Stereoselective Metabolism of Cisapride and Enantiomer-Enantiomer Interaction in Human Cytochrome P450 Enzymes: Major Role of CYP3A

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Abstract

Cisapride is a chiral molecule that is marketed as a racemate consisting of two optical isomers, but little is known about its stereoselective metabolism. Studies with (−)-, (+)-, and (±)-cisapride were undertaken in human liver microsomes (HLMs) and recombinant cytochrome P450s (P450s) to determine the stereoselective metabolism and enantiomer-enantiomer interaction. Each enantiomer and racemic cisapride were N-dealkylated to norcisapride (NORCIS) and hydroxylated to 3-fluoro-4-hydroxycisapride (3-F-4-OHCIS) and 4-fluoro-2-hydroxycisapride (4-F-2-OHCIS). The kinetics for the formation of NORCIS from (−)-cisapride (K_m = 11.9 ± 4.8 μM; V_max = 203 ± 167 pmol/min/mg of protein) or (+)-cisapride (K_m = 18.5 ± 4.7 μM; V_max = 364 ± 284 pmol/min/mg of protein) in HLMs exhibited simple Michaelis-Menten kinetics, while a sigmoidal model characterized those of 3-F-4-OHCIS and 4-F-2-OHCIS. In vitro, NORCIS appears to be the major metabolite of both enantiomers. NORCIS and 3-F-4-OHCIS were preferentially formed from (+)-cisapride rather than (−)-cisapride, but that of 4-F-2-OHCIS was the reverse, suggesting regional and stereoselective metabolism. The formation rate of each metabolite from each enantiomer (20 μM) in 18 HLMs was highly variable (e.g., NORCIS, >35-fold) and correlated with the activity of CYP3A (r = 0.6–0.85; p < 0.05). Coincubation of troleandomycin (50 μM) with cisapride enantiomers (15 μM) in HLMs resulted in potent inhibition of NORCIS formation (by 75–80%), while other inhibitors showed negligible effect. Of 10 recombinant human P450s tested, CYP3A4 catalyzed the formation of NORCIS, 3-F-4-OHCIS, and 4-F-2-OHCIS from each enantiomer and racemic cisapride (15 μM) with the highest specific activity (K_m values close to those in HLMs). We noted that the rate of racemic cisapride metabolism by HLMs and recombinant human CYP3A4 is slower compared with equimolar concentrations of each enantiomer. When incubated simultaneously in HLMs, the enantiomers inhibit each other’s metabolism. In conclusion, our data demonstrate for the first time the stereoselective metabolism and enantiomer-enantiomer interaction of cisapride. Provided that the potency or the response of the enantiomers differ, understanding the factors that control their disposition as opposed to that of racemic cisapride may better predict adverse drug interactions and the resulting prokinetic efficacy and cardiac safety of cisapride.

Cisapride is a gastrointestinal prokinetic agent that has been widely used in adults and children for the treatment of gastrointestinal motility disorders, including dyspepsia, gastrointestinal reflux diseases, and gastroparesis (McCallum et al., 1988; Wiseman and Faulds, 1994). When cisapride was introduced as a prokinetic, it gained popularity over the older prokinetic drugs because it is devoid of dopamine receptor blockade and thus of neurological adverse effects (McCallum et al., 1988).

However, the cardiac safety of cisapride has become a serious concern in recent years. Although cisapride-induced tachycardia (Bateman, 1986) and dizziness resulting in clinical dropout (Francois and Nutte, 1987) have been reported as early as 1986 and reports of cardiac adverse effects continued when Olsson and Edwards (1992) reported seven cases of tachycardia and palpitation with cisapride administration, the seriousness of the problem was not recognized until 1996 when the United States Food and Drug Administration, through its MedWatch reporting program, received in the period between 1993 to 1996 a total of 57 cases of arrhythmias associated with cisapride use (Wysowski and Bacsanyi, 1996). Experimental evidence suggests that cisapride delays cardiac repolarization (Rampe et al., 1997) and prolongs the action potential duration as well as QT interval (Puisieux et al., 1996; Carlsson et al., 1997), partly through blockage of the rapid component of the delayed rectifier potassium current (I_{Kr}) (Rampe et al., 1997). Although epidemiological studies (Wager et al., 1997; Walker et al., 1999) have failed to

Abbreviations: NORCIS, norcisapride; 3-F-4-OHCIS, 3-fluoro-4-hydroxycisapride; 4-F-2-OHCIS, 4-fluoro-2-hydroxycisapride; P450, cytochrome P450; HPLC, high-performance liquid chromatography; HLM, human liver microsome; RT, retention time.
identify cisapride cardiac risk, several subsequent clinical cases and studies have implicated this drug as the cause of serious cardiac arrhythmias, including torsade de points that may precipitate syncope and sudden cardiac death (for review, see Michalets and Williams, 2000). As a result, broad marketing of cisapride in the United States and some other countries was suspended (Ferriman, 2000), but it continues to be available to patients who meet eligibility criteria in the United States, and it is still widely used in other countries.

The cardiac risk of cisapride appears to be rare in the absence of other factors, particularly the use of medications that inhibit CYP3A (for reviews, see Bedford and Rowbotham, 1996; Michalets and Williams, 2000). Cisapride is extensively metabolized in humans, with only less than 7% of the total dose appearing as unchanged in urine and feces (Meuldermans et al., 1988). N-Dealkylation to norcisapride (NORCIS) and aromatic hydroxylation of the fluoroophenoxy moiety to 3-fluoro-4-hydroxycisapride (3-F-4-OHCIS) and 4-fluoro-2-hydroxycisapride (4-F-2-OHCIS) has been reported to be the main in vivo and in vitro human metabolic pathways of cisapride (Fig. 1) (Meuldermans et al., 1988; Bohets et al., 2000; Desta et al., 2000a), although other minor primary metabolic routes (e.g., oxidative O-dealkylation and N-glucuronidation) and secondary metabolic routes have been also identified. Recently, we (Desta et al., 2000) and other authors (Bohets et al., 2000) had shown that CYP3A is the major isoform responsible for the formation of NORCIS, 3-F-4-OHCIS, and 4-F-2-OHCIS from racemic cisapride in vitro, and this appears to be the case in vivo in humans (van Haarst et al., 1998; Kivisto et al., 1999).

Unfortunately, studies dealing with cisapride pharmacology have so far been confined to the racemic mixture. As shown in Fig. 1, cisapride has asymmetric carbons at the positions 3 and 4 of the piperidinyl ring, and, as with many chiral drugs (Eichelbaum, 1988), its pharmacological properties may be enantiospecific. We have evidence that the pharmacokinetics of cisapride in normal volunteers is stereoselective (Desta et al., 2000b, 2001). This might be due to differences in the rate of enzymatic oxidation of the enantiomers, although other mechanisms (e.g., effect of transport proteins such as P-glycoproteins) cannot be excluded. Stereoselective metabolism can arise from metabolism of the enantiomers via different routes catalyzed by different enzymes or via the same route and the same enzyme at different rates (Testa, 1988). As has been shown for a number of chiral drugs (Testa, 1988; Kroemer et al., 1991, 1994), substrate stereoselectivity can cause in vitro and in vivo interactions between enantiomers as a result of interference at the binding and/or catalytic step. We know that racemic cisapride is mainly catalyzed by CYP3A (Bohets et al., 2000; Desta et al., 2000a) and the enantiomers may compete for each other’s metabolic step, provided CYP3A plays an important role in the oxidation of the enantiomers. In the present study, the stereoselectivity and the enantiomer-enantiomer interaction of cisapride metabolism in microsomes from human livers and recombinant P450 isoforms were investigated. A chiral HPLC method with UV detection was developed to separate cisapride enantiomers for study and this method was modified to measure NORCIS enantiomers.

**Materials and Methods**

**Chemicals.** Racemic cisapride, (±)-cis-4-amino-5-chloro-N-[1-[3-(4-fluorophenoxy)-propyl]-3-methoxy-4-piperidinyl]-2-methoxybenzamide, was purchased from Research Diagnostic, Inc. (Flanders, NJ). Cisapride enantiomers were obtained by means of collecting chirally separated fractions after injecting racemic cisapride solution into an HPLC system described below (purity was >95%). Quinidine, tolbutamide, quercetin, diethylthiocarbamate, troleandomycin, ketoconazole, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, and β-NADP were purchased from Sigma Chemical Co. (St. Louis, MO). Sulfaphenazole, S-mephenytoin, and furafylline were obtained from Ultrafine Chemicals (Manchester, England). Omeprazole was a generous gift from Dr. Tommy Anderson (Clinical Pharmacology, Astra Hässle AB, Möln达尔, Sweden). Authentic synthetic metabolites used to identify peaks on the chromatograms were racemic NORCIS, 3-F-4-OHCIS, and 4-F-2-OHCIS and were generously supplied by Dr. Russell Gotschall (Department of Clinical Pharmacology and Therapeutics, Children’s Mercy Hospital, Kansas City, MO). (+)-NORCIS and (−)-NORCIS were generously provided by Dr. Steve Ebert, Department of Pharmacology, Georgetown University. Paroxetine was a generous gift from Dr. Jae-Gook Shin, Department of Pharmacology, Inje University College of Medicine and Clinical Pharmacology Center, Pusan Paik Hospital, South Korea. All other reagents were of HPLC grade.

**Human Liver Microsomes (HLMs) and Recombinant Human P450s.** The HLMs used 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 (Desta et al., 1998, 2000a). Protein concentrations were determined using the Bradford method (Bradford, 1972). Baculovirus-insect cell expressed human P450s (1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4) (with reductase) were purchased from GENTEST (Woburn, MA) and stored at −80°C. Microsomes were thawed on ice before use.

**Chiral Separation of Cisapride Enantiomers from Racemic Mixture.** Synthetic enantiomers of cisapride were not available to

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**Fig. 1.** Chemical structure of cisapride and its human metabolites. The two asymmetric carbons are shown with asterisks.
us. The enantiomers used in this study were fractions separated using a chiral column and collected after injecting racemic cisapride onto HPLC. The separation system consists of ChiralCel OJ column (4.6 x 250 mm) (Chiral Technologies, Inc., Exton, PA) and a mobile phase containing ethanol/hexane/diethylamine (35:64.5:0.5, v/v/v) (Desta et al., 2000a). Under these conditions, two chromatographic peaks were successfully separated (Fig. 2) at retention times of 7.3 ± 0.2 and 10.9 ± 0.5 min and were temporarily designated as EI and EII, respectively. HPLC eluates corresponding to each peak were collected in glass test tubes using an automatic fraction collector (Foxy Jr Fraction Collector; ISCO, Lincoln, NE) and photodiode array were the same as those of racemic cisapride. The enantiomers in the HPLC eluates showed a UV spectrum similar to that of racemic cisapride, having a minimum at 215 nm and a maximum at 274 nm. EI and EII were reinjected into chiral column to determine enantiomeric purity. The ratio of the area under the response-time curve of the chromatographic peaks of EI and EII after injection of racemic cisapride solutions into a chiral HPLC column was close to 1 (E1/EII: 1:1.0014).

**Identification of Cisapride Enantiomers.** After successful separation and collection of adequate amounts of EI and EII from racemic cisapride, we determined the stereoisomers of EI and EII as follows. EI and EII (20 μM) were separately incubated with HLMs and an NADPH-generating system using incubation conditions described in our previous work with the racemic cisapride (Desta et al., 2000a), as pilot experiments have indicated that the metabolic pathways of EI, EII, and racemic cisapride are qualitatively similar. After termination of the reaction with 1 N NaOH, the microsomal incubate was extracted in a basic pH (see below), dried samples were reconstituted with 200 μl ethanol, and 100 μl was injected onto a chiral HPLC system. The chiral HPLC assay method developed to measure cisapride enantiomers in plasma (Desta et al., 2000b) was slightly modified and adapted to determine enantiomers of NORCIS in microsomal incubates. In brief, the separation system consists of ChiralCel OJ column (4.6 x 250 mm) (Chiral Technologies, Inc.) and a mobile phase containing ethanol/hexane/diethylamine (30:69.5:0.5, v/v/v). The flow rate was 0.7 ml/min and the metabolites were monitored by UV set at λ = 275 nm. The retention times of NORCIS formed from EI and EII were then compared with the retention times of authentic synthetic (+)-NORCIS and (-)-NORCIS. The retention times of NORCIS formed from EI (8.3 min) and EII (9.2 min) corresponded with the retention times of (-)-NORCIS (8.3 min) and (+)-NORCIS (9.2 min), respectively. We also performed coelution experiments by spiking the microsomal incubate of EI and EII with synthetic standards of (-)-or (+)-NORCIS. We noted that the HPLC peaks of NORCIS from EI and EII microsomal incubate were coeluted with that of synthetic (-)- and (+)-NORCIS, respectively, supporting the retention time data. Thus, EI was designated as (-)-cisapride and EII was assigned as (+)-cisapride (Fig. 2). The absolute chemical configuration (Fig. 2) was derived by comparative analysis to the stereochemistry of piperidine-based analogs of cocaine as described elsewhere (Kozikowski et al., 1998).

**Metabolism of Cisapride Enantiomers in HLMs.** To test the stereoselective metabolism of cisapride, we used incubation conditions that we developed earlier to study the metabolism of racemic cisapride in HLMs (Desta et al., 2000a). In all experiments, cisapride enantiomers were dissolved and serially diluted with methanol to the required concentrations, and any methanol was removed through evaporation using vacuum centrifugation. The incubation mixture (final volume 250 μl in phosphate buffer, pH 7.4) consisted of an NADPH-generating system (13 mM NADP, 33 mM glucose 6-phosphate, 33 mM MgCl2, and 4 U/ml glucose-6-phosphate dehydrogenase), 5 mg/ml microsomal protein and cisapride enantiomers. After a 5-min preincubation at 37°C, reaction was initiated by addition of 25 μl of microsomes (5 mg/ml). The incubation was performed at 37°C for 30 min. The reaction was terminated by addition of 100 μl of acetonitrile, the incubation mixture was vortex-mixed and centrifuged at 14,000 rpm for 5 min in an Eppendorf model 5415C centrifuge (Brinkman Instruments, Westbury, NY), and aliquots of supernatant (100 μl) were injected into an achiral HPLC system (see below) without further extraction. In our previous work with racemic cisapride, we had shown that cisapride was oxidized to three primary metabolites in vitro (Desta et al., 2000a). These metabolites have been identified as major oxidative metabolites in human urine and feces (Meuldermans et al., 1998). To allow qualitative comparison between the metabolites formed from racemic cisapride and the two enantiomers, we incubated each enantiomer and the racemate separately in HLMs and monitored the metabolite peaks appearing in an achiral HPLC. Three metabolite peaks were formed from each enantiomer and the racemate mixture. The formation of these peaks was dependent on an NADPH-generating system, time of incubation, and microsomal protein and substrate concentrations. Linear conditions selected were a 30-min incubation period (37°C) at a final protein concentration of 0.5 mg/ml. The identities of the metabolite peaks were determined by comparing the retention times of each of the metabolite peaks formed from the enantiomers and the racemate with that of reference peaks of three synthetic racemic cisapride metabolites: NORCIS, 3-F-4-OHCIS, and 4-F-2-OHCIS (Fig. 1). The retention times of the reference metabolites were tested after direct injection or after adding them to incubation mixtures that did not contain active microsomes.

After confirming the major primary metabolic pathways of cisapride, experiments were designed to characterize the human P450 isoforms catalyzing these reactions from each enantiomer using HLMs or recombinant human P450s. We first optimized the HPLC

![Fig. 2. Chiral HPLC separation of cisapride enantiomers from racemic cisapride solutions. The two peaks were temporarily designated as EI and EII until the identification of the specific (-)- and (+)-enantiomers were made (see Materials and Methods).](https://example.com/figure2)
separation of the metabolites. Since the chromatographic peak of NORCIS reported in our earlier work with racemic cisapride was close to the solvent front (Desta et al., 2000a), we first developed an extraction method that substantially improved the separation of NORCIS. The method of extraction was as follows. The incubation reaction was stopped by adding 100 μl of 1 N NaOH (instead of acetonitrile) and the sample was extracted with 1 ml of tert-butylmethyl ether and centrifuged at 14,000 for 5 min. The organic phase was transferred into a separate Eppendorf tube, dried by vacuum centrifugation, reconstituted in 200 μl of mobile phase, and 100 μl was injected onto HPLC. This new extraction method was superior and used for the isolation of NORCIS as well as 4-F-2-OH CIS. Because we lost the third metabolite, 3-F-4-OHCIS, in the water negative control experiments were included (Desta et al., 1998, 2000a). Incubation and HPLC conditions are as described above. All incubations were run in duplicate and appropriate negative control experiments were included (Desta et al., 1998, 2000a). Concentrations of metabolites in microsomal incubates were quantitated from linear calibration plots based on the peak area under the response-time curves of known racemic NORCIS concentrations (0.1–10 μM).

Kinetic Analysis. The formation of NORCIS, 3-F-4-OH CIS, and 4-F-2-OH CIS from (−), ( ), and ( )-cisapride (1–50 μM) were determined in HLM (0.5 mg/ml of protein) preparations in the presence of an NADPH-generating system (incubation: at 37°C for 30 min). Appropriate enzyme kinetic models were selected to estimate apparent kinetic parameters (K and V) (see Data Analysis).

Correlation Experiments in HLMs. The formation rates of the primary metabolites described for each cisapride enantiomer (20 μM) were determined in a panel of HLMs prepared from 18 different human organ donors and one inactive human liver that we used as a positive control. The rates of formation of the metabolites for each cisapride enantiomer were compared with the catalytic activities of P450 1A2, 2D6, 3A, 2C9, and 2C19, as measured by specific substrate reaction probes. The probes used to test the activity of individual isoforms and the methods involved are described in our previous publications (Desta et al., 1998, 2000a). Incubation and sample preparation were carried out as described above (30-min incubation at 37°C, 0.5 mg/ml of protein). The ratio for the formation rate of metabolites from (+)-cisapride to the corresponding formation rate of metabolite formed from (−)-cisapride in each HLM was calculated to determine the variability in the stereoselective metabolism of cisapride across liver panels.

Chemical Inhibition Experiments. The individual enantiomers of cisapride (15 μM) were incubated in HLMs and an NADPH-generating system in the absence (control) and presence of known P450 isoform-specific inhibitors/substrates. The following compounds were examined for their ability to inhibit the microsomal metabolism of (−)-cisapride and (+)-cisapride: quinidine and paroxetine (CYP2D6, and 2 μM, respectively); furafylline (CYP1A2, 10 μM); sulfaphenazole (CYP2C9, 20 μM); ketonazole and troleandomycin (CYP3A, 1 and 50 μM, respectively); tolbutamide and quercetin (CYP2C8, 100 and 20 μM, respectively); omeprazole and (S)-mephentoin (CYP2C19, 10 and 50 μM, respectively); and diethyldithiocarbamate (CYP2E1, 50 μM). The incubation reactions consisted of cisapride enantiomers (with or without P450 isoform-specific inhibitor) and an NADPH-generating system was preincubated for 5 min at 37°C. HLMs (0.5 mg/ml) were added to initiate the reaction and incubated for 30 min at 37°C (final incubation volume of 250 μl). Troleandomycin and furafylline are mechanism-based inhibitors of CYP3A and CYP1A2, respectively, and were first preincubated in the presence of an NADPH-generating system and HLMs at 37°C for 15 min before initiating the reaction by addition of the enantiomers. All isoform-specific inhibitors were studied at concentrations chosen to be selective for the respective P450 isoforms on the basis of published K values of the inhibitor probes (Desta et al., 1998, 2000a). Inhibitors were dissolved in water where appropriate or in suitable organic solvents (ethanol, methanol, or dimethyl sulfoxide). The organic solvents were removed through evaporation by vacuum centrifugation or stock solutions were serially diluted with water to the required concentration containing <0.1% of solvents in the final volume. Rates of metabolite formation were compared with those of controls in which the inhibitor was replaced with an appropriate concentration of vehicle. To construct Dixon plots for the inhibition of metabolism of cisapride enantiomers by troleandomycin, we preincubated the inhibitor (5–35 μM) with HLMs and an NADPH-generating system for 15 min and reaction was initiated by adding the enantiomers (5–40 μM).

Metabolism of Cisapride Enantiomers by Recombinant Human P450s. To further identify the specific P450 isoforms catalyzing the metabolism of cisapride enantiomers, 25 μl of microsomes of recombinant human P450 1A1, 1A2, 2A6, 2C19, 2C8, 2C9, 2D6, 2B6, 2E1, and 3A4 (250–500 pmol of P450/ml in phosphate reaction buffer, pH 7.4) was incubated with each cisapride enantiomer (15 μM) and an NADPH-generating system (same composition as noted above) at 37°C for 30 min. All other incubation conditions and HPLC assay of the metabolites were the same as described for HLMs. Full kinetics for the formation of metabolites from cisapride enantiomers was determined by incubating each enantiomer (0–50 μM) with recombinant human CYP3A4 and CYP2C8 (25 μM of 250 pmol of P450/ml). Data on the rate of formation of the metabolites were given as picomoles per minute per picomoles of P450.

HPLC Assay of Metabolites of Cisapride Enantiomers. Cisapride enantiomers and their metabolites were measured by an HPLC system as described in our previous work (Desta et al., 2000). Aliquots (100 μl) of unextracted supernatants of the centrifuged incubates or the extracted and reconstituted samples (see above) were injected into the HPLC. The HPLC system consisted of a Waters model 600 dual-piston pump (Milford, MA), a Waters model 717 autosampler, a Waters model 996 PDA detector, and a Waters model 470 scanning fluorescence detector. The separation column consisted of a (150 × 3.9-mm i.d.) stainless steel symmetry column (Symmetry) packed with 5-μm particle size RP-C (Waters) and a Waters NovaPack C guard column (4 μm, 60 A). The mobile phase was composed of 20% methanol, 17% acetonitrile, and 0.5% triethylamine in 50 mM NaHP 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 eluate was monitored using fluorescence at an excitation wavelength of 247 nm and emission wavelength of 350 nm.

Cisapride Enantiomer-Enantiomer Interaction. Since we had information that indicated that CYP3A is the major isoform responsible for the metabolism of both cisapride enantiomers, we performed the following microsomal studies to determine whether the enantiomers would compete with each other’s metabolism. Preliminary experiments that involved incubation of equimolar concentrations of each enantiomer and racemic cisapride separately in HLMs have shown that the rate of formation of NORCIS from racemic cisapride was lower than that from each enantiomer. Subsequently, we determined the apparent kinetic parameters for the formation of metabolites from each cisapride enantiomer and the racemic mixture by incubating a range of substrate concentrations (1–50 μM) in HLMs (0.5 mg/ml protein) in the presence of an NADPH-generating system at 37°C for 30 min. We tested whether the enantiomer-enantiomer interaction observed in HLMs occurs in recombinant human CYP3A4 by incubating each cisapride enantiomer and the racemic cisapride (10 μM) separately in HLMs and recombinant human CYP3A4 (under the same incubation condition) and monitoring the formation of NORCIS, 3-F-4-OH CIS, and 4-F-2-OHCIS. The incubation and HPLC conditions are as described above. The ability of one enantiomer to inhibit the metabolism of the other was further tested by incubating (−)-cisapride and (+)-cisapride simultaneously in HLMs and measuring the formation of (−)- and (+)-NORCIS. Although both enantiomers of the metabolite were clearly separated, (+)-NORCIS was formed from (+)-cisapride at the highest rate and for analytical reasons we chose to test inhibition of (+)-cisapride (25 μM) metabolism in the absence and presence of...
increasing concentrations of (-)-cisapride (1–50 μM) from which an IC50 was estimated. NORCIS enantiomers in the microsomal incubates were measured as described above. When (-)-cisapride was used as a substrate, the HPLC sensitivity of (-)-NORCIS was very low. Thus, we tested inhibition of (-)-cisapride metabolism by (+)-cisapride (25 μM) at a relatively higher substrate concentration (50 μM).

**Data Analysis.** After obtaining initial kinetic parameters from Lineweaver-Burk plots, precise estimates of enzyme kinetic variables were obtained by nonlinear regression analysis (WinNonlin software, version 1.5; Scientific Consulting Inc., Apex, NC). The single ($V = V_{\text{max}} + C(K_m + C)$) or two-site Michaelis-Menten equation $V = V_{\text{max1}} + C(K_m1 + C) + V_{\text{max2}} + C(K_m2 + C)$ and Hill equation $V = V_{\text{max}} + C(K_m + C)^n$ were fitted to the average of duplicate formation rates ($V$) obtained in HLMs or recombinant enzymes versus the substrate concentrations. $V$ is the velocity of the reaction at substrate concentration $C$. Apparent $V_{\text{max}}$ is the maximum velocity, and apparent $K_m$ represent the substrate concentration at which the reaction velocity is 50% of $V_{\text{max}}$. The models that best fit were selected based on the dispersion of residuals and standard errors of the parameter estimates. In vitro intrinsic clearance (Clint) was given as $V_{\text{max}}/K_m$ (or $K_m$). Correlation coefficients between the formation of cisapride metabolites and the activities of P450 isozymes from different human livers were calculated by nonparametric regression analysis (Spearman’s rank correlation test) with GraphPad Prism software (version 3.1; GraphPad, San Diego, CA). A $p$ value less than 0.05 was considered significant.

**Results**

**Stereoselective Metabolism of Cisapride by HLMs.** We tested in HLMs whether the metabolic routes of racemic cisapride and its enantiomers are the same. Each substrate was incubated separately in HLMs and the resulting metabolite peaks were monitored by achiral HPLC system. Three metabolite peaks (temporarily designated as M1, M2, and M3) were identified in the microsomal incubate of each substrate. The identities of these metabolite peaks were confirmed by comparing their retention times with those of authentic synthetic racemic cisapride metabolites. Accordingly, the retention times of M1, M2, and M3 peaks noted from each substrate were selected based on the dispersion of residuals and standard errors of the parameter estimates. In vitro intrinsic clearance (Clint) was given as $V_{\text{max}}/K_m$ (or $K_m$). Correlation coefficients between the formation of cisapride metabolites and the activities of P450 isozymes from different human livers were calculated by nonparametric regression analysis (Spearman’s rank correlation test) with GraphPad Prism software (version 3.1; GraphPad, San Diego, CA). A $p$ value less than 0.05 was considered significant.

The enzyme kinetic parameters for each individual metabolite in different HLMs were estimated from the formation rate of metabolites versus substrate concentrations according to the Michaelis-Menten or Hill equation (see Data Analysis) after (-)- and (+)-cisapride were incubated separately in HLMs. Comparison of goodness-of-fit values generated from NORCIS velocity data after modeling to single- or two-site Michaelis-Menten equations showed that a single-enzyme system provided a better regression than a two-enzyme system. Representative Michaelis-Menten kinetics for the formation of NORCIS as a function of each cisapride enantiomer concentration (0–50 μM) in HLMs is shown in Fig. 3. The corresponding Eadie-Hofstee plots revealed a linear relationship between the rate of NORCIS formation ($V$) and $V/S$ (substrate concentration) (see inset of Fig. 3). Although a slight curvature of the Eadie-Hofstee plot was observed when (+)-cisapride was used as a substrate and this may lead to slight over estimation of the apparent $K_m$ value, this process was not marked enough to suggest substrate activation. In fact, the plot of metabolite formation versus substrate concentration data showed hyperbolic curve and were fit to a single-enzyme Michaelis-Menten rather than to Hill equation. The microsomal formation rate of 3-F-4-OHCIS and 4-F-2-OHCIS from each enantiomer in HLMs diverged from that of NORCIS. None of these metabolites were detected at substrate concentrations less than 2.5 μM. Figure 4, A and B, shows velocity values of 3-F-4-OHCIS and 4-F-2-OHCIS versus substrate concentrations, respectively, and are better described by a sigmoidal curve (Hill equation). The rate was slow at substrate concentrations less than 10 μM, but it accelerated at higher substrate concentrations. It is unlikely that this process represents a lag phase. Instead, it could be that the hydroxylated metabolites formed during the microsomal incubation are further N-dealkylated to norcisapride, making HPLC detection difficult at lower substrate concentrations. The Eadie-Hofstee graphical analysis of these kinetic data was consistent with positive cooperativity (plots not shown).

The mean (± S.D.) of the computer-derived kinetic parameters (apparent $V_{\text{max}}$ and $K_m$) for the formation of NORCIS and the corresponding in vitro Clint ($V_{\text{max}}/K_m$) are summarized in Table 1. NORCIS (Fig. 3) and 3-F-4-OHCIS (Fig. 4A) were formed at a higher rate from (+)- than from (-)-cisapride, while 4-F-2-OHCIS was formed at a higher rate from (-)- than (+)-cisapride (Fig. 4B), suggesting stereoselective metabolism of cisapride. The average $V_{\text{max}}$, $K_m$, and Clint values of (+)-cisapride metabolism to NORCIS were higher (1.79-, 1.55-, and 1.23-fold, respectively) than those from (-)-cisapride. Kinetic parameters for the formation of 3-F-4-OHCIS and 4-F-2-OHCIS were presented in Table 1. The Clint of 3-F-4-OHCIS formation from (+)-cisapride in this HLM was 1.78 higher than that from (-)-cisapride, largely due to changes in affinity, while the Clint of 4-F-2-OHCIS formation from (-)-cisapride was 1.63-fold greater than that from (+)-cisapride. However, the kinetic estimates of these two metabolites should be interpreted carefully. Be-cause of the very steep slope of velocity versus substrate concentration curves, we were not able to accurately estimate the parameters and thus the values may be artificially lower or higher. This was also the reason for our inability to estimate reliable kinetic parameters from velocity data obtained in the other HLMs tested (Table 1). In addition, HPLC de-
tection of these metabolites was unreliable in HLMs with lower global enzyme activity.

The formation of NORCIS appears to represent the major metabolic pathway of both (−)- and (+)-cisapride. First, incubation of lower concentrations of each enantiomer (2.5 μM) in HLMs has revealed that NORCIS is the only metabolite HPLC peak detectable (Fig. 3). Second, in HL9A where the kinetic parameters of all metabolites were estimated, the in vitro Clint was higher for the formation of NORCIS than that of 3-F-4-OHCIS or 4-F-2-OHCIS (Table 1). Moreover, the rate of NORCIS formation from both enantiomers (20 μM) in 17 HLMs was greater than the formation rate of 3-F-4-OHCIS or 4-F-2-OHCIS (Fig. 5). However, it is not known whether the origin of NORCIS we measured in the microsomal incubate was from the N-dealkylation of the parent substrates only and/or from the hydroxylated metabolites.

Correlation Studies. The rates of metabolism of each cisapride enantiomer (20 μM) in microsomes from 18 different human liver donors were determined with the intention of correlating these data with previously measured isoenzyme-specific P450-catalytic activity and of estimating the sample-to-sample variation in the relative stereoselective metabolism of cisapride to NORCIS, 3-F-4-OHCIS, and 4-F-2-OHCIS. The formation rate of these metabolites revealed high interindividual variability among the livers tested. The formation rate (mean ± S.D. pmol/min/mg protein) of metabolites from (−)-cisapride and (+)-cisapride, respectively, were as follows: NORCIS, 173.4 ± 144.3 (range 11.9–489, 41-fold) and 304.5 ± 230.3 (range 20–714, 35.7-fold); 3-F-4-OHCIS, 83 ± 48.4 (range 20.3–163.3, 8-fold) and 162.7 ± 89.3 (range 38.2–285, 7.5-fold); and 4-F-2-OHCIS, 39.9 ± 26.1 (range 3.6–84, 23-fold) and 35.9 ± 15.6 (range 3.6–69.7, 19.4-fold). The formation rate of each metabolite from (−)-cisapride was compared with that from (+)-cisapride. In each HLM tested, NORCIS and 3-F-4-OHCIS were formed consistently at the highest rate when (+)-cisapride was used as a substrate, indicating that the stereoselectivity we observed in a limited number of human livers (Figs. 3 and 4A) is maintained across livers of different activities (Fig. 5, A and B). The average ratio (±S.D.) of NORCIS and 3-F-4-OHCIS formation rate in 17 HLMs from (+)-cisapride to the formation rate of these metabolites from (−)-cisapride were 1.9 ± 0.4 (range 1.5–2.5) and 2 ± 0.4 (range 1.5–2.5), respectively. The average ratio of 4-F-2-OHCIS formation rate from (−)-cisapride to that from (+)-cisapride was slightly >1 (1.2 ± 0.6), but the range (0.4–2.7) clearly indicated that the stereoselective formation of this metabolite varies from human liver to human liver (Fig. 5C). When HLMs with high global P450 activity, or specifically CYP3A, were used (e.g., HL16, HL9A, HL9B, and HL2), the formation rate of 4-F-2-OHCIS from (−)-cisapride was clearly higher than those obtained from (+)-cisapride (Fig. 5C), but it appears to be diminished/abolished in livers with low activity. In Table 2, the correla-
tion between the activity of individual P450 isoforms (as measured by isoform-specific substrate reaction probe in HLMs) and the formation rate of the metabolites from each cisapride enantiomer is illustrated. The formation rate of all three metabolites from each enantiomer showed significant correlation with the activity of CYP3A and CYP2C19 (Table 2).

**Chemical Inhibition of Cisapride Metabolism.**

The effects of isoform specific substrates/inhibitors of P450 (1A2, 2C8, 2C19, 2C9, 2D6, 2E1, and 3A) on the metabolism of cisapride enantiomers (15 μM) were investigated in HLMs. Troleandomycin (50 μM) markedly inhibited the formation rate of NORCIS from 15 μM (−)- and (+)-cisapride by 75 ± 2 and 80 ± 2%, respectively (Fig. 6A). Similarly, ketoconazole (1 μM) was a potent inhibitor of this metabolite [48 ± 3 and 48 ± 4% when (−)- and (+)-cisapride were used as substrates, respectively] (Fig. 6A). The degree of inhibition by ketoconazole and troleandomycin of (−)- and (+)-cisapride was similar. Other isoform-specific inhibitors/substrates tested had negligible effect on the rate of NORCIS from either of the enantiomers (Fig. 6A). The marked decrease in the formation of NORCIS by ketoconazole and troleandomycin (but not by other inhibitors) was associated with a decrease in disappearance rate of both enantiomers (parent drugs) (Fig. 6B). To estimate precise Ki values for the inhibition of NORCIS formation by troleandomycin, we incubated each cisapride enantiomer (5–50 μM) with troleandomycin (5–35 μM) in HLMs. Representative Dixon plots for the inhibition of NORCIS formation from each cisapride enantiomer by troleandomycin in HLMs are shown in Fig. 7. The Ki values calculated by nonlinear regression using competitive enzyme inhibition model were 11.9 and 13.7 μM, respectively. The percentage of inhibition and the Ki values derived indicate that troleandomycin equipotently inhibits the formation of NORCIS from each enantiomer.

On the other hand, the hydroxylation of each cisapride enantiomer to 3-F-4-OHCIS and 4-F-2-OHCIS was minimally affected by any of the isoform-specific inhibitors/substrates tested (Table 3). In fact, there appeared activation (data not shown), particularly with respect to the formation rate of 4-F-2-OHCIS from (+)-cisapride, when lower concentrations of the enantiomers were incubated with certain inhibitors (e.g., troleandomycin).

**Metabolism of Cisapride by Recombinant Human P450s.**

Microsomes derived from 10 baculovirus-infected insect cell lines expressing human P450 isoforms were used to evaluate the potential of each enzyme to metabolize each enantiomer of cisapride (15 μM). As demonstrated in Fig. 8, CYP3A4 resulted in the formation of all the three primary metabolites (Fig. 8A). The effects of various substrate concentrations on each enantiomer were studied in HLMs. The kinetic parameters for the formation of cisapride metabolites from each enantiomer in HLMs are shown in Table 1.

### Table 1: Kinetic parameters for the formation of cisapride metabolites from each enantiomer in HLMs

<table>
<thead>
<tr>
<th>Enantiomer</th>
<th>HLMs</th>
<th>Vmax (pmol/min/mg of protein)</th>
<th>Km (μM)</th>
<th>Vmax/Km</th>
<th>Vmax (pmol/min/mg of protein)</th>
<th>Km (μM)</th>
<th>Vmax/Km</th>
<th>Vmax (pmol/min/mg of protein)</th>
<th>Km (μM)</th>
<th>Vmax/Km</th>
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<tr>
<td>(+)-Cisapride</td>
<td>HL9A</td>
<td>632.2</td>
<td>21.7</td>
<td>29.1</td>
<td>364 ± 284</td>
<td>18.5 ± 5</td>
<td>17.6 ± 13</td>
<td>203 ± 167</td>
<td>11.9</td>
<td>18.3</td>
</tr>
<tr>
<td></td>
<td>HL9B</td>
<td>471.4</td>
<td>22.9</td>
<td>20.6</td>
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<td>HL16</td>
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<tr>
<td></td>
<td>HLD</td>
<td>29.5</td>
<td>11</td>
<td>2.7</td>
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<td></td>
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<td></td>
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<td></td>
<td>HL29</td>
<td>92.2</td>
<td>17.4</td>
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<tr>
<td>Mean ± S.D.</td>
<td></td>
<td>364 ± 284</td>
<td>18.5 ± 5</td>
<td>17.6 ± 13</td>
<td>203 ± 167</td>
<td>11.9</td>
<td>18.3</td>
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<tr>
<td>(--)-Cisapride</td>
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<td></td>
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<tr>
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<td>HL16</td>
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<td>HLD</td>
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<td>HL29</td>
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<tr>
<td>Mean ± S.D.</td>
<td></td>
<td>203 ± 167</td>
<td>11.9 ± 5</td>
<td>14.3 ± 10</td>
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<tr>
<td>(±)-Cisapride</td>
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*Vmax (pmol/min/mg of protein), Km (μM), and Vmax/Km (μl/min/mg of protein).*
TABLE 2

Correlation of formation rates of NORCIS, 3-F-4-OHCIS, and NORCIS from 20 μM cisapride enantiomers with the activities of different human P450 isozymes in 18 HLMs.

Data were analyzed using the nonparametric correlation test (Spearman r). The values represent correlation coefficient (r). Isoform substrate probes were phenacetin (1A2), tolbutamide (2C9), omeprazole (2C19), and dextromethorphan (2D6 and 3A) (see Materials and Methods for the specific reactions tested).

<table>
<thead>
<tr>
<th>P450 Isoforms</th>
<th>Metabolites Formed from (−)-Cisapride</th>
<th>Metabolites Formed from (+)-Cisapride</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NORCIS r (p)</td>
<td>3-F-4-OHCIS r (p)</td>
</tr>
<tr>
<td>CYP1A2</td>
<td>0.50 (0.07)</td>
<td>0.49 (0.1)</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>0.22 (0.05)</td>
<td>0.82 (0.001)</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>0.64 (0.01)</td>
<td>0.57 (0.05)</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>0.38 (0.2)</td>
<td>0.70 (0.01)</td>
</tr>
<tr>
<td>CYP3A</td>
<td>0.60 (0.02)</td>
<td>0.85 (0.0005)</td>
</tr>
</tbody>
</table>

* Statistically significant at p = 0.05.

Fig. 6. Inhibition by P450 isoform-specific inhibitors/substrates of metabolism of each cisapride enantiomer to NORCIS in HLMs. Each cisapride enantiomer (15 μM) was incubated with or without the specific inhibitors/substrates. A, percentage of control of formation rate of NORCIS remaining. B, percentage of control of each parent cisapride enantiomer remaining in the microsomal incubates. The numbers in front of the each isoform-specific inhibitor/substrate in the x-axis represent the final concentrations (μM) used in the inhibition experiments. The data are mean ± S.D. (n = 6 determinations each).

4-F-2-OHCIS was 0.21 ± 0.04 and 0.15 ± 0.04. The activity of other recombinant isoforms toward the metabolism of each cisapride enantiomer is generally minimal. CYP2C8 participated in the formation of all cisapride metabolites, but this was at a very low rate (V < 0.09 pmol/min/pmol of P450) (Fig. 8). Although other recombinant enzymes catalyze the formation of one or more cisapride metabolites [e.g., CYP2B6, NORCIS formation from (−)- and (+)-cisapride; CYP2A6, 4-F-2-OHCIS formation from (−)-cisapride], the rate was very small compared with that observed with CYP3A4. Consistent with the data from HLMs, the pattern of stereoselective metabolism of cisapride to the respective metabolites was similar irrespective of the isoforms involved, i.e., the formation of NORCIS by CYP3A4, CYP8 and CYP2B6 (Fig. 8A) and 3-F-4-OHCIS by CYP3A4 and CYP2C8 (Fig. 8B) favored (+)-over (−)-cisapride, while the formation of 4-F-2-OHCIS by CYP3A, CYP2A6 and CYP2C8 was formed at the highest rate from (−)-than (+)-cisapride (Fig. 8C).

Subsequently, we determined the kinetics for the formation of NORCIS, 3-F-4-OHCIS, and 4-F-2-OHCIS from each cisapride enantiomer (0–50 μM) by recombinant human CYP3A4 and CYP2C8 and compared these data with the kinetic parameters of cisapride metabolism in HLMs. We chose these isoforms for study on the basis of our preliminary data (Fig. 8). The kinetic profiles obtained with recombinant enzymes (Fig. 9; Table 4) essentially reflect those kinetic profiles and stereoselective metabolism observed in HLMs (Figs. 9–5). Recombinant CYP3A4 formed NORCIS (Fig. 9A) and 3-F-4-OHCIS (Fig. 9B) preferentially from (+)-cisapride and 4-F-2-OHCIS preferentially from (−)-cisapride (Fig. 9C). The Vmax values for the formation of NORCIS and 3-F-4-OHCIS by CYP3A4 from (+)-cisapride, respectively, were 2.25- and 2.35-fold higher than those from (−)-cisapride (Vmax/Km, 1.8- and 2.3-fold), with no change in the Km values. CYP2C8 was more efficient catalyst of 3-F-4-OHCIS from (+)-enantiomer (Vmax/Km, 1.6- and 1.8-fold higher, respectively) than from the (−)-enantiomer (Fig. 9D; Table 4). In HLMs, the stereoselective metabolism of cisapride enantiomers to 4-F-2-OHCIS was lost in microsomes of some human livers (Fig. 5D), but when microsomes from recombinant human CYP3A4 were used, the formation rate of 4-F-2-OHCIS at any given (−)-cisapride concentration was consistently higher than that of (+)-cisapride, but generally the degree of stereoselectivity for the formation of this metabolite appears very small. The kinetics for the formation of NORCIS by CYP3A4 was consistent with that observed in HLMs. The Km values for the formation of NORCIS from (−)-cisapride (5.6 μM) and (+)-cisapride (6.8 μM) derived from recombinant enzymes (Table 4) were close to those obtained in HLMs (average 11.9 and 18.5 μM) (Table 1). On the other
hand, the kinetics for the formation of 3-F-4-OHCIS and 4-F-2-OHCIS in recombinant P450 isoforms was described by a simple Michaelis-Menten equation (Fig. 9, B–D; Table 4), while in HLMs they were characterized by Hill equation (Fig. 4; Table 1).

Cisapride Enantiomer-Enantiomer Interaction. In vitro HLMs, the metabolic pathways of each enantiomer and the enzymes involved were similar to those described for racemic cisapride. In pilot experiments in HLMs, however, we noted that the rate of formation of metabolites was considerably lower when racemic cisapride was used as a substrate than that obtained from an equimolar concentrations of (+)- or (−)-cisapride. This observation was extended when a range of substrate concentrations was incubated in HLMs and the rate of formation of N-OHCIS (Fig. 3), 3-F-4-OHCIS, and 4-F-2-OHCIS (data not shown) was monitored.

![Fig. 8. Formation rates (pmol product/min/pmol of P450) of N-OHCIS (A), 3-F-4-OHCIS (B), and 4-F-2-OHCIS (C) from each cisapride enantiomer (15 μM) by a panel of recombinant human P450s. The data presented are not corrected for abundance of each P450 in the liver. N-OHCIS and 4-F-2-OHCIS were measured after further extraction of incubates (see Materials and Methods). Data are given as mean (±S.D., n = 6 measurements). 3-F-4-OHCIS was measured without extraction and represent average of duplicate determinations.](image)

![Fig. 9. Kinetics for the formation (pmol product/min/pmol of P450) of N-OHCIS, 3-F-4-OHCIS, and 4-F-2-OHCIS from cisapride enantiomers (0–50 μM) by recombinant human CYP3A4 and CYP2C8. A–C, formation rates of N-OHCIS, 3-F-4-OHCIS, and 4-F-2-OHCIS by CYP3A4, respectively. D, formation rate of 3-F-4-OHCIS by CYP2C8. Each point represents average of duplicate measurements. Kinetic parameters derived from these data are listed in Table 4.](image)

### TABLE 3

<table>
<thead>
<tr>
<th>Inhibitors/substrates</th>
<th>P450 Isoforms</th>
<th>(−)-Cisapride Metabolites</th>
<th>(+)-Cisapride Metabolites</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>3-F-4-OHCIS</td>
<td>4-F-2-OHCIS</td>
</tr>
<tr>
<td>(μM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paroxetine (2)</td>
<td>CYP2D6</td>
<td>81 ± 11</td>
<td>98 ± 21</td>
</tr>
<tr>
<td>Tolbutamide (100)</td>
<td>CYP2C9</td>
<td>82 ± 9</td>
<td>99 ± 9</td>
</tr>
<tr>
<td>Quercetin (20)</td>
<td>CYP2C8</td>
<td>88 ± 6</td>
<td>101 ± 10</td>
</tr>
<tr>
<td>DEDTC (50)</td>
<td>CYP2E1</td>
<td>88 ± 8</td>
<td>92 ± 9</td>
</tr>
<tr>
<td>S-Mephenytoin (50)</td>
<td>CYP2C19</td>
<td>107 ± 7</td>
<td>88 ± 3</td>
</tr>
<tr>
<td>Sulfaphenazole (20)</td>
<td>CYP2C9</td>
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<td>86 ± 6</td>
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<tr>
<td>Furafylline (10)</td>
<td>CYP1A2</td>
<td>82 ± 2.6</td>
<td>92 ± 8</td>
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<td>Omeprazole (10)</td>
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<td>90 ± 9</td>
<td>89 ± 4</td>
</tr>
<tr>
<td>Ketoconazole (1)</td>
<td>CYP3A</td>
<td>89 ± 10</td>
<td>68 ± 6</td>
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<tr>
<td>Troleandomycin (50)</td>
<td>CYP3A</td>
<td>99 ± 3</td>
<td>51 ± 23</td>
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</table>

DEDTC, diethyldithiocarbamate.
cemic cisapride (Fig. 3; Table 1). We also noted that the $K_m$ of NORCIS formation from (±)-cisapride was 2- to 3-fold lower than that from (−)-cisapride and (+)-cisapride, respectively. This suggested to us a possible enantiomer-enantiomer interaction. Since CYP3A4 was the major isozyme involved in the metabolism of cisapride (present data; Desta et al., 2000a), we tested whether this isozyme might be responsible for this process. We incubated (−), (+), and (±)-cisapride (10 μM) separately under the same incubation and HPLC conditions in HLMs as well as recombinant human CYP3A4. The results summarized in Fig. 10 indicate that the stereoselective metabolism and enantiomer-enantiomer interaction of cisapride observed in HLMs clearly correlate with those obtained in recombinant human CYP3A4. In both microsomal systems, the rate of formation of all three metabolites from (±)-cisapride was consistently slower than that from the enantiomers.

The ability of one enantiomer of cisapride to inhibit the metabolism of the other was tested after incubation of both enantiomers (pseudoracemate) simultaneously in HLMs and by measuring the specific NORCIS enantiomer formed using a chiral HPLC separation method. The effect of (−)-cisapride (1–50 μM) on the metabolism of (+)-cisapride (25 μM) to (+)-NORCIS is demonstrated in Fig. 11A. (−)-Cisapride markedly reduced the formation of (+)-NORCIS from (+)-cisapride (IC$_{50}$ of ~15 μM; ~90% inhibited at 50 μM (~)-cisapride). Clearly, (−)-cisapride markedly decreased the rate of disappearance of (+)-cisapride compared with no (−)-cisapride in the microsomal incubates (Fig. 11B). Often, it is difficult or not sensitive enough to separate and detect the small amounts of chiral metabolites formed via chiral HPLC, with subsequent UV detection. The enantiomers of NORCIS in the microsomal incubate were clearly separated using the chiral analysis. The HPLC peak in the microsomal incubates corresponding to (±)-NORCIS was sensitive enough to carry out inhibition studies at a wide range of concentrations, but the peak that corresponds to (−)-NORCIS was very small and did not allow us to conduct inhibition studies at lower concentrations. For that reason, we only tested inhibition of (−)-cisapride metabolism by (+)-cisapride at a relatively higher substrate concentration (50 μM). The formation of (−)-NORCIS from 50 μM (−)-cisapride was strongly inhibited by 25 μM (+)-cisapride (by ~70%) (Fig. 11), while the rate of disappearance of the parent substrate was slowed by about 15% (Fig. 11C).

**Table 4**

<table>
<thead>
<tr>
<th>P450</th>
<th>NORCIS</th>
<th>3-F-4-OHCIS</th>
<th>4-F-2-OHCIS</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>$V_{max}$ (pmol/min/pmol of P450)</td>
<td>$K_m$ (μM)</td>
<td>$V_{max}$ (pmol/min/pmol of P450)</td>
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<tr>
<td>(−)-Cisapride</td>
<td>CYP3A4</td>
<td>0.52 ± 0.2</td>
<td>5.6 ± 2.8</td>
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<tr>
<td>(+)-Cisapride</td>
<td>CYP2C8</td>
<td>1.17 ± 0.1</td>
<td>6.8 ± 2.9</td>
</tr>
<tr>
<td></td>
<td>(+)-Cisapride</td>
<td>CYP3A4</td>
<td>0.19 ± 0.02</td>
</tr>
</tbody>
</table>

$V_{max}$ (pmol/min/pmol of P450), $K_m$ (μM), and $V_{max}/K_m$ (μl/min/pmol of P450).

**Discussion**

We present here a detailed characterization of the in vitro human metabolism of cisapride enantiomers. The proposed human metabolism of cisapride and its enantiomers (present data; Desta et al., 2000a) is illustrated in Fig. 12. We have shown that 1) each cisapride enantiomer and the racemic mixture undergo N-dealkylation (major) and 2- and 4-hydroxylation (minor); 2) N-dealkylation and 4-hydroxylation reactions favor (+) over (−)-cisapride, while 2-hydroxylation favors (−) over (+)-cisapride; 3) these reactions are principally catalyzed by CYP3A (with minor contribution of other isoforms); and 4) there is evidence for an enantiomer-enantiomer interaction, although the in vivo relevance is yet to be determined. These data provide an important part of the information needed to predict factors that alter the disposition of each cisapride enantiomer to help identify patients at...
risk for cisapride-induced cardiac arrhythmia or loss of prokinetic efficacy.

\[ \text{N-Dealkylation to NORCIS and hydroxylation to 3-F-4-OHCIS and to 4-F-2-OHCIS represent the primary metabolic pathways of each enantiomer of cisapride (present data) and the racemic mixture (Desta et al., 2000a; present data). NORCIS appears to be the principal metabolite of each cisapride enantiomer: the CL_{int} from each enantiomer in HLMs and recombinant enzymes was higher than that of the other metabolites identified, NORCIS was the only metabolite detected in the HPLC chromatograms at lower concentrations, and inhibition of NORCIS formation (but not of 3-F-4-OHCIS and 4-F-2-OHCIS) by an isoform-specific CYP3A inhibitor was associated with a significant decrease in the rate of disappearance of the parent enantiomers in microsomal incubates compared with those without inhibitors. Since the chiral metabolism of cisapride enantiomers in vivo is unknown, it is difficult to compare our in vitro data to in vivo situations. However, it is worthwhile to suggest that NORCIS is the major metabolite identified both in vitro as well as in vivo. Based on in vitro CL_{int} estimate, NORCIS accounts for \approx 56\% of the total metabolic clearance of racemic cisapride, while the contribution of 3-F-4-OHCIS and 4-F-2-OHCIS appears to be small (\approx 25\%) (Desta et al., 2000a). In the excreta of humans (urine and feces), NORCIS has been identified as the major metabolite of racemic cisapride, accounting for 41 to 45\% of the administered dose (Meuldermans et al., 1988). Here, we did not study the secondary metabolism of cisapride, but we suspect that the source of the NORCIS we measured in the microsomal incubates may not be solely the product of N-dealkylation of the parent enantiomers. Further metabolism of the primary metabolites such as 3-F-4-OHCIS and 4-F-2-OHCIS may also contribute. Whatever the sources of NORCIS in vitro and in vivo might be, it is reasonable to suggest that the metabolic clearance of each cisapride enantiomer as well as the racemic mixture could be predicted from the rate of formation of NORCIS.

Evidence from our correlation analysis (Table 2), inhibition studies with isoform-specific chemical inhibitors (Fig. 6), and kinetic analysis (Fig. 3) in HLMs strongly suggest that CYP3A is the major isoform responsible for the metabolism of both (+)- and (-)-cisapride. This conclusion was supported by data obtained from incubation of cisapride enantiomers with a panel of recombinant human P450 isoforms (Figs. 8 and 9) where the formation of all three metabolites (NORCIS, 3-F-4-OHCIS, and 4-F-2-OHCIS) from each enantiomer was catalyzed by CYP3A4 at the highest specific activity. Further evidence for the role of CYP3A4 in cisapride stereoselective metabolism is provided by the fact that the \( K_m \) values for formation of NORCIS from each enantiomer in HLMs (Table 1) were close to those in recombinant human CYP3A4 (Table 4). The role of other isoforms in cisapride stereoselective metabolism appears to be small. Although we noted a significant correlation between the metabolism of the enantiomers and the activity of CYP2C19, subsequent experiments with specific inhibitors and recombinant P450s failed to support a significant involvement of CYP2C19. This could be simply due to a significant correlation between CYP2C19 and CYP3A activities in the panel of liver microsomes used (Spearman \( r = 0.91, p < 0.0001, \) data not shown). We noted that recombinant human CYP isoforms other than CYP3A4 (e.g., CYP2C8, 2B6, and 2A6) catalyze the formation of one or more metabolites from cisapride enantiomers. These findings are consistent with earlier studies with racemic cisapride (Bohets et al., 2000; Desta et al., 2000a). However, the contribution of these isoforms to the overall metabolic clearance of cisapride enantiomers would be minor, if any, because of the very low rate of metabolism formation by these isoforms (Fig. 8) and because of the major involvement of CYP3A in

\[ \begin{align*}
(+)-Cisapride & \rightarrow (+)-NORCIS \\
& \rightarrow (+)-3-F-4-OHCIS \\
(-)-Cisapride & \rightarrow (-)-NORCIS \\
& \rightarrow (-)-3-F-4-OHCIS \\
(\pm)-Cisapride & \rightarrow (-)-4-F-2-OHCIS \\
& \rightarrow (+)-4-F-2-OHCIS \\
& \rightarrow (+)-3-F-4-OHCIS
\end{align*} \]
cisapride metabolism and its relative abundance in the liver and intestine. Of note, concurrent administration of several medications that have inhibition of CYP3A in common increases the plasma concentrations of cisapride and/or its cardiac risk (Bedford and Rowbotham, 1996; van Haarst et al., 1998; Kivisto et al., 1999; Dresser et al., 2000; Michelets and Williams, 2000).

We have demonstrated that cisapride metabolism is stereoselective. In HLMs and recombinant human P450 isoforms, we observed that (+)-cisapride is more efficiently metabolized to NORCIS and 3-F-4-OHCIS than (−)-cisapride. This was maintained across a panel of different HLMs and the recombinant enzymes involved. We noted that 4-F-2-OHCIS is preferentially formed from (−)-cisapride in (certain) HLMs and recombinant human P450s. However, this stereoselectivity was generally less marked compared with that of NORCIS and 3-F-4-OHCIS, and it was not observed in all HLMs (Fig. 5C), probably due to effects of secondary metabolism or involvement of other unknown isoforms that might offset the stereoselective formation of 4-F-2-OHCIS. Altogether, our in vitro data suggest that cisapride stereoselective metabolism is determined primarily by the formation of NORCIS and 3-F-4-OHCIS. Because the metabolic pathways of (−)- and (+)-cisapride are qualitatively similar and CYP3A appears to be the major enzyme catalyzing them, differences in their respective orientations relative to the enzyme active site may explain the difference in the efficiency of regio- or stereoselectivity we observed here.

After oral administration, less than 50% of the dose of racemic cisapride reaches systemic circulation unchanged, suggesting first-pass elimination in the liver and intestine. This is not surprising given that CYP3A is the most abundant human CYP isoform in both these tissues and plays an important role in the oral bioavailability and systemic clearance of a vast number of drugs (Thummel and Wilkinson, 1998). CYP3A exhibits highly variable expression and is susceptible to induction and inhibition by a large variety of drugs (Pelkonen et al., 1998; Thummel and Wilkinson, 1998). If our in vitro data, indicating that (+)-cisapride is more efficiently metabolized relative to (−)-cisapride, can be extrapolated to in vivo conditions, it is likely that (+)-cisapride is more susceptible to CYP3A-mediated presystemic and systemic metabolism than (−)-cisapride. Recently, we have tested this hypothesis in normal volunteers who received racemic cisapride (Desta et al., 2000b, 2001) and found that the Cmax and area under the response-time curve of (−)-cisapride were ~2.9-fold higher than those of (+)-cisapride, essentially confirming our in vitro data. The degree of metabolic drug interaction is often large for drugs with high presystemic metabolism (Dresser et al., 2000). Whether (+)-cisapride may be more susceptible to metabolic inhibition, which could shift the plasma ratio of the enantiomers without marked effect on the total concentrations of the racemate, remains to be determined.

We have provided evidence for cisapride enantiomer-enantiomer interaction. Metabolic interaction between enantiomers may be expected when 1) both enantiomers, being metabolized by the same enzyme at different rates, either mutually compete for the same catalytic site of the enzyme [e.g., (R)- and (S)-propafenone 5-hydroxylation by CYP2D6 (Kroemer et al., 1991, 1994)] or only one enantiomer acts as a competitive inhibitor (unidirectional interaction) of the other’s metabolism [e.g., (S)-propranolol inhibition of (R)-propranolol glucuronidation (Wilson and Thompson, 1984)]; and 2) one of the enantiomer binds to the enzyme that metabolizes the other without itself being metabolized to any appreciable extent by that enzyme [e.g., (S)-warfarin 7-hydroxylation by CYP2C9 and (R)-warfarin (Kunze et al., 1991)]. Because our findings support that CYP3A acts on both cisapride enantiomers as a different substrate and since the enantiomers inhibit each other’s metabolism, the mechanism of enantiomer-enantiomer is likely to predominantly involve mutual competition of the enantiomers for the active site(s) that could be understood by a mixed alternative substrate model suggested elsewhere (Segel, 1993). A similar phenomenon has been reported with the antiarrhythmic drug propafenone where the (R)- and (S)-enantiomers underwent 5-hydroxylation by CYP2D6 and both enantiomers inhibit each other’s 5-hydroxylation by this isoform (Kroemer et al., 1991, 1994).

The clinical consequence of cisapride enantiomer-enantiomer interaction remains to be tested through appropriate pharmacokinetic and pharmacodynamic analysis after the individual enantiomers and the racemate are administered separately. Provided the pharmacological activities of cisapride exhibit stereoselectivity, we would expect that metabolic drug interaction and the effect of racemate cisapride therapy differ than would be predicted based on the summation of the effects observed with the individual enantiomers. According to Kroemer et al. (1994) and Li et al. (1998), (R)-propafenone has been shown to reduce the clearance of the more potent β-blocker (S)-propafenone when the racemic mixture was administered to normal volunteers. As a result, 75 mg of (S)-propafenone contained in the racemic drug was about as equieffective as 150 mg of (S)-propafenone when administered separately with respect to β-blocking effect. It is often attempted to develop the homochiral as a drug from the currently available racemates, provided one enantiomer exhibits favorable pharmacological properties over the other. In such cases, it is important to consider enantiomer-enantiomer interactions during evaluation of the pharmacology of racemic cisapride and its enantiomers.

Although the use of cisapride in the United States is suspended owing to its cardiac toxicity, the drug continues to be available to patients who meet eligibility criteria for a limited-access protocol in the United States, and it is still widely used in other countries. At present, there is no data on whether cisapride prokinetic and cardiac actions are stereoselective. It may be possible that one enantiomer is more cardiotoxic while the other is mainly responsible for the prokinetic action, or the enantiomers have similar effects with different potency, paving the way to develop the relatively safe enantiomer as a prokinetic. To our knowledge, this is the first report of enantiospecific cisapride metabolism and interactions. Understanding the individual enantiomers as opposed to racemic cisapride may be more a reliable predictor for therapeutic failure by drug interactions with inducers or for toxicity with inhibitors of CYP3A. In addition, the enantiomer-enantiomer interaction observed here should be taken into account when the pharmacology of racemic cisapride is compared with its enantiomers.
References


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