ABSTRACT:

Vanoxerine (1-[2-[bis(4-fluorophenyl)methoxy]ethyl]-4-(3-phenylpropyl)piperazine; GBR12909) is a promising agent for the treatment of cocaine dependence. Knowledge of the major pathway for GBR12909 metabolism is important for the prediction of the likelihood of drug-drug interactions, which may affect the therapeutic clinical outcome, when this agent is used in cocaine-dependent individuals receiving multiple drug therapy. We studied biotransformation of GBR12909 in human liver microsomes (n = 4), human hepatocytes, and microsomes containing cDNA-expressed human P450 isoforms with GBR12909 concentrations within the range of steady-state plasma concentrations detected in healthy volunteers. A high-pressure liquid chromatography assay was used to measure parent GBR12909 and its primary metabolite. GBR12909 was metabolized by human liver microsomes, hepatocytes, and microsomes containing cDNA-expressed human P450 isoforms with GBR12909 concentrations within the range of steady-state plasma concentrations detected in healthy volunteers. A high-pressure liquid chromatography assay was used to measure parent GBR12909 and its primary metabolite. GBR12909 was metabolized by human liver microsomes, hepatocytes, and microsomes containing cDNA-expressed human P450 isoforms with GBR12909 concentrations within the range of steady-state plasma concentrations detected in healthy volunteers.

Ketoconazole, a selective inhibitor of CYP3A, reduced GBR12909 biotransformation in human liver microsomes and primary hepatocytes by 92 ± 2 and 92.4 ± 0.4%, respectively. Quercetin (an inhibitor of CYP2C8/3A4) was a less effective inhibitor producing 62 ± 22% inhibition in human liver microsomes and 54 ± 35% in hepatocytes. Other P450 selective inhibitors did not decrease GBR12909 biotransformation more than 29% in either human liver microsomes or hepatocytes with the exception of chlorzoxazone (CYP2E1), which inhibited GBR12909 biotransformation by 71.4 ± 18.5% in primary human hepatocytes. Ciprofloxacin (CYP1A2), sulfaphenazole (CYP2C9), quinidine (CYP2D6), chlorzoxazone (CYP2E1), and mefenoxam (CYP2C19) did not demonstrate statistically significant inhibition (p > 0.05) of GBR12909 biotransformation in liver microsomes. cDNA-expressed P450 3A4 metabolized GBR12909 to a greater extent than 2C8 and 2E1. These data suggest the possibility that multiple P450 isoforms may be involved in human GBR12909 metabolism but that CYP3A appears to be the major enzyme responsible for human GBR12909 biotransformation.

Cocaine is generally acknowledged as one of the most addictive substances (Das, 1993), and drug abuse with cocaine continues to be a major public health concern (Musto, 1992). The development of high-affinity dopamine (DA) reuptake inhibitors as cocaine antagonists or substitutes represents a significant advance in the search for effective treatments of cocaine addiction (Baumann et al., 1994; Rothman and Glowa, 1995).

Vanoxerine (1-[2-[bis(4-fluorophenyl)methoxy]ethyl]-4-(3-phenylpropyl)piperazine; GBR12909) (Fig. 1) is a long-acting inhibitor of DA uptake, which selectively binds to the DA transporter with a higher affinity than cocaine itself and has very low abuse liability (Ritz et al., 1987; Wise and Bozarth, 1987; Koob and Bloom, 1988; Kuhar et al., 1991). Considerable effort has been expended in recent years in the investigation of behavioral and physiological effects as well as molecular events that occur after exposure to cocaine versus GBR12909 (Tella et al., 1996). Numerous studies have shown that
pretreatment with GBR12909 reduces episodes of cocaine self-administration in animal models (Skjoldager et al., 1993; Głow et al., 1995a,b; Tella, 1995; Villemagne et al., 1999), and GBR12909 appears to alleviate withdrawal symptoms (Van Der Zee et al., 1980; Heikila and Manzino, 1984; Westerink et al., 1987; Andersen, 1989; Nissbrandt et al., 1991; Rothman et al., 1991). The proposed mechanism for GBR12909 effect involves slow dissociation of GBR12909 from the DA transporter, which prolongs the action of drug and may be useful as a cocaine antagonist. These characteristics of GBR12909 have made it a candidate for a potential medication that limits the use of cocaine.

Multiple drug therapy is common in drug dependence treatment, and may lead to the occurrence of deleterious drug-drug interactions. It is important to determine which enzymes are responsible for the metabolism of medications during drug development to be able to effectively plan clinical trials and ultimately produce a more informed drug label. Therefore it is necessary to assess the enzymatic pathways (inhibition spectrum) for drug candidates such as GBR12909, which could become an effective treatment for cocaine addiction. Incubation of new drug candidates with substrates/inhibitors of specific cytochrome P450 isoforms with human liver microsomes and hepatocytes is a powerful tool in the characterization of their P450-mediated metabolism. There are no studies reported for elucidation of metabolic pathways involved in GBR12909 metabolism. The goal of this study was to assess which human P450 enzymes metabolize GBR12909 in vitro.

Materials and Methods

Reagents. GBR12909, GBR12935 [1-[2-(diphenylmethoxy)ethyl]-4-(3-phenylpropyl)piperazine] (Fig. 1), chloroxazone, sulfaphenazole, ketocoonazole, quinidine sulfate, and quercetin were purchased from Sigma Chemical Co. (St. Louis, MO), as were constituents of the NADPH-generating system (0.1 M Na2HPO4, glucose 6-phosphate, glucose-6-phosphate dehydrogenase). Methylenedioxyphenylmethylglycine (MDP) was obtained from the United States Pharmacopeia Convention (Rockville, MD). Reaction buffer (0.1 M NaH2PO4, glucose 6-phosphate, 1 mM NADP+) was preincubated for 3 min at 37°C before use. Mephenytoin was obtained from the United States Pharmacopeia Convention (Rockville, MD). Reaction buffer (0.1 M Na+ and K+ phosphate, 1 mM EDTA, 5.0 mM MgCl2; pH 7.4) was purchased from Quality Biological, Inc. (Gaithersburg, MD). Ciprofloxacin was obtained from U.S. Food and Drug Administration. Human hepatocytes were obtained from In Vitro Technologies, Inc. (Baltimore, MD). Human B-lymphoblastoid-derived cytochrome microsomes, CYP3A4, CYP2C8, and CYP2E1 were purchased from GENTEST Corp. (Woburn, MA). HPLC grade acetonitrile, water, and phosphoric acid were obtained from Fisher Scientific (Pittsburgh, PA).

Preparation of Microsomes. Human livers, medically unsuited for transplantation, were obtained through the Washington Regional Transplant Consortium, Washington, DC. Microsomes were prepared by differential centrifugation of liver homogenates. Liver microsomes from three individual liver donors and one pooled microsomal suspension from four additional donors were used in this study. Data describing the concurrent medications given to the donor for each liver sample were reviewed for potential inhibitors of P450 metabolism, and none were found. Microsomal protein concentration was measured using the Bio-Rad protein assay kit, Bio-Rad Laboratories (Richmond, CA). Microsomes were stored at −80°C until use.

Microsomal Incubation. Human liver microsomal suspension (1 ml), prepared in reaction buffer at a concentration of 0.5 mg microsomal protein/ml, was preincubated for 3 min at 37°C with an NADPH-generating system (10 mM glucose 6-phosphate, 1 mM NADP+, and 1 unit/ml yeast glucose-6-phosphate dehydrogenase). For inhibition studies, 10 μM of 100-fold concentrated cytochrome P450 inhibitor solutions prepared in ethanol were added to 0.92 ml of the microsomal suspension before incubation. The reactions were initiated with the addition of varying concentrations of aqueous solutions of GBR12909. The final incubation concentrations for GBR12909 utilized in this study were 0.1, 0.2, 0.5, and 1 μM. Incubation samples were placed on ice (4°C) after 10 min of incubation in a shaking water bath (37°C). The incubation reaction with human B-lymphoblastoid microsomes started with the addition of microsomes to the incubation mixture and was terminated after 2 h of incubation by placing the incubation mixture on ice. Control reactions for all experiments included the same solvent (10 μl of ethanol) without inhibitors.

Incubation with Human Hepatocytes. Human hepatocytes at 1 × 106 cells/ml were placed in a 24-well plate and incubated at 5% CO2, 37°C. Hepatocyte culture medium (Dulbecco’s modified Eagle’s medium based media, In Vitro Technologies, Inc.) was replaced with 500 μl of the solutions of GBR12909 and inhibitors prepared in hepatocyte culture medium. After incubation for 4 h at 37°C in 5% CO2, the medium was harvested from the wells to the glass tubes, and sample preparation was initiated.

Sample Preparation. GBR12935 in water (100 μl of 0.5 μg/ml) was added to the incubation mixture as an internal standard, followed by liquid-liquid extraction with 5 ml of heptane/ethyl acetate mixture (9:1), mixing (speed 5) for 10 min with a multiblade vortexer S/P (Baxter Diagnostic Inc., Deerfield, IL), and centrifuging (3500 revolutions/min) (Sorvall RT 6000D, PerkinElmer Life Sciences, Boston, MA) at 0°C for 10 min. After freezing the lower aqueous layer of the samples (15 min, −80°C), the organic layer was decanted into a glass tube and evaporated to dryness with a Heto Speed-Vac apparatus (Heto Laboratory Equipment A/S, Copenhagen, Denmark) for approximately 30 min at 60°C. The evaporative residues were reconstituted with 30 μl of water/acetonitrile (1:1, v/v) and mixed for 5 min with a multiblade vortexer S/P (Baxter Diagnostic Inc.). The HPLC injection volume for each sample was 5 μl of the reconstituted solution.

Chromatography. All analyses were conducted on a Hewlett Packard 1100 with 1046A fluorescence detector set at an excitation wavelength of 200 nm and emission wavelength of 284 nm; 100 μl column 150 × 2.1 mm by Phenomenex (Torrance, CA) and guard column Javelin/Prism (Keystone Scientific Inc., Bellefonte, PA)); column heater temperature, 35°C; injection volume, 5 μl; run time, 50 min. Samples were eluted with water (A) and 0.2% phosphoric acid in acetonitrile (B) as follows: the initial eluent profile was 80% A at 0.20 ml/min, then both B and the flow rate increased linearly to 98% and 0.25 ml/min, respectively, over 40 min; B and the flow rate decreased linearly to 20% and 0.2 ml/min, respectively, over 10 min; the column was equilibrated with 80% A for 10 min. Retention times averaged 16.8, 17.7, and 19.3 min for GBR12909 metabolite, GBR12935, and GBR12909, respectively.

Statistical Analysis. Statistical differences between observed effects of selective P450 inhibitors on metabolism of GBR12909 were determined using one-way analysis of variance with the Scheffe’s post hoc test. The level of statistical significance was set at p < 0.05.

Results

Biotransformation of GBR12909 by Human Liver Microsomes and Hepatocytes. Three individual human livers (1 male, 2 female), one pooled microsomal suspension from four additional human livers (2 male, 2 female), and human hepatocytes (female) used in this study were from donors 16 to 49 years old. Incubations with human liver microsomes, human B-lymphoblastoid microsomes, and primary hepatocytes showed that GBR12909 was metabolized to a single metabolite (Fig. 2). These findings reflect the same pattern of GBR12909 biotransformation observed in healthy volunteers. GBR12909 biotransformation was limited to microsomal fractions, was protein and NADPH-dependent, and was time-linear for 12 min (data not shown).

Effect of Selective Cytochrome P450 Inhibitors on GBR12909 Metabolism in Human Liver Microsomes. We used a range of GBR12909 concentrations (0.2–1 μM) with recommended concentrations of inhibitors known to be relatively selective for individual P450 isoforms (Fitzsimmons and Collins, 1997). A summary of the effects of seven specific CYP450 isoforms on GBR12909 metabolism in liver microsomes is presented in Table 1. Inhibition of CYP3A and CYP2C8 (ketocoza) and quercetin) produced concentration-dependent decreases in metabolite production (Fig. 3). Both inhibitors demonstrated statistically significant inhibition of GBR12909 metabolism (p < 0.05). Ketocoza at a concentration of 3 μM inhibited the formation of GBR12909 metabolite by 92 ± 2% with respect to
control incubations (Table 1), which is a strong indication of CYP3A4 primacy in GBR12909 metabolism. Quercetin at concentrations of 10 and 100 µM was a less effective inhibitor of GBR12909 metabolism producing a 37 ± 22 and 62 ± 22% of inhibition, respectively. The remaining cytochrome P450 inhibitors tested [ciprofloxacin (CYP1A2), sulfaphenazole (CYP2C9), quinidine (CYP2D6), chlorzoxazone (CYP2E1), and mephenytoin (CYP2C19)] did not demonstrate statistically significant inhibition (p > 0.05) of GBR12909 biotransformation in liver microsomes compared with the respective control incubations.

Effect of Selective Cytochrome P450 Inhibitors on GBR12909 Metabolism in Human Hepatocytes.

The same inhibitors chosen for study with human liver microsomes were also investigated for their ability to inhibit GBR12909 metabolism in cultured human hepatocytes. Among the tested inhibitors, ketoconazole (3 µM) was the most potent, with 92 ± 0.4% of inhibition of GBR12909 metabolite formation with respect to controls (p < 0.05). Chlorzoxazone (10 µM) significantly inhibited GBR12909 metabolite formation by 71 ± 19% with respect to controls. Inhibition of GBR12909 metabolite by 100 µM quercetin was 54 ± 35% of controls (p < 0.05). The other P450 inhibitors tested in this experiment did not inhibit GBR12909 biotransformation more than 25% (Table 1). These data indicate a predominant role for CYP3A4 in GBR12909 metabolism in these human drug-metabolizing systems with the possible involvement of other P450 isoforms (2E1, 2C8).

Human Recombinant Cytochrome P450-Dependent Biotransformation of GBR12909. Incubation with human recombinant cytochrome P450 3A4 (CYP3A4) resulted in production of GBR12909 metabolite with a qualitatively similar profile to that obtained with both human liver microsomes and hepatocytes (Fig. 2) and was substrate concentration-dependent (Fig. 4). Human recombinant CYP2C8 and CYP2E1 appeared to metabolize GBR12909 to a lesser extent. The product of GBR12909 biotransformation by CYP3A4 was 8 and 8.5 times greater than that seen with CYP2C8 and CYP2E1 when expressed per milligram of protein, respectively (Fig. 4). Because P450 3A4 contributes up to 40% of the total cytochrome P450 in human livers, and all of our data was normalized per milligram of protein, we may expect that CYP3A4 has an even greater impact on GBR12909 metabolism in vivo.

Discussion

Due to the high likelihood of use of concomitant medications by patients being treated for cocaine abuse, it is important to understand the potential for metabolism-based drug-drug interactions for any agent being considered for use as a therapy for this condition. The DA reuptake blocker GBR12909 is a promising cocaine antagonist (Tella et al., 1996). Important metabolism-based pharmacokinetic issues need to be considered prior to the widespread clinical testing of this cocaine antagonist.

GBR12909 has previously been suggested to be extensively metabolized in man presumably due to a saturable first-pass metabolism (Ingwersen et al., 1993). The reported Cmax obtained at steady state during oral dosing of 125 mg of vanoxerine (GBR12909) in 14 healthy male volunteers was 0.24 ± 0.09 µM. The in vitro metabolism of GBR12909 has not been studied previously in tissues from any species. We studied the metabolism of GBR12909 via cytochrome P450 in vitro in human liver microsomes, heterologously expressed P450s, and primary hepatocytes using GBR12909 concentrations similar to the reported range of circulating plasma levels (240 nM) in patients taking this agent at doses of 125 mg daily. This report, for the first time, establishes that CYP3A4 is involved in GBR12909 metabolism.

The study of chemical inhibition performed in human liver microsomes showed a concentration-dependent decrease in GBR12909 metabolite production by a CYP3A4-selective inhibitor ketoconazole at concentrations of 1 and 3 µM by more than 70% over the range of concentrations of GBR12909 examined. Quercetin, a specific inhibitor of CYP3A4, was also effective in decreasing GBR12909 biotransformation (Fig. 3). This indicates primacy of CYP3A4 in GBR12909 metabolism. Furthermore, recombinant CYP3A4 demonstrated the most activity of all recombinant enzymes studied in GBR12909 metabolism (Fig. 4). A role of CYP3A4 in the GBR12909 biotransformation was also shown in the experiments with primary human hepatocytes, containing a full complement of human drug-metabolizing enzymes and therefore representing a relevant experimental system most appropriate for in vivo modeling of drug metabolism (Table 1) (Li, 1997; Li et al., 1999). The substantive inhibition of GBR12909 biotransformation by low concentration of ketoconazole is solid evidence for the involvement of the CYP3A family.

The extent of involvement of P450 isoforms other than 3A4 is not completely understood and may involve CYP2C8 and CYP2E1. Recombinant CYP2C8 and CYP2E1 metabolized GBR12909 with quan-
titatively small production of metabolite (Fig. 4) and therefore can be considered as candidates in the catalysis of GBR12909 biotransformation. In addition, chlorzoxazone, a selective inhibitor of CYP2E1, inhibited GBR12909 biotransformation by 71% in primary hepatocytes. However, based on the relatively low concentrations of CYP2C8 and CYP2E1 in human liver microsomes, it can reasonably be concluded that CYP3A4 is the major enzyme involved in GBR12909 biotransformation.

Ciprofloxacin (CYP1A2 inhibitor), sulfaphenazole (2C9), quinidine (2D6), and mephenytoin (2C19) did not significantly modify GBR12909 in vitro biotransformation in human liver microsomes, and produced less than 25% of inhibition in hepatocytes, suggesting that the respective P450 isoforms are not major enzymes for this substrate.

GBR12909 may be eventually shown to be a useful pharmacotherapeutic agent for cocaine addiction. The management of substance abuse patients being treated with GBR12909 requires a knowledge of potential drug-drug interactions.

Cocaine is metabolized by both hydrolytic and oxidative pathways with the major route of hydrolysis by liver esterases and plasma cholinesterase (Kloss et al., 1984) to the main metabolites ecgonine methyl ester and benzoylecgonine. CYP3A4 has been identified in humans as the enzyme responsible for sequential cocaine oxidation to norcocaine, N-hydroxynorcocaine, and norcocaine nitroxide (Bornheim, 1998). The fact that both GBR12909 and cocaine are substrates of CYP3A4 (Ladona et al., 2000) may raise concerns for a potential of cocaine-GBR12909 interaction leading to possible inhibition of each substrate’s CYP3A metabolism. However, norcocaine is a minor metabolite that accounts for only 2 to 6% of the administered cocaine dose (Inaba et al., 1978), and thus the effect of GBR12909 on cocaine metabolism will probably be a negligible one if any.

The findings of the present study suggest possible interactions between GBR12909 and drugs known to interact with P450 3A4 producing metabolic inhibition or induction of GBR12909. Coadministration of GBR12909 with specific inhibitors/inducers of P450 3A4 such as ketoconazole, ritonavir, and rifampin could alter expected therapeutic effects in cocaine-abusing individuals. Our results suggest that clinical studies of GBR12909 with CYP3A inhibitors and inducers are necessary to fully characterize potential of in vivo interactions.

### TABLE 1

Effect of cytochrome P450-selective inhibitors on GBR12909 (0.5 μM) biotransformation in human liver microsomes and primary hepatocytes

Values are the mean ± S.D. for triplicate incubations. Statistical differences between treatments compared with GBR12909 controls (incubations without inhibitors).

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Inhibitor Concentration</th>
<th>CYP450</th>
<th>Percentage of Control (Primary Human Hepatocytes)</th>
<th>Percentage of Control (Human Liver Microsomes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ciprofloxacin</td>
<td>200 μM</td>
<td>1A2</td>
<td>74.9 ± 2.2</td>
<td>121.8 ± 53.4</td>
</tr>
<tr>
<td>Sulfaphenazole</td>
<td>100 μM</td>
<td>2C9</td>
<td>87.5 ± 0.9</td>
<td>71.7 ± 25.5</td>
</tr>
<tr>
<td>Quinidine</td>
<td>1 μM</td>
<td>2D6</td>
<td>82.5 ± 4.3</td>
<td>95.8 ± 12.1</td>
</tr>
<tr>
<td>Chlorzoxazone</td>
<td>10 μM</td>
<td>2E1</td>
<td>83.1 ± 19.9</td>
<td>76.9 ± 30.5</td>
</tr>
<tr>
<td>Quercetin</td>
<td>100 μM</td>
<td>2C8/3A4</td>
<td>28.5 ± 18.5*</td>
<td>66.1 ± 17.8*</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>1 μM</td>
<td>3A4</td>
<td>46.5 ± 35.3*</td>
<td>39.2 ± 21.6*</td>
</tr>
<tr>
<td>Mephenytoin</td>
<td>10 μM</td>
<td>2C19</td>
<td>7.6 ± 0.4*</td>
<td>7.9 ± 2.3*</td>
</tr>
<tr>
<td></td>
<td>100 μM</td>
<td></td>
<td>100.7 ± 18.8</td>
<td>88.2 ± 33.9</td>
</tr>
</tbody>
</table>

* Statistically significant (p < 0.05).

### FIG. 3.

Substrate concentration dependence and effect of 3 μM ketoconazole and 100 μM quercetin on GBR12909 biotransformation in human liver microsomes.

The y-axis represents GBR12909 metabolite formation expressed as ratio = peak height of metabolite/peak height of internal standard.

### FIG. 4.

Biotransformation of GBR12909 at 0.5 and 1 μM in microsomes containing c-DNA expressed in human P450.

All incubations were for 2 h. Values are the mean ± S.D. for triplicate incubations. The y-axis represents GBR12909 metabolite formation expressed as ratio = peak height of metabolite/peak height of internal standard.
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References


