

**Mechanism-Based Inhibition Screening.** Triplicate samples for mechanism-based inhibition screening were preincubated for 15 min at 37°C with 1 μM lisdexamfetamine with or without NADPH. The percentage of remaining activity of CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 was measured against peak areas of the m/z 372→176 product ion of the internal standard.

**Results**

**Estimated IC₅₀.** Incubation of lisdexamfetamine in microsomal suspensions at concentrations ranging from 0.01 to 100 μM showed no concentration-dependent inhibition for any of the isoenzymes under investigation (Table 3). Because there was no inhibition from 0.01 to 100 μM, IC₅₀ values were not determined. Percentage of remaining activity ranged from 82.4% for CYP3A4 (6βT), with lisdexamfetamine at a concentration of 1 μM, to 111% for CYP3A4 (MDZ), with lisdexamfetamine at a concentration of 0.1 μM.

**Mechanism-Based Inhibition.** Comparison of microsomes preincubated for 15 min with 1 μM lisdexamfetamine and NADPH with microsomes preincubated with lisdexamfetamine without NADPH indicated no mechanism-based inhibition (Table 4). Whereas results
results in this study, however, indicate that any interactions would likely be caused by amphetamine and its metabolites rather than the intact lisdexamfetamine prodrug.

Potential drug-drug interactions are a serious concern with any compound. Problems with P450 inhibition can result in increased serum levels of unmetabolized drug, possibly leading to adverse events or even toxic effects. Therefore, failure to detect any significant inhibition of the P450 system by lisdexamfetamine is a significant finding. No significant inhibition of the P450 system by lisdexamfetamine suggests that this drug could be a safe therapeutic option for ADHD.

Results from this study show that P450 inhibition does not occur to any significant degree with lisdexamfetamine. There is good reason to believe that the results of this in vitro study will correlate well with in vivo effects. Therefore, the data presented here for lisdexamfetamine suggest that this agent has low potential for interactions with other drugs.

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