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Article

Potential of Hydroxyebastine and Terfenadine Alcohol to Inhibit the Human Cytochrome P450 2J2 Isoform

Yune-Jung Yoon[1] and Kwang-Hyeon Liu[2,3]*

[1]*Department of Clinical Phamacology and Clinical Trial Center, Inje University Busan Paik Hospital, Busan 614-735, Republic of Korea*

[2]*Department of Pharmacology and PharmacoGenomics Research Center, Inje University College of Medicine, Busan, Republic of Korea*

[3]*College of Pharmacy and Research Institute of Pharmaceutical Sciences, Kyungpook National University, Daegu 702-701, Republic of Korea*

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Although selective inhibitors of cytochrome P450 enzymes can be used to determine relative contributions of the enzymes to xenobiotic metabolism, characterization of CYP2J2 in drug metabolism is more challenging due to lack of selective, well-characterized inhibitors. Thus, selectivity of hydroxyebastine, which has high affinity for recombinant CYP2J2, was studied. The IC_{50} value of hydroxyebastine in CYP2J2-mediated astemizole *O*-demethylation activity was lower than that of its structural analog, terfenadine alcohol. Terfenadine alcohol inhibited several other P450 activities, such as CYP2D6, more potently than CYP2J2, and is thus not suitable as a CYP2J2-selective inhibitor. Inhibitory potential values of hydroxyebastine in CYP2J2-catalyzed astemizole *O*-demethylation, tolbutamide hydroxylation (CYP2C9), *S*-mephenytoin hydroxylation (CYP2C19), and dextromethorphan *O*-demethylation (CYP2D6) were 0.45, 2.74, 10.22, and 3.83 μM, respectively. The inhibitory potential of other P450 enzymes, such as CYP1A2, CYP2B6, CYP2E1, and CYP3A, was negligible. Although hydroxyebastine was a relatively potent inhibitor of CYP2J2, it provided a selectivity of only > 6-fold (CYP2J2 vs. other P450s). However, hydroxyebastine can serve as a relatively selective inhibitor of CYP2J2 and can be used to characterize the contribution of CYP2J2 to xenobiotic metabolism due to the lack of a more specific inhibitor.

Key words: CYP2J2, hydroxyebastine, selective inhibitor, terfenadine alcohol

Cytochrome P450 (P450) enzymes play the most important role in drug metabolism and are responsible for clearance of a majority of xenobiotics, including drugs and pesticides [Rendic, 2002]. Alteration in the activity of these enzymes represents the major underlying mechanism behind pharmacokinetic or pharmacodynamic drug-drug interactions [Clarke and Jones, 2002]. Five of these P450 enzymes, CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A, are considered to be important drug-metabolising enzymes in humans, and they have been studied extensively with regard to their role in metabolic clearance and drug interactions [Bjornsson *et al*., 2003]. However, recent investigations have shown that some other human P450 enzymes, such as CYP2J2, also play a major role in the metabolism of some xenobiotics [Matsumoto *et al*., 2003; Liu *et al.,* 2006]. Thus, CYP2J2 could be a target of interactions for the drugs for which it plays an important metabolic role. CYP2J2 has been shown to be involved in the metabolism of endogenous metabolites, such as epoxyeicosatrienoic acid [Scarborough *et al*., 1999; Spiecker *et al*., 2004], as well as drugs such as astemizole [Matsumoto *et al*., 2003], apixaban [Wang *et al*., 2010], and ebastine [Liu *et al*., 2006].

Inhibition of the *in vitro* metabolism of a drug in human liver microsomes by isoform-selective chemical inhibitors is a valuable approach for reaction phenotyping to identify P450 isoforms responsible for drug metabolism [Rodrigues, 1999]. Some chemicals, such as terfenadine

*Corresponding author
Phone: +82-53-950-8567; Fax: +82-53-950-8557
E-mail: dstlkh@knu.ac.kr

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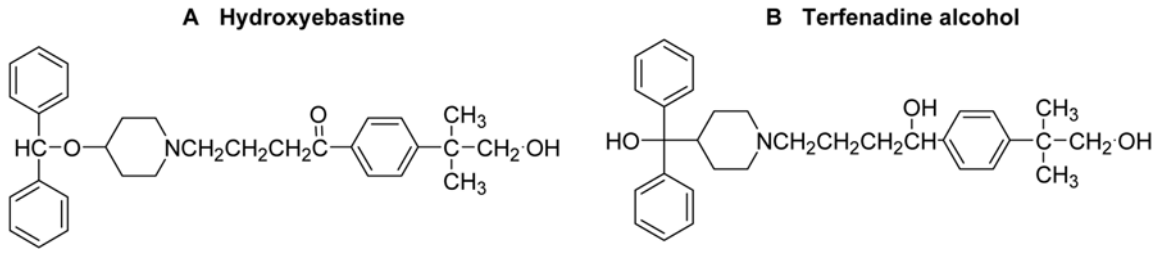

Fig. 1. Chemical structures of hydroxyebastine (A) and terfenadine alcohol (B).

and terfenadone, have already been reported to inhibit CYP2J2 [Lafite *et al*., 2006]. Terfenadone potently inhibits CYP2J2-catalyzed ebastine hydroxylation, with an IC_{50} value of 0.7 μM [Lafite *et al*., 2007b]. However, there have been only limited reports on CYP2J2 inhibitors compared with those of other P450 isoforms.

In our previous study, the formation of carebastine from hydroxyebastine by recombinant CYP2J2 was found to exhibit strong substrate inhibition [Liu *et al*., 2006]. This finding indicated that hydroxyebastine has strong affinity for the CYP2J2 isoform, thus opening the possibility of the chemical inhibiting CYP2J2-mediated biotransformation. In the present study, hydroxyebastine (Fig. 1A) and its structural analog, terfenadine alcohol, were investigated (Fig. 1B) for their ability to inhibit CYP2J2 activity in human liver microsomes, and recombinant P450 isoforms and their selectivity for CYP2J2 versus other human P450 enzymes was evaluated.

Materials and Methods

Chemicals and reagents. Hydroxyebastine and ebastine were provided by Almirall Prodesfarma, S.A. (Barcelona, Spain). Phenacetin, acetaminophen, bupropion, tolbutamide, dextromethorphan, chlorzoxazone, reduced form of β-nicotinamide adenine dinucleotide phosphate (NADPH), $MgCl_2$, glucose 6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from Sigma-Aldrich (St. Louis, MO). 4-Hydroxytolbutamide, *S*-mephenytoin, 4'-hydroxymephenytoin, dextrorphan, 6-hydroxychlorzoxazone, midazolam, and 1'-hydroxy-midazolam were purchased from Ultrafine Chemical Co. (Manchester, England). Hydroxybupropion, pooled human liver microsomes, eight different human recombinant P450 isoforms 1A2, 2B6, 2C9, 2C19, 2D6, 2J2, 2E1, and 3A4, were purchased from BD Gentest Co. (Woburn, MA). These recombinant P450s (supersomes) were coexpressed with human P450 reductase and cytochrome b_5. The manufacturer supplied information regarding protein concentration and P450 content. All other reagents and chemicals were of analytical or high-performance liquid chromatography (HPLC) grade.

Inhibition studies using human liver microsomes and recombinant P450s. The inhibitory potency of hydroxyebastine and terfenadine alcohol was determined using cytochrome P450 assays in the absence and presence of both compounds using pooled human liver microsomes (H161; Gentest) and cDNA-expressed P450 isoforms. The incubation mixtures (final volume, 250 μL) contained 0.25 mg/mL microsomal protein (H161) or cDNA-expressed P450 (diluted to 200 pmol/mL with phosphate buffer, pH 7.4), 0.1 M phosphate buffer (pH 7.4), 1 mM NADPH, various P450 isoform-specific substrates (phenacetin for CYP1A2, bupropion for CYP2B6, tolbutamide for CYP2C9, *S*-mephenytoin for CYP2C19, dextromethorphan for CYP2D6, chlorzoxazone for CYP2E1, astemizole, ebastine or terfenadine for CYP2J2, and midazolam for CYP3A), and an inhibitor (hydroxyebastine or terfenadine alcohol). The substrates were used at concentrations approximately equal to their respective K_m values: 50 μM for phenacetin, 50 μM for bupropion, 100 μM for tolbutamide, 100 μM for *S*-mephenytoin, 5 μM for dextromethorphan, 50 μM for chlorzoxazone, 10 μM for astemizole, 5 μM for ebastine, 5 μM for terfenadine, and 5 μM for midazolam [Yuan *et al*., 2002; Liu *et al*., 2006]. In all experiments, substrates were dissolved and serially diluted with methanol to the required concentrations. The final concentration of methanol for the incubation conditions was <1.0%. After a 5-min pre-incubation at 37°C, the reactions were initiated by the addition of 1 mM NADPH. The incubations were performed at 37°C in a shaking water bath. The reactions were terminated by placing the incubation tubes on ice, adding 100 μL of cold methanol that contained 20 μM chlorpropamide as an internal standard after 15 min, and vortexing before centrifugation. The samples for each enzyme assay were centrifuged (1,000 g, 5 min, 4°C) and aliquots of the supernatants were analyzed by liquid chromatography-tandem mass spectrometry (LC/MS/MS) as described previously [Kim *et al*., 2005; Liu *et al*., 2006], with some modifications.

LC/MS/MS analysis. Samples were analyzed using an API QTRAP 4000 LC/MS/MS system (Applied Biosystems, Foster City, CA) equipped with an electrospray ionization

Table 1. MRM parameters for the major metabolites of the P450 probe substrates used in all the assays

P450 enzyme	Substrate	Concentration (μM)	Metabolite	Transition (*m/z*)	Polarity	Collision energy (eV)
1A2	Phenacetin	50	Acetaminophen	152>120	ESI^+	25
2B6	Bupropion	50	Hydroxybupropion	256>238	ESI^+	25
2C9	Tolbutamide	100	4-Hydroxytolbutamide	287>89	ESI^+	55
2C19	*S*-Mephenytoin	100	4'-Hydroxymephenytoin	235>150	ESI^+	25
2D6	Dextromethorphan	5	Dextrorphan	258>157	ESI^+	55
2E1	Chlorzoxazone	50	6-Hydroxychlorzoxazone	184>120	ESI^-	-28
	Astemizole	10	Desmethylastemizole	445>204	ESI^+	35
2J2	Ebastine	5	Hydroxyebastine	486>167	ESI^+	42
	Terfenadine	5	Terfenadine alcohol	488>452	ESI^+	36
3A	Midazolam	5	1'-Hydroxymidazolam	342>203	ESI^+	25
IS	Chlorpropamide			277>175	ESI^+	25

interface used to generate positive ions $[M+H]^+$ and negative ions $[M-H]^-$. Aliquots were injected onto a reversed-phase column (Luna C18, 2×50 mm i.d., 3 μm particle size; Phenomenex, Torrance, CA). The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) at a flow rate of 0.2 mL/min. For the analysis of acetaminophen, hydroxybupropion, 4-hydroxytolbutamide, 4'-hydroxymephenytoin, dextrorphan, 6-hydroxychlorzoxazone, and 1-hydroxymidazolam, the mobile phase A was linearly increased from 10 to 80% over 0.1 min, held at 80% for an additional 3.5 min, and then immediately stepped back down to 10% for re-equilibration. The total run time was 7 min. After 0.5 min, the LC eluent was diverted from waste to the ion source of the mass spectrometer. For analysis of desmethylastemizole, hydroxyebastine, and terfenadine alcohol, the isocratic mobile phase composition (50% A) was used. The total run time was 2 min.

The turbo ion spray interface was operated in positive-ion mode at 5,500 V and in the negative ion mode at 4,500 V. The ion source temperature was 550°C. Quantitation was performed by multiple reaction monitoring (MRM) of the precursor ion and the related product ion for each metabolite, using an internal standard to establish peak area ratios. The MRM transitions and collision energies determined for each metabolite and internal standard are listed in Table 1. Quadrupoles Q1 and Q3 were set at unit resolution. The analytical data were processed using the Analyst software (ver. 1.4; Applied Biosystems).

Data analysis. The IC_{50} values were determined from the following equation using the WinNonlin software (Pharsight, Mountain View, CA): percentage of control activity $=100A\times[1-(I/(I+IC_{50}))]$, where A is the maximum activity, I is the concentration of inhibitor, and IC_{50} is the inflection point on the curve [Kim *et al*., 2006]. The apparent kinetic parameters for the inhibitory potential (K_i) were first estimated by graphical methods, such as Lineweaver-Burk, Dixon, and secondary reciprocal plots, and were finally determined by non-linear least squares regression analysis, based on the best enzyme inhibition model [Segel, 1975], using the WinNonlin software. In our experiment, the inhibition data were consistently best fitted by the competitive inhibition model, via the Akaike information criterion and Schwartz criteria. The models tested included pure and partial competitive inhibition, non-competitive inhibition, mixed-type inhibition, and uncompetitive inhibition [Liu *et al*., 2004; Seo *et al*., 2008].

Results

Inhibitory effects of hydroxyebastine and terfenadine alcohol on P450s. The effects of hydroxyebastine and terfenadine alcohol on the catalytic activities of human P450s were investigated in human liver microsomes and recombinant P450 isoforms. At a 100 μM concentration of hydroxyebastine, inhibition of CYP2C9, CYP2C19, CYP2D6, and CYP2J2-related activity was observed. Terfenadine alcohol (100 μM) also inhibited CYP2C19, CYP2D6, CYP3A, and CYP2J2-related activity (Fig. 2). Essentially similar findings were obtained with recombinant expressed P450 enzymes (Fig. 2). The IC_{50} of hydroxyebastine was 1.5 and 7.6 μM against CYP2J2-mediated astemizole *O*-demethylation and terfenadine hydroxylation, respectively, in recombinant CYP2J2 (Table 2). Inhibition of CYP2D6 (IC_{50}=7.5 μM), CYP2C9 (IC_{50}=10.2 μM), and CYP2C19 (IC_{50}=19.1 μM) were also seen with hydroxyebastine (Fig. 3). Terfenadine alcohol inhibited dextromethorphan *O*-demethylation (CYP2D6) in recombinant CYP2D6, as shown by an IC_{50} of 5.4 μM for incubation with 5 μM

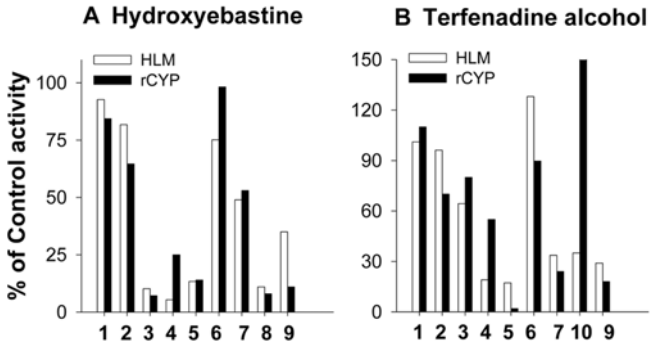

Fig. 2. Effects of hydroxyebastine (A) and terfenadine alcohol (B) on P450 enzyme activities (CYP1A2-mediated phenacetin *O*-deethylation (1), CYP2B6-mediated bupropion hydroxylation (2), CYP2C9-mediated tolbutamide hydroxylation (3), CYP2C19-mediated *S*-mephenytoin hydroxylation (4), CYP2D6-mediated dextromethorphan *O*-demethylation (5), CYP2E1-mediated chlorzoxazone hydroxylation (6), CYP3A-mediated midazolam 1'-hydroxylation (7), CYP2J2-mediated terfenadine hydroxylation (8), CYP2J2-mediated astemizole *O*-demethylation (9), and CYP2J2-mediated ebastine hydroxylation (10)) in human liver microsomes (□) and recombinant enzymes (■).

dextromethorphan (Table 2). Compared with CYP2D6, the IC_{50} of terfenadine alcohol on CYP2J2-catalyzed astemizole *O*-demethylation reaction (IC_{50}=30.9 μM) was > 5-fold higher (Fig. 4). Thus, terfenadine alcohol is not sufficiently selective as a CYP2J2 inhibitor.

Inhibitory effect of hydroxyebastine on CYP2J2, CYP2C9, CYP2C19, and CYP2D6. As hydroxyebastine showed inhibition of CYP2J2, CYP2C9, CYP2C19, and CYP2D6, we sought to clarify the mechanism of inhibition. The inhibitory potential (K_i values) of hydroxyebastine on P450 isoforms was in the order: CYP2J2 > CYP2C9 > CYP2D6 > CYP2C19 (Table 3). The Lineweaver-Burk, Dixon, and secondary reciprocal plots indicated that hydroxyebastine competitively inhibited CYP2J2-, CYP2C9-, CYP2D6-, and CYP2C19-mediated oxidation, with K_i values of 0.45, 2.74, 3.83, and 10.22 μM, respectively (Fig. 5, Table 3).

Discussion

CYP2J2 is expressed in the human liver, heart, kidney, lung, and small intestine [Wu *et al*., 1997; Zeldin *et al*., 1997]. Human CYP2J2 is regarded as a minor hepatic drug-metabolizing enzyme; however, recent studies have shown that CYP2J2 represents 1.4% of the total intestinal P450 content [Paine *et al*., 2006] and CYP2J2 is particularly abundant in the heart [Scarborough *et al*., 1999]. CYP2J2 is important in the metabolism of drugs such as astemizole [Matsumoto *et al*., 2003], apixaban [Wang *et al*., 2010], and ebastine [Liu *et al*., 2006].

In a previous study, we found that the biotransformation of carebastine from hydroxyebastine by recombinant CYP2J2 exhibited very strong substrate inhibition kinetics, with a K_{si} (substrate inhibition constant) value of 5.55 μM [Liu *et al*., 2006]. We hypothesized that hydroxyebastine has strong affinity for the CYP2J2 isoform, and it inhibits CYP2J2-catalyzed metabolism. In the present study, we investigated the inhibitory potential of hydroxyebastine and its structural analog terfenadine alcohol on other major P450 activities, as well as CYP2J2 activity.

Hydroxyebastine and terfenadine alcohol inhibited CYP2J2-catalyzed biotransformations in a substrate-

Table 2. Effects of hydroxyebastine and terfenadine alcohol on cytochrome P450 metabolic activities in recombinant P450 enzymes

P450 enzyme	Enzyme activity	IC_{50} values (μM) [a]	
		Hydroxyebastine	Terfenadine alcohol
CYP1A2	Penacetin *O*-deethylation	>100.0	>400.0
CYP2B6	Bupropion hydroxylation	>100.0	258.0
CYP2C9	Tolbutamide hydroxylation	10.2	137.2
CYP2C19	*S*-Mephenytoin hydroxylation	19.1	131.5
CYP2D6	Dextromethorphan *O*-demethylation	7.5	5.4
CYP2E1	Chlorzoxazone hydroxylation	>100.0	>400.0
CYP3A4	Midazolam 1'-hydroxylation	83.9	48.2
CYP2J2	Astemizole *O*-demethylation	1.5	30.9
	Terfenadine hydroxylation	7.6	-[b]
	Ebastine hydroxylation	-[b]	16.1[c]

[a]Average of duplicate determinations
[b]Not determined
[c]EC_{50} (Concentration effective in causing 50% of maximal activation)

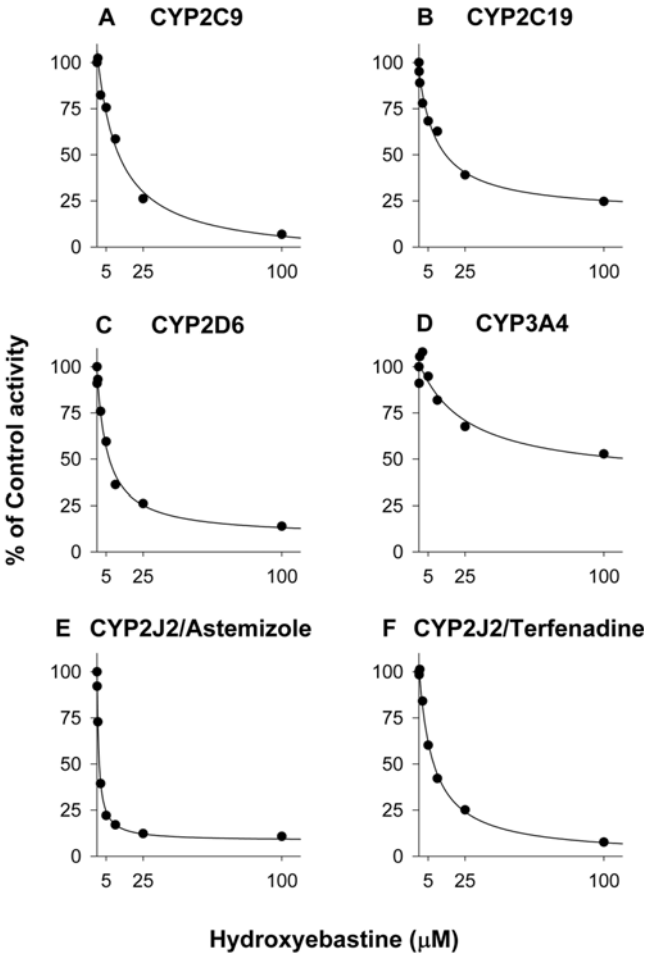

Fig. 3. Inhibitory effects of hydroxyebastine on CYP2C9-catalyzed tolbutamide hydroxylation (A), CYP2C19-catalyzed *S*-mephenytoin hydroxylation (B), CYP2D6-catalyzed dextromethorphan *O*-demethylation (C), CYP3A4-catalyzed midazolam 1'-hydroxylation (D), CYP2J2-catalyzed astemizole *O*-demethylation (E), and CYP2J2-catalyzed terfenadine hydroxylation (F) in recombinant P450 enzymes. Recombinant P450 enzymes were incubated with each P450-selective substrate. The substrates were used at concentration approximately equal to their respective K_m value.

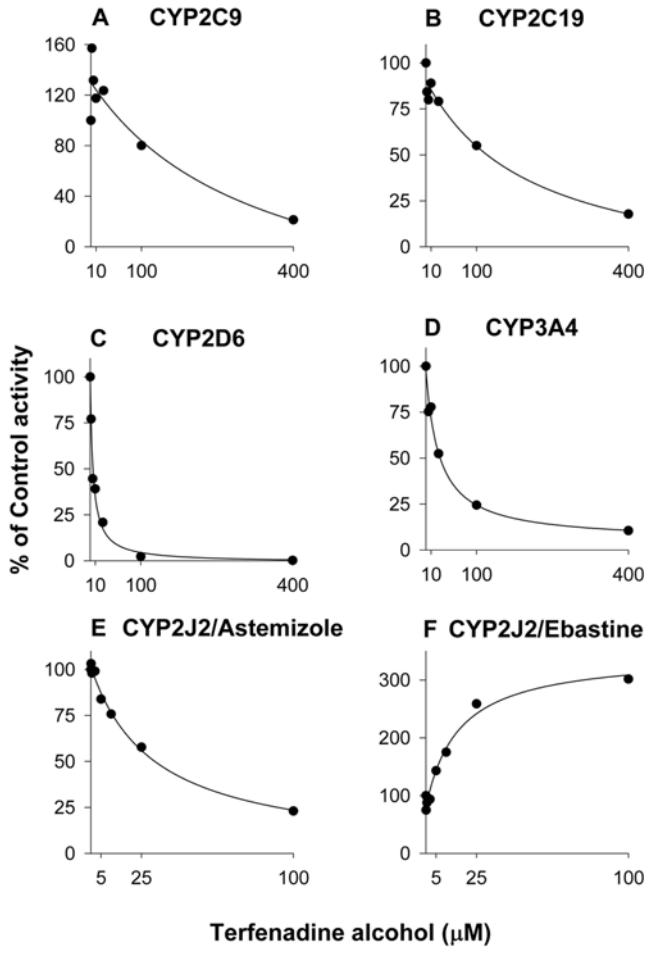

Fig. 4. Inhibitory effects of terfenadine alcohol on CYP2C9-catalyzed tolbutamide hydroxylation (A), CYP2C19-catalyzed *S*-mephenytoin hydroxylation (B), CYP2D6-catalyzed dextromethorphan *O*-demethylation (C), CYP3A4-catalyzed midazolam 1'-hydroxylation (D), CYP2J2-catalyzed astemizole *O*-demethylation (E), and CYP2J2-catalyzed ebastine hydroxylation (F) in recombinant P450 enzymes. Recombinant P450 enzymes were incubated with each P450-selective substrate. The substrates were used at concentration approximately equal to their respective K_m value.

dependent manner (Table 2). Hydroxyebastine was a more potent inhibitor of CYP2J2 than was terfenadine alcohol. This result was consistent with earlier work [Lafite *et al*., 2006] with terfenadine and terfenadone, which showed that the inhibitory effect of terfenadone towards recombinant CYP2J2 was much higher than towards terfenadine (IC_{50} 0.7 vs. 8.1 μM, respectively). Previously, it was reported that the keto functional group is well-positioned to establish hydrogen bonds with the guanidine moiety of an arginine residue of the CYP2J2 isoform [Lafite *et al*., 2007a]. Terfenadone and hydroxyebastine have the same keto functional group. This indicates that the ketone structure in the alkylphenylbutanone group is important and leads to high-affinity binding of CYP2J2 inhibitors.

Terfenadine alcohol inhibited CYP2D6 activity (IC_{50}= 5.4 μM) more strongly than CYP2J2 activity (IC_{50}=48.2 μM), which means terfenadine alcohol cannot be used as a CYP2J2 isoform-selective inhibitor (Table 2). Additionally, terfenadine alcohol caused the activation of CYP2J2-mediated ebastine hydroxylation, whereas it inhibited CYP2J2-catalyzed astemizole *O*-demethylation. Activation of CYP2J2 by terfenadine alcohol is not an unusual finding, given that evidence of atypical enzyme kinetics, such as activation, substrate inhibition, and partial inhibition, is common [Korzekwa *et al*., 1998].

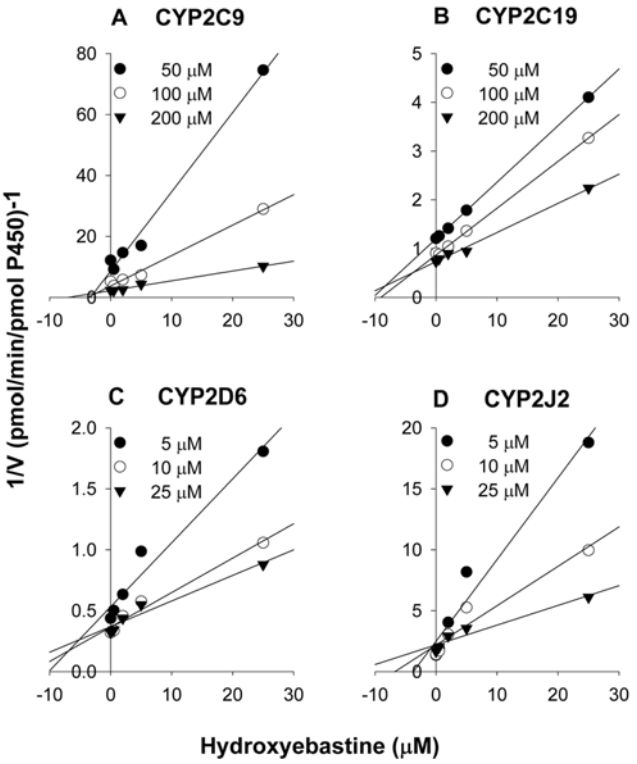

Fig. 5. Representative Dixon plots for inhibition of CYP2C9-mediated tolbutamide hydroxylation (A), CYP2C19-mediated *S*-mephenytoin hydroxylation (B), CYP2D6-mediated dextromethorphan *O*-demethylation (C), and CYP2J2-mediated astemizole *O*-demethylation (D) by hydroxyebastine (0-25 μM) in recombinant P450 enzymes on P450-specific substrate metabolic activity in recombinant P450 enzymes. The substrates were used at three different concentrations of tolbutamide (50, 100, and 200 μM), *S*-mephenytoin (50, 100, and 200 μM), dextromethorphan (5, 10, and 25 μM), and astemizole (5, 10, and 25 μM) for recombinant CYP2C9, CYP2C19, CYP2D6, and CYP2J2 enzymes, respectively.

Many studies have demonstrated activation of metabolism by P450 isoforms, such as CYP3A4 [Shou *et al*., 1994; Galetin *et al*., 2002] and CYP2C9 [Hutzler *et al*., 2002; Liu *et al*., 2005]. The activation observed for CYP2J2 might be explained by different binding domains for substrate and modifiers within the active site of the enzyme [Liu *et al*., 2005]. A 3D model of CYP2J2, built using a combination of the X-ray structures of CYP2A6, CYP2B4, CYP2C5, CYP2C8, and CYP2D6 as a template, has indicated that the active site volume (945 $Å^3$) is larger than that reported for CYP2D6 (540 $Å^3$) [Lafite *et al*., 2007a] and CYP2C9 (670 $Å^3$) [Williams *et al*., 2003]. This means that multiple substrates/ligands might be bound to CYP2J2 simultaneously, consistent with a report that suggested a "multiple-site model" for CYP2J2 activity [Liu *et al*., 2006].

The significance of identifying P450-isoform-selective inhibitors lies in the fact that, in screening of molecules during drug development, enzyme-specific inhibitors are convenient tools for the characterization of the metabolizing enzymes [Taavitsainen *et al*., 2001]. Thus, model inhibitors specific to each P450 isoform are important when metabolic interactions are evaluated. The most frequently used inhibitors are furafylline (CYP1A2; [Tassaneeyakul *et al*., 1994], tranylcypromine (CYP2A6; [Taavitsainen *et al*., 2001], thio-TEPA (CYP2B6; [Walsky and Obach, 2007], montelukast (CYP2C8; [Schoch *et al*., 2008], sulphaphenazole (CYP2C9; [Eagling *et al*., 1998], *S*-benzylnirvanol (CYP2C19; [Walsky and Obach, 2003], quinidine (CYP2D6; [Muralidharan *et al*., 1991], diethyldithiocarbamate (CYP2E1; [Chang *et al*., 1994], and ketoconazole (CYP3A; [Stresser *et al*., 2004]. Currently, however, there is no selective inhibitor for CYP2J2, which is involved in the metabolism of astemizole, apixaban, ebastine, and terfenadine. An early report on CYP2J2 described terfenadine derivatives as potential CYP2J2 inhibitors [Lafite *et al*., 2006]; however, their selectivity for this enzyme versus other human P450 enzymes has since been evaluated for some P450s (CYP2B6, CYP2C8, CYP2C9, and CYP3A4). In the present study, hydroxyebastine strongly inhibited CYP2J2-catalyzed astemizole *O*-demethylation (K_i=0.45 μM); however, it also slightly inhibited other P450s, such as CYP2C9 (K_i= 2.74 μM), CYP2C19 (K_i=10.22 μM), and CYP2D6 (K_i= 3.83 μM; Table 3, Fig. 5). Other P450 activities were not significantly inhibited by hydroxyebastine (Table 2).

Table 3. Inhibition constants (K_i values, μM) of hydroxyebastine on cytochrome P450 metabolic activities in recombinant P450 enzymes

P450 enzyme	Enzyme activity	K_i (μM) [a]	Type of inhibition
CYP2C9	Tolbutamide hydroxylation	2.74	Competitive
CYP2C19	*S*-Mephenytoin hydroxylation	10.22	Competitive
CYP2D6	Dextromethorphan *O*-demethylation	3.83	Competitive
CYP2J2	Astemizole *O*-demethylation	0.45	Competitive

[a]K_i values were determined by nonlinear least squares regression analysis based on the best enzyme inhibition model using WinNonlin software.

Based on these results, hydroxyebastine is not a good selective inhibitor for CYP2J2; however, it could still be useful as a CYP2J2 inhibitor in the absence of a better one, because terfenadone which has higher inhibitory potential on CYP2J2 activity than hydroxyebastine is commercially unavailable.

In conclusion, these data show that hydroxyebastine is a more potent competitive inhibitor of CYP2J2 than its structural analog terfenadine alcohol. Hydroxyebastine should be a useful tool in *in vitro* P450 phenotyping studies, although it shows moderate inhibition of CYP2C9, CYP2C19, and CYP2D6, and there is a lack of commercially available CYP2J2 inhibitors.

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