INTERACTION OF CISAPRIDE WITH THE HUMAN CYTOCHROME P450 SYSTEM: METABOLISM AND INHIBITION STUDIES

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ABSTRACT:

Using human liver microsomes (HLMs) and recombinant cytochrome P450s (CYP450s), we characterized the CYP450 isoforms involved in the primary metabolic pathways of cisapride and documented the ability of cisapride to inhibit the CYP450 system. In HLMs, cisapride was N-dealkylated to norcisapride (NORCIS) and hydroxylated to 3-fluoro-4-hydroxycisapride (3-F-4-OHCIS) and to 4-fluoro-2-hydroxycisapride (4-F-2-OHCIS). Formation of NORCIS, 3-F-4-OHCIS, and 4-F-2-OHCIS in HLMs exhibited Michaelis-Menten kinetics (Kₘ; 23.4 ± 8.6, 32 ± 11, and 31 ± 23 μM; Vₘₐₓ; 155 ± 91, 52 ± 23, and 31 ± 23 pmol/min/mg of protein, respectively). The average in vitro intrinsic clearance (Vₘₐₓ/Kₘ) revealed that the formation of NORCIS was 3.9- to 5.9-fold higher than that of the two hydroxylated metabolites. Formation rate of NORCIS from 10 μM cisapride in 14 HLMs was highly variable (range, 4.9-133.6 pmol/min/mg of protein) and significantly correlated with the activities of CYP3A (r = 0.86, P = .0001), CYP2C19, and 1A2. Of isoform-specific inhibitors, 1 μM ketoconazole and 50 μM troleandomycin were potent inhibitors of NORCIS formation from 10 μM cisapride (by 51 ± 9 and 44 ± 17%, respectively), whereas the effect of other inhibitors was minimal. Of 10 recombinant human CYP450s tested, CYP3A4 formed NORCIS from 10 μM cisapride at the highest rate (V = 0.56 ± 0.13 pmol/min/pmol of P450) followed by CYP2C8 (V = 0.29 ± 0.08 pmol/min/pmol of P450) and CYP2B6 (0.15 ± 0.04 pmol/min/pmol of P450). The formation of 3-F-4-OHCIS was mainly catalyzed by CYP2C8 (V = 0.71 ± 0.24 pmol/min/pmol of P450) and that of 4-F-2-OHCIS by CYP3A4 (0.16 ± 0.03 pmol/min/pmol of P450). Clearly, recombinant CYP2C8 participates in cisapride metabolism, but when the in vitro intrinsic clearances obtained were corrected for abundance of each CYP450 in the liver, CYP3A4 is the dominant isoform. Cisapride was a relatively potent inhibitor of CYP2D6, with no significant effect on other isoforms tested, but the Kᵢ value derived (14 ± 16 μM) was much higher than the clinically expected concentration of cisapride (<1 μM). Our data suggest that CYP3A is the main isoform involved in the overall metabolic clearance of cisapride. Cisapride metabolism is likely to be subject to interindividual variability in CYP3A expression and to drug interactions involving this isoform.

Cisapride, (±)cis-4-amino-5-chloro-N-[3-(4-fluorophenoxypyrol]-3-methoxy-4-piperidinyl]-2-methoxybenzamide (Fig. 1), is a gastrointestinal prokinetic agent that has been widely used in adults and children for the treatment of dyspepsia, gastrointestinal reflux diseases, and other upper gastrointestinal motility disorders (McCallum et al., 1988; Vandenplas et al., 1999). In contrast to the older prokinetic drugs (e.g., metoclopramide), cisapride is devoid of antidiopaminergic properties and does not affect psychomotor function or induce central depressant adverse effects (McCallum et al., 1988).

The most frequently reported adverse effect of cisapride is transient abdominal pain (McCallum et al., 1988). However, several clinical case reports and studies have linked the use of cisapride to rare, but life-threatening ventricular arrhythmias, some of which are fatal (reviews: Wysowski and Bacsanyi, 1996; Tonini et al., 1999; Walker et al., 1999). The mechanism of proarrhythmic action of cisapride is probably due to its ability to lengthen cardiac action potential and Q-T intervals as a result of a blockade of the rapid component of the delayed rectifier potassium current (Carlsson et al., 1997; Drolet et al., 1998). The association of cisapride with cardiac toxicity has become a major safety concern in recent years to researchers, regulatory authorities, prescribing physicians, and, not least, to patients. Alarmed by this, the United States Food and Drug Administration has warned physicians against the use of cisapride except as a last resort (Josephson, 1998), and very recently released a renewed strong warning for cisapride after it reviewed 250 heart rhythm disorders (including 70 fatalities) associated with cisapride use (FDA, 2000).

Although cardiac toxicity of cisapride has been observed in patients taking therapeutic doses of the drug, this risk appears to be rare in the absence of other confounding factors. Accumulation of unmetabolized cisapride probably resulting from coadministration of drugs that slows its elimination (Bedford and Rowbotham, 1996) or from overdose (Bran et al., 1995) appear to be important determinants of cisapride cardiac toxicity. The potential for cisapride to bring about cardiotoxicity appears to be directly proportional to its plasma concentrations. Recently, it has been shown that repeated administration of clarithromycin to human volunteers increased the plasma concentrations of cisapride, which was associated with Q-Tc interval prolongation (van Haarst et al., 1998). Although clarithromycin is known to inhibit CYP3A and transport proteins such as P-glycoproteins, cisapride does

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Cisapride

Fig. 1. Chemical structure of cisapride and sites of N-dealkylation and hydroxylation pathways.

not seem to be a P-glycoprotein substrate (Abdel-Rahman et al., 2000). It follows that variability of cisapride metabolism in subsets of patients due to genetic polymorphism in metabolic pathways or exposure to other drugs, foods, and herbal medicines may expose patients to the clinical occurrence of Q-T prolongation and ventricular arrhythmia.

Indirect evidence from the literature implicates the cytochrome P450 (CYP450) system in the metabolism of cisapride, but, despite the clinical use of the cisapride for over a decade and the implication of altered metabolism in its cardiac risk, the specific isoforms involved are not fully characterized. Based on urinary excretion data in humans, cisapride appears to undergo primarily hepatic N-dealkylation to norcisapride (NORCIS), which accounts for 41 to 45% of the administered dose, aromatic hydroxylation to 3-fluoro-4-hydroxycisapride (3-F-4-OHCIS), and 4-fluoro-2-hydroxycisapride (4-F-2-OHCIS) (Meuldermans et al., 1988). Warning in the label states that cisapride is metabolized by CYP3A and advises against prescribing cisapride; HLMs, human liver microsomes; Cl int, intrinsic clearance.

Materials and Methods

Chemicals. Cisapride was purchased from Research Diagnostics, Inc. (Flanders, NJ). Dextromethorphan HBr, phenytoin, chlorpropamide, quinidine sulfate, tolbutamide, quercetin, diethyldithiocarbaminate, troleanomycin, ketoconazole, phenacetin, acetaminophen, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, NADP, and EDTA were purchased from Sigma Chemical Co. (St. Louis, MO). Sulfaphenazole, furafylline, S-mephenytoin, 4-hydroxymephenytoin, and 4'-methylhydroxytolbutamide were obtained from Ultrafine Chemicals (Manchester, England). Levallorphan was obtained from U.S. Pharmacopeia Convention (Rockville, MD). Dextrophan and 3-methoxyphorphin were purchased from Hoffman-La Roche, Inc. (Nutley, NJ). Omeprazole was a gift from Dr. Tommy Anderson (Clinical Pharmacology, Astra Hässle AB, Möln达尔, Sweden). N-(4-hydroxyphenyl)butamide was provided by Dr. John Strong (Division of Clinical Pharmacology, Center for Drug Evaluation and Research, U.S. Food and Drug Administration, Rockville, MD). Cisapride metabolites, NORCIS, 3-F-4-OHCIS, and 4-F-2-OHCIS were supplied by Dr. Russell Gotschall (Department of Clinical Pharmacology and Therapeutics, Children’s Mercy Hospital, Kansas City, MO). Other reagents were of HPLC grade.

Human Liver Microsomes (HLMs) and Recombinant Human CYP450s. The HLMs were prepared from human liver tissues that were medically unsuitable for liver transplantation and frozen at −80°C within 3 h of cross-clamp time. Microsomal fractions were prepared and pellets were suspended in a reaction buffer to a protein concentration of 10 mg/ml (stock) and were kept at −80°C until used (see Desta et al., 1998). Protein concentrations were determined using the Bradford method (Pillard et al., 1978). The activity of each isoform in the HLMs used was determined using isoform-specific substrate reaction probes and the apparent kinetic parameters (Km and Vmax) of these probes were documented for the human livers used as previously described (Ko et al., 1997; Desta et al., 1998). Baculovirus insect cell-expressed human CYP450s (1A1, 1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1, and 3A4) (with reductase) and CYP2C8 antibody (with nonimmune serum) were purchased from Gentest Corporation (Woburn, MA) and stored at −80°C. Protein concentrations, CYP450 contents, and specific activity were as supplied by the manufacturer. Microsomes were thawed on ice before use.

Incubation Conditions. To define suitable conditions for incubation and HPLC analysis, a range of cisapride concentrations (0–200 μM) and a NADPH-generating system (13 mM NADP, 33 mM glucose-6-phosphate, 33 mM MgCl2, and 0.4 U/ml glucose-6-phosphate dehydrogenase) in potassium phosphate reaction buffer (pH 7.4) were prewarmed for 5 min at 37°C. A stock solution of 10 mg/ml of cisapride (21.5 mM) was prepared in 1 ml of 0.1 N HCl/methanol mixture (86 μl of concentrated HCl and 9.914 ml of 100% methanol) and serially diluted with water to the required concentration (final methanol and HCl concentrations <0.1 and 0.05%, respectively). Reactions were initiated by adding 25 μl of microsomes (0.1–1 mg protein/ml) or 25 μl of recombinant human CYP450 isoforms (diluted to 250–500 pmol of P450/ml with buffer, pH 7.4). The mixture was then incubated for 0 to 150 min at 37°C (final incubation volume, 250 μl). The reactions were terminated by placing tubes on ice, immediately adding 200 μl of acetonitrile, and vortex mixing. The samples were then centrifuged at 14,000 rpm for 5 min in an Eppendorf model 5415C centrifuge (Brinkman Instruments, Westbury, NY). Aliquots of supernatant (100 μl) were injected into the HPLC system without additional extraction. All subsequent incubations were run in duplicate, and less than 5% of the substrate was consumed during the incubations. Negative control incubations for each experiment were carried out by excluding either the substrate, NADPH-generating system, or microsomes (BSA was used instead) in the incubation mixture.

Assay of Racemic Cisapride and Its Metabolites. An HPLC method with fluorescent detection for plasma cisapride assay was modified to measure cisapride and its metabolites in human liver microsomal incubations (Preecha- goon and Charles, 1995). The HPLC system consisted of a Waters Associates model 600 dual piston pump (Milford, MA), a Waters Associates model 717 autosampler, a Waters model 996 PDA detector, and an FD-300 Dual Monochromator fluorescence detector (GTI Holding Co., Concord, MA). The separation column consisted of a (150 mm x 3.9 mm i.d.) stainless steel symmetry column (Symmetry) packed with 5-μm particle size (100 Å pore size) RP-C-8 (Waters) and a Waters Nova-Pack C18 guard column (4 μm, 60 Å). The mobile

1 Abbreviations used are: CYP450, cytochrome P450; NORCIS, norcisapride; 3-F-4-OHCIS, 3-fluoro-4-hydroxycisapride; 4-F-2-OHCIS, 4-fluoro-2-hydroxycisapride; HLMs, human liver microsomes; Cl int, intrinsic clearance.
Fig. 2. HPLC traces of cisapride and its metabolites (NOR CIS, 3-F-4-OH CIS, and 4-F-2-OH CIS) from in vitro incubation with HLMs.

Retention times of NOR CIS, 3-F-4-OH CIS, 4-F-2-OH CIS, and cisapride were about 2.3, 4.4, 6.9, and 8.8 min, respectively. Reference synthetic metabolites were injected onto HPLC either directly or after spiking them to incubation mixture containing inactive microsomes.
phase was composed of 20% methanol, 17% acetonitrile, and 0.5% triethyl-
amine in 50 mM NaH$_2$PO$_4$ buffer (adjusted to pH 3.0 using 1% phosphoric
acid). The operating temperature was 20°C and the flow rate 1.0 ml/min. The
column elute was monitored using fluorescence detection at an excitation
wavelength of 247 nm and emission wavelength of 350 nm.

We noted three fluorescent metabolite peaks at retention times of 2.3, 4.4,
and 6.9 min (cisapride at 9.8 min); their formation was dependent on a
NADPH-generating system, cisapride, and microsomal protein concentrations
as well as time of incubation. An incubation time of 30 min, a protein
concentration of 0.5 mg/ml, and a cisapride concentration of 10 μM represent
linear ranges for the formation of these metabolites and were used in the
ensuing experiments unless stated otherwise.

The identities of the metabolite peaks were determined by comparing their
retention times to those of reference peaks of synthetic cisapride metabolites:

\[
\begin{array}{cccc}
\text{HLMs} & \text{NORCIS} & 3\text{-F-4-OHCIS} & 4\text{-F-2-OHCIS} \\
& K_m & V_{max} & V_{max}/K_m & K_m & V_{max} & V_{max}/K_m & K_m & V_{max} & V_{max}/K_m \\
8 & 11.3 & 52 & 4.6 & 33 & 28.1 & 0.85 & 7.7 & 14.6 & 1.9 \\
13 & 23.6 & 278 & 11.8 & 29.4 & 40.2 & 1.4 & 33.2 & 15.6 & 0.47 \\
16A & 23.1 & 210 & 9.1 & 43.7 & 85.7 & 2.0 & 69.5 & 73.7 & 1.1 \\
16B & 23.6 & 141 & 6.0 & 14.6 & 42.5 & 2.9 & 19.4 & 38.5 & 1.8 \\
29 & 35.6 & 93 & 2.6 & 40 & 65 & 1.6 & 26.7 & 9.9 & 0.37 \\
\text{Mean} & 23.4 & 154.8 & 6.6 & 32.1 & 52.3 & 1.74 & 31.3 & 30.5 & 1.16 \\
\pm \text{S.D.} & 8.6 & 90.6 & 3.6 & 11.3 & 22.9 & 0.78 & 23.4 & 26.6 & 0.70 \\
\end{array}
\]

$K_m$ (micromoles), $V_{max}$ (picomoles/minute/milligram of protein), and $V_{max}/K_m$ (microliters/minute/milligram)

\[\text{Fig. 3. Interindividual variation of cisapride metabolites formation from 10 μM cisapride in microsomes from 15 different human livers.}\]

Microsomes from HL1 are inactive and served as negative controls. Data are averages of duplicates.

TABLE 1

<table>
<thead>
<tr>
<th>HLMs</th>
<th>NORCIS</th>
<th>3-F-4-OHCIS</th>
<th>4-F-2-OHCIS</th>
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<td>$K_m$</td>
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<td>Mean</td>
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<tr>
<td>± S.D.</td>
<td>8.6</td>
<td>90.6</td>
<td>3.6</td>
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$K_m$ (micromoles), $V_{max}$ (picomoles/minute/milligram of protein), and $V_{max}/K_m$ (microliters/minute/milligram)
NORCIS, 3-F-4-OHCIS, and 4-F-2-OHCIS. The retention times of the reference metabolites were tested after direct injection and after adding them to incubation mixture that did not contain active microsomes. The first peak on the chromatograms corresponded with the retention time of NORCIS, the second with 3-F-4-OHCIS, and the third with 4-F-2-OHCIS. Subsequent experiments were designed to characterize the human CYP450 isoforms responsible for the formation of these three metabolites in HLMs or recombinant human CYP450s. Due to limited availability of the metabolites, the concentrations of metabolite were measured by comparing the metabolite peaks to standard curves obtained using known cisapride concentrations. The difference between the fluorescent activity of the metabolites and that of cisapride, determined by constructing standard curves from direct injections of known equimolar concentrations of synthetic reference metabolites and cisapride was very small (<10% at any concentration).

**Determination of Km and Vmax of Cisapride Metabolism.** Kinetic parameters for the formation of cisapride metabolites were obtained by incubating a range of cisapride concentrations (0–200 μM) with HLMs (or human recombinant CYP450) and an NADPH-generating system. An appropriate model for each function was selected to calculate kinetic parameters (see data analysis below).

**Correlation Experiments.** Cisapride (10 μM) was incubated with microsomes from 14 different HLMs (and one inactive HLM as a control) to test the correlation of cisapride metabolism with the activity of CYP1A2 measured by the O-deethylation of phenacetin (Tassaneeyakul et al., 1993), CYP2D6 measured by the O-demethylation of dextromethorphan (Broly et al., 1989), CYP3A measured by the 4-hydroxylation of midazolam (Thummel et al., 1994), CYP2C19 measured by the 4-hydroxylation of S-mephenytoin (Wrighton et al., 1993), and CYP2C9 measured by the 4-methylhydroxylation of tolbutamide (Relling et al., 1990).

**Inhibition of Cisapride Metabolism.** Formation of metabolites from 10 μM cisapride was evaluated in the absence (control) and presence of known isoform-specific inhibitors. The isoform-specific inhibitors of CYP450 isoforms we used and the appropriate concentrations are listed elsewhere (Clarke 1998; Desta et al., 1998). Tolbutamide and quercetin were tested as potential...
inhibitors of CYP2C8 (Desai et al., 1998). Cisapride was preincubated for 5 min at 37°C with or without CYP450 isoform specific inhibitor and with a NADPH-generating system. HLMs were added to initiate the reaction and incubated for 30 min at 37°C (final incubation volume, 250 μl). Troleandomycin and furafylline were first preincubated in the presence of a NADPH-generating system and HLMs at 37°C for 15 min, and the reaction was initiated by the addition of cisapride. All isoform specific inhibitors were studied at concentrations chosen to be selective for the respective CYP450 isoforms on the basis of published Ki values of the inhibitor probes. Rates of metabolite formation were compared with those of controls in which the inhibitor was replaced with an appropriate concentration of vehicle. Immunoinhibition of CYP2C8 was examined by incubating 10 μM cisapride with HLMs in the presence of increasing concentrations of polyclonal anti-CYP2C antibody or no immune serum.

**Metabolism of Cisapride by Recombinant Human CYP450s.** To further test the specific isoforms catalyzing cisapride metabolism, 10 μM cisapride was incubated with 25 μl of microsomes from recombinant human CYP450s 1A1, 1A2, 2A6, 2C19, 2C8, 2C9, 2D6, 2B6, 2E1, and 3A4 (250–500 pmol of P450/ml in potassium phosphate reaction buffer, pH 7.4) at 37°C for 30 min. All other conditions were the same as described for the incubations using HLMs. Because our preliminary data suggested the contribution of CYP3A4 and CYP2C8 in cisapride metabolism, full kinetics of cisapride metabolism were determined by incubating a range of cisapride concentrations (0–50 μM) with recombinant human CYP3A4 and CYP2C8 isoforms.

**Inhibition of CYP450 Isoforms by Cisapride.** The inhibitory effect of cisapride on the activities of five common drug-metabolizing CYP450 isoforms was tested in HLMs using probes selective for each isoform. The reaction probes used were phenacetin O-deethylation for CYP1A2 (Tassaneeyakul et al., 1993), tolbutamide 4-methylhydroxylation for CYP2C9 (Relling et al., 1990), omeprazole 5-hydroxylation for CYP2C19 (Ko et al., 1997), dextromethorphan O-demethylation for CYP2D6 (Broly et al., 1989), and dextromethorphan N-demethylation for CYP3A (Gorski et al., 1994). The incubation conditions and analytical assays for the activity of these isoforms are used routinely in our laboratory and have been described in more detail in our earlier works (Ko et al., 1997; Desta et al., 1998). In short, using incubation conditions specific for each isoform that were linear for time, substrate, and protein concentrations, isoform specific substrate probes were incubated in duplicate at 37°C with HLMs and a NADPH-generating system in the absence (control) or presence of varying concentrations of cisapride (0–100 μM). Because inhibition by cisapride of CYP2D6 was marked at the fixed substrate concentration (around the Km), the preliminary data obtained were simulated and used to generate optimal substrate and inhibitor concentrations for constructing Dixon plots in two different HLMs. From these data, precise inhibition constants (Ki values) for CYP2D6 (and CYP3A) were calculated.

**Data Analysis.** Kinetic parameters for the formation of each cisapride metabolite were performed by fitting the experimental data to appropriate one- or two-site models using nonlinear least square regression analysis (WinNonlin Software, version 1.5; Scientific Consulting Inc., Apex, NC). Initial parameter
The dominant fluorescent metabolite was NORCIS formed by gram from a microsomal incubation of cisapride is depicted in Fig. 2. with Michaelis-Menten kinetics. The apparent kinetic parameters (\( V_{\text{max}}/K_m \)) of cisapride metabolites by HLMs had a kinetic pattern consistent 3-F-4-OHCIS and 4-F-2-OHCIS represents minor peaks. The forma-
dedalkylation of cisapride, whereas aromatic hydroxylation to form
Correlation Studies. The formation rates of the individual metabolites from 10 \( \mu \)M cisapride were determined in 14 different HLMs (Fig. 3). The mean (\( \pm S.D. \)) for the formation rate (picomoles/minute/milligram of protein) of NORCIS, 3-F-4-OHCIS, and 4-F-2-OHCIS were 52.9 \( \pm \) 40.7, 11.7 \( \pm \) 8.9, and 13.5 \( \pm \) 9.9, respectively. These formation rates showed considerable individual variation among the HLMs (e.g., 27-fold variation for NORCIS, range: 4.9–133.6 pmol/min/mg of protein). In all the samples examined, NORCIS was a predominant fluorescent metabolite (Fig. 3). The formation rate of NORCIS significantly correlated with the activity of CYP3A (Spearman \( r = 0.86, P = .0001 \)), CYP2C19 (\( r = 0.8, P = .0009 \)), and CYP1A2 (\( r = 0.69, P = .007 \)). The formation rates of 3-F-4-OHCIS were significantly correlated with the activity of CYP2C19 (\( r = 0.73, P = .005 \)) and CYP3A (and \( r = 0.58, P = .03 \)), whereas the formation rates of 4-F-2-OHCIS showed significant correlation with CYP2C19 activity (\( r = 0.57, P = .04 \)). We also noted significant correlation between the formation rates of the three metabolites (\( r = 0.66–0.8; P < .0001 \)).

Chemical Inhibition of Cisapride Metabolism. To characterize the potential contribution of CYP450 isoforms to cisapride metabolism, 10 \( \mu \)M cisapride was incubated in HLMs with or without isoform-specific inhibitors of CYP1A2, 3A, 2C19, 2C9, 2C8, 2E1, and 2D6. As demonstrated in Fig. 4, 1 \( \mu \)M ketoconazole and 50 \( \mu \)M troleandomycin inhibited cisapride metabolism to NORCIS by ~51 \( \pm \) 9 and 44 \( \pm \) 17%, respectively. The effect of increasing concentrations of ketoconazole and troleandomycin on cisapride metabolism is shown in Fig. 5. The average estimated \( K_i \) values for the inhibition of cisapride metabolism to NORCIS, 3-F-4-OHCIS, and 4-F-2-OHCIS by ketoconazole were 0.84, 1.5, and 3.7 \( \mu \)M and by troleandomycin were 22, 25, and 7 \( \mu \)M, respectively. The inhibitory effect of troleandomycin was characterized by a significant residual activity, especially with regard to the formation of NORCIS and 3-F-4-OHCIS. The marked residual activity after inhibition with troleandomycin was consistently observed in a panel of microsomes from six different human livers (Fig. 6).

Metabolism of Cisapride by Human Recombinant CYP450s. To further evaluate the potential of each CYP isoform to metabolize cisapride, we incubated microsomes from 10 recombinant human CYP450 isoforms with 10 \( \mu \)M cisapride (Fig. 7). Recombinant CYP3A4 formed NORCIS from cisapride at the highest rate (\( V = 0.56 \pm 0.13 \) pmol/min/pmol of P450) followed by CYP2C8 (\( V = 0.29 \pm 0.08 \) pmol/min/pmol of P450) and CYP2B6 (\( V = 0.145 \pm 0.04 \) pmol/min/pmol of P450). The formation of 3-F-4-OHCIS was mainly catalyzed by CYP2C8 (\( V = 0.71 \pm 0.24 \) pmol/min/pmol of P450) and to some degree by CYP3A4 (\( V = 0.099 \pm 0.01 \) pmol/min/pmol of P450). The formation of 4-F-2-OHCIS was solely mediated by CYP3A4 (0.16 ± 0.03 pmol/min/pmol of P450).

The kinetics for the formation of NORCIS, 3-F-4-OHCIS, and 4-F-2-OHCIS by recombinant human CYP3A4 and CYP2C8 are demonstrated in Fig. 8. The apparent kinetic parameters derived using nonlinear regression analysis clearly demonstrate the relative contributions of these two isoforms in the formation of each metabolite (Fig. 9). When calculated on the basis of picomoles of P450, CYP2C8 is an effective catalyst of the metabolism of cisapride to NORCIS and
3-F-4-OHCIS, but when the abundance adjusted Cl_{int} is calculated, CYP3A4 is clearly the dominant isoform. This estimation assumes that CYP3A and CYP2C8 represent 30 and 6% of the total immuno-blottable CYP450 proteins in the liver, respectively (Lin and Lu, 1998).

A significant problem in characterizing the contribution of CYP2C8 to the metabolism of a drug is the lack of specific inhibitor probe. We tested quercetin and tolbutamide as inhibitors of CYP2C8-catalyzed cisapride metabolism to NORCIS (Fig. 4) and 3-F-4-OHCIS (data not shown) in HLMs, but the effects were marginal. We also tested a polyclonal anti-CYP2C antibody that is considered to be relatively selective (at lower concentrations) toward this isoform than other CYP2C family members. The percentage of immunoinhibition for the formation of NORCIS and 3-F-4-OHCIS in HLMs we observed was less than 15% at any concentration used (data not shown).

**Inhibition of P450 by Cisapride.** Preliminary experiments were conducted to test the inhibitory effect of cisapride on different CYP450 isoforms. A range of cisapride concentrations was incubated...
with a single concentration (around its observed $K_m$ in HLMs) of each isoform-specific substrate reaction probe. As shown in Fig. 10, cisapride is a relatively potent inhibitor of CYP2D6 ($K_i = 70 – 84\%$ at concentrations $25 \mu M$ cisapride), whereas there was little inhibition ($<30\%$) of the other isoforms tested. These data were used to obtain an initial estimate of the inhibition constant ($K_i$ value) to choose appropriate substrate and inhibitor concentrations for constructing full Dixon plots for the inhibition of CYP2D6 and CYP3A. A representative Dixon plot was constructed by incubating a range of cisapride concentrations ($5–50 \mu M$) and dextromethorphan ($10–150 \mu M$) in HLMs (Fig. 11A) and the exact $K_i$ value was calculated by a nonlinear regression analysis after initial estimates were obtained from the Dixon plots. Consistent with the data in Fig. 10, cisapride showed potent and competitive inhibition of CYP2D6-catalyzed dextromethorphan $O$-demethylation reaction (Fig. 11A), with a mean $K_i$ of $14\pm16 \mu M$ ($n = 2$ HLMs). In the same incubations used to determine CYP2D6 inhibition, we simultaneously determined CYP3A-mediated dextromethorphan $N$-demethylation. Cisapride was a weak competitive inhibitor of this reaction ($K_i = 142 \pm 27 \mu M$) (Fig. 11B). Because there was no meaningful cisapride effect on other CYP450 isoforms tested (Fig. 10), we did not perform detailed experiments to estimate the inhibition constant for each isoform.

**Discussion**

In this study, we report the in vitro interaction of cisapride with the CYP450 system. Specifically, we characterized the metabolic pathways and the CYP450 isoforms involved in cisapride metabolism and determined the ability of cisapride to inhibit drug-metabolizing CYP450 isoforms. The data obtained should allow physicians to identify and predict drug interactions with cisapride.

We noted that cisapride is $N$-dealkylated to NORCIS and hydroxy-
lated to 3-F-4-OHCIS and 4-F-2-OHCIS. On the basis of the in vitro CL\textsubscript{int} (V\textsubscript{max} / K\textsubscript{m}) in HLMs, N-dealkylation of cisapride to NORCIS appears to be the major metabolic pathway of cisapride, accounting for \approx 56% of the total metabolic clearance, whereas both hydroxylation pathways seem to be minor (\approx 25%). These findings are consistent with the observation in humans that NORCIS is the major urinary metabolite of cisapride in humans, accounting for 41 to 45% of the administered dose (Meuldermans et al., 1988).

Our in vitro data strongly suggest that CYP3A is the major enzyme responsible for cisapride metabolism. First, the formation rates of the major cisapride metabolite, NORCIS, in 14 different HLMs showed significant correlation with the activity of CYP3A. Second, ketoconazole and troleandomycin were potent inhibitors of cisapride metabolism. Third, the formation of NORCIS and 4-F-2-OHCIS were catalyzed by recombinant human CYP3A4 with the highest specific activity. CYP3A also participates in the formation of 3-F-4-OHCIS. Fourth, cisapride was a competitive inhibitor of CYP3A-catalyzed dextromethorphan N-demethylation (Fig. 11B). Lastly, consistent with involvement of CYP3A in cisapride metabolism, several drugs that inhibit this isoform including clarithromycin (van Haarst et al., 1998), erythromycin (Bedford and Rowbotham, 1996), azole antifungals (Bedford and Rowbotham, 1996; Hoover et al., 1996), certain antidepressants (Owen and Nemeroff, 1998), diltiazem (Thomas et al., 1998), and grapefruit juice (Kivisto et al., 1999) have been suggested to increase the risk of cisapride cardiac toxicity. It follows that the safety and efficacy of cisapride is largely determined through modulation of CYP3A activity.

This study was conducted using HLMs, and thus reflects hepatic CYP450 activity. Significant CYP3A catalytic activity exists in the intestine, and for some drugs it is the major site that determines presystemic elimination of orally administered drugs and drug interactions (Lin et al., 1999). After oral administration of cisapride, only 40 to 50% of the dose is available to the systemic circulation (McCallum et al., 1988), suggesting first-pass metabolism. Expression of CYP3A in intestinal tissues is likely to contribute to cisapride presystemic metabolism, although this effect may not be as large as drugs exhibiting extensive first-pass metabolism (e.g., terfenadine and mizolastine), where modulation of CYP3A could lead to a severalfold difference in the area under the curve of the parent drugs (Backman et al., 1998; Thummel and Wilkinson, 1998; Lin et al., 1999).

Besides CYP3A, screening of cisapride metabolism with a panel of recombinant human CYP450 suggests that CYP2C8 is involved. Calculated on the basis of picomoles of P450, CYP2C8 was an efficient catalyst of 3-F-4-OHCIS formation, and significantly participated in the formation of NORCIS. However, the data obtained with recombinant human CYP2C8 is not in total agreement with those obtained from HLMs because inhibition of cisapride metabolism by quercetin, which has been used as a specific CYP2C8 inhibitor by some authors (Desai et al., 1998), and the effect of anti-CYP2C antibodies, was negligible. There are a number of possible explanations for these observations. First, despite the contribution of CYP2C8 to cisapride metabolism, inhibition of this isoform might not change the overall cisapride metabolism. Given the major involvement of CYP3A, it is likely that CYP3A takes over when the pathway mediated by CYP2C8 is inhibited. Second, it is important to take into account that CYP3A is the most abundant isoform in human liver.
accounting for about 30% of the total CYP450 content, whereas that of CYP2C8 is estimated to be ~6% (Lin and Lu, 1998). Although the $C_{\text{int}}$ for the calculation of NORCIS was greater for CYP2C8 (320 nM/pmol of P450) than for CYP3A (230 nM/pm mol of P450), the abundance-adjusted $C_{\text{int}}$ shows that the contribution of CYP2C8 is 3.6-fold lower than that of CYP3A4. Thus, only one-fourth (~11% of the total cisapride dose) of $C_{\text{int}}$ for NORCIS formation may be expected to be accounted for by CYP2C8-mediated metabolism. The contribution of CYP2C8 toward the $C_{\text{int}}$ for formation of 3-F-4-OHCIS appears to be high when calculated on the basis of picomoles of P450, the abundance-adjusted rates, however, were similar (53% by CYP2C8 and 47% by CYP3A4). Moreover, this metabolic pathway appears to be minor. Based on these assumptions, CYP2C8 may be responsible for only <20% of the total $C_{\text{int}}$ for cisapride metabolism. However, significant caveats must accompany this conclusion. Although recombinant human CYP2C8 appears capable of metabolizing several drugs including cisapride (present data), carbamazepine, and taxol (Clarke, 1998), the quantitative relevance of CYP2C8 enzyme in humans in terms of its contribution to drug interactions remains difficult to predict. This is because the data generated with recombinant CYP2C8 cannot be supplemented with detailed kinetic studies in HLMs, as specific chemical and immunoinhibitors of CYP2C8 have not been fully delineated. For these reasons, our conclusion regarding the contribution of CYP2C8 are solely dependent on data obtained from recombinant human CYP2C8. The clinical relevance of the in vitro CYP2C8 involvement we observed in this study in cisapride metabolism remains to be studied.

We tested the inhibitory effect of cisapride on the activity of drug-metabolizing CYP isoforms. Except for the activity of CYP2D6 in which cisapride was a relatively potent CYP2D6 inhibitor ($K_i \sim 15 \mu M$), our in vitro results show that cisapride is a weak inhibitor of other isoforms tested. Although cisapride is known to alter the pharmacokinetics of several coadministered drugs including morphine, diazepam, cyclosporin, ethanol, levodopa, and nifedipine (Bedford and Rowbotham, 1996), this is probably related to its ability to accelerate gastric emptying and thus increased absorption from the gastrointestinal site. Cisapride has been suggested to elevate plasma concentrations of bromperidol and its reduced metabolite through inhibition of CYP450s (Ishida et al., 1997), but a subsequent clinical study failed to reproduce this claim (Mihara et al., 1999). Similarly, cisapride has been reported to have no effect on the activity of CYP450s, as assessed by the clearance of antipyrine (Bedford and Rowbotham, 1996). Thus inhibition of CYP450 appears to play a limited role in cisapride-induced pharmacokinetic changes of coadministered drugs. Whether the inhibition of CYP2D6 we observed in this study is clinically relevant remains to be determined. The $K_i$ we estimated for the inhibition of this isoform is severalfold higher than the therapeutic plasma concentrations of cisapride, which does not normally exceed 200 nM (McCullum et al., 1988). The concentration of cisapride present in human liver after administration of therapeutic doses is difficult to predict. In rats, the concentration of cisapride in the liver is 35 times higher (~6 μM) than that measured in plasma (Michiels et al., 1987; McCullum et al., 1988). Even under this assumption, the $K_i$ we obtained is high and suggests no meaningful drug interactions. Moreover, we are unaware of any drug interaction between CYP2D6 substrates and cisapride that can be understood in terms of inhibition by cisapride of CYP2D6. However, the possibility that CYP2D6 inhibition coupled with the ability of cisapride to increase absorption of drugs by increasing gastrointestinal motility may result in important clinical interactions cannot be ruled out.

This study demonstrates that cisapride is metabolized in humans via N-dealkylation and aromatic hydroxylation, and these steps are catalyzed primarily by CYP3A. Of note, this isoform is the major CYP450 in the liver and intestine, exhibits highly variable expression, and is amenable to induction and inhibition by a large variety of xenobiotics (Lin and Lu, 1998; Thummel and Wilkinson, 1998). Patients may be placed at greater risk for therapeutic failure by drug interactions with inducers or toxicity by inhibitors of CYP3A than with other drugs that modify the levels and activity of other CYP450 enzymes. It is unlikely that cisapride is an important inhibitor of CYP450-mediated metabolism of coadministered drugs in vivo. Cisapride is a chiral molecule (Fig. 1) and we recognize the pharmacological and disposition properties of many chiral drugs are enantiomeric (Eichelbaum, 1988). Work is in progress in our laboratory to further characterize the stereoselective metabolism of cisapride.

References


