Involvement of CYP1A2 in mexiletine metabolism

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Aims Mexiletine has been reported to be hydroxylated by cytochrome P450 2D6 (CYP2D6) in humans. However, the involvement of CYP1A2 in the metabolism of mexiletine has been proposed based on the interaction with theophylline which is mainly metabolized by CYP1A2. The aim of this study was to clarify the role of human CYP1A2 in mexiletine metabolism.

Methods Human CYP isoforms involved in mexiletine metabolism were investigated using microsomes from human liver and B-lymphoblastoid cells expressing human CYPs. The contributions of CYP1A2 and CYP2D6 to mexiletine metabolism were estimated by the relative activity factor (RAF).

Results Mexiletine *p*- and 2-hydroxylase activities in human liver microsomes were inhibited by ethoxyresorufin and furafylline as well as quinidine. Mexiletine *p*- and 2-hydroxylase activities in microsomes from nine human livers correlated significantly with bufuralol 1'-hydroxylase activity ($r=0.907$, $P<0.001$ and $r=0.886$, $P<0.01$, respectively). Microsomes of B-lymphoblastoid cells expressing human CYP1A2 exhibited lower mexiletine *p*- and 2-hydroxylase activities than those expressing human CYP2D6. It was estimated by RAF that the major isoform involved in mexiletine metabolism was CYP2D6, and the contribution of CYP1A2 to both mexiletine *p*- and 2-hydroxylase activities was 7–30% in human liver microsomes. However, the K_m values of the expressed CYP1A2 ($\sim 15 \mu\text{M}$) were almost identical with those of the expressed CYP2D6 ($\sim 22 \mu\text{M}$) and human liver microsomes.

Conclusions Mexiletine is a substrate of CYP1A2. The data obtained in this study suggest that the interaction of mexiletine with theophylline might be due to competitive inhibition of CYP1A2.

Keywords: cytochrome P450, CYP2D6, bufuralol, ethoxyresorufin, furafylline, cDNA-expressed microsomes, relative activity factor

Introduction

Mexiletine is a class 1B antiarrhythmic agent used for the control of ventricular arrhythmias. It is of particular value because of its long time course of action and its suitability for either parenteral or oral administration [1]. Numerous studies have shown that there is marked interindividual variability in mexiletine plasma concentrations after a given dose. As the antiarrhythmic activity and toxicity of mexiletine is strongly correlated with its plasma concentration and as the therapeutic index is narrow [2], monitoring of the plasma concentration is desirable to ensure efficacy and to reduce the side effects of the drug. Mexiletine is eliminated by both renal excretion of the unchanged compound and extensive hepatic metabolism [1, 3]. Less than 10% of mexiletine is recovered unchanged in urine over 48 hr [4]. As shown in Figure 1, the major metabolic routes are aromatic hydroxylation, forming *p*-hydroxymexiletine, and aliphatic hydroxylation, forming 2-hydroxymexiletine [3].

These metabolites have no significant antiarrhythmic activity [5]. Several *in vitro* [6, 7] and *in vivo* studies [8, 9] have suggested that *p*- and 2-hydroxymexiletine formation are mediated by CYP2D6 in humans.

Mexiletine is occasionally used together with theophylline in patients who have both cardiac and pulmonary diseases. Recent reports have suggested that mexiletine increases the theophylline plasma concentration resulting in theophylline

Figure 1 Primary metabolic pathways of mexiletine in humans.

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toxicity [10, 11]. Theophylline is extensively metabolized to 1-methylxanthine (1X), 3-methylxanthine (3X), and 1,3-dimethyluric acid (13U). It has been reported that the formation of these metabolites is catalyzed by CYP1A2 in humans [12, 13]. Mexiletine was reported to reduce theophylline clearance and subsequently increase its plasma concentration by inhibiting *N*-demethylation of theophylline [14]. Therefore, the involvement of CYP1A2 in mexiletine metabolism is suggested. The aim of this study was to clarify the role of CYP1A2 in mexiletine *p*- and 2-hydroxylations in human livers.

Methods

Chemicals

Mexiletine [1-(2, 6-dimethylphenoxy)-2-aminopropane] hydrochloride, *p*-hydroxmexiletine [1-(2, 6-dimethyl-4-hydroxyphenoxy)-2-aminopropane] hydrochloride and 2-hydroxmexiletine [1-(2-hydroxymethyl-6-methylphenoxy)-2-aminopropane] oxalate were kindly provided by Nippon Boehringer Ingelheim (Hyogo, Japan). 5-Methoxyindole-3-acetic acid was purchased from Aldrich Chemical (Milwaukee, WI). (±)-Bufuralol hydrochloride and 1'-hydroxybufuralol maleate were purchased from ULTRAFINE chemicals (Manchester, England). 7-Ethoxyresorufin and resorufin were from Sigma (St Louis, MO), furafylline was from Funakoshi (Tokyo, Japan). NADP⁺, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were from Oriental Yeast (Tokyo, Japan). Other chemicals were of the highest grade commercially available. Microsomes from human B-lymphoblastoid cell lines expressing various human cytochrome P450s and control microsomes which were not transfected were obtained from Gentest (Woburn, MA).

Tissue samples and preparation of microsomes

Human liver samples were obtained at autopsy. The use of human liver for the study had been approved by the Institutional Committee of Tokyo Medical Examiner's Office. Liver tissues were rapidly frozen in liquid nitrogen immediately after excision and were stored at -80°C. Microsomes from human liver were prepared as described previously [15] and were stored at -80°C until use. Protein concentration was measured according to the method of Lowry *et al.* [16]. Cytochrome P450 content was estimated by the method of Johannesen & Depierre [17].

Mexiletine hydroxylase activities

The hydroxylated metabolites of mexiletine were determined by h.p.l.c. A typical incubation mixture (0.2 ml total volume) contained 100 mM potassium phosphate buffer (pH 7.4), an NADPH-generating system (0.5 mM NADP⁺, 5 mM glucose-6-phosphate, 5 mM MgCl₂, 1 U ml⁻¹ glucose-6-phosphate dehydrogenase), 0.5 mg ml⁻¹ microsomal protein and mexiletine as substrate. The reaction was initiated by the addition of the NADPH-generating system following a 2 min pre-incubation at 37°C. The reaction was terminated by addition of 0.1 ml of ice-cold methanol,

and 5-methoxyindole-3-acetic acid (5 ng) was added as an internal standard. After removal of protein by centrifugation at 10 000 *g* for 5 min, 100 µl portion of the supernatant was subjected to h.p.l.c. H.p.l.c. analyses were performed using a DG-980-50 degasser (Jasco, Tokyo, Japan), a PU-980 intelligent pump (Jasco), an AS-950 intelligent sampler (Jasco), an 807-IT integrator (Jasco), a model L-7480 fluorescence detector (Hitachi, Tokyo, Japan) equipped with an analytical Capcell Pak C₁₈ UG120 column (4.6 mm i.d. × 250 mm, 4 µm, Shiseido, Tokyo, Japan). The hydroxylated metabolites were detected fluorometrically (excitation; 270 nm, emission; 312 nm). The mobile phase was 10% acetonitrile containing 10 mM potassium dihydrogen phosphate (pH 5.0); the flow rate was 1.0 ml min⁻¹; the column temperature was 35°C. Accuracy and precision of the assay were determined with 15–150 pmol authentic standards/tube. Accuracy ranged from 98–102% and 96–123% for *p*- and 2-hydroxmexiletine, respectively. Coefficients of variation were 0.4–4.9% and 4.8–10.5%, respectively. The detection limits were 0.5 pmol and 5 pmol for *p*- and 2-hydroxmexiletine produced, respectively.

Inhibition analysis

The effects of CYP inhibitors on mexiletine *p*- and 2-hydroxylase activities in human liver microsomes at a substrate concentration of 20 µM were investigated. The inhibitors studied were furafylline [18], ethoxyresorufin [19], quinidine [20], and bufuralol [21]. The range of concentrations used were 0.25–10 µM for furafylline, ethoxyresorufin and quinidine, 1–100 µM for bufuralol. The incubation mixture including chemical inhibitors was pre-incubated for 2 min before the reaction was initiated by the addition of an NADPH-generating system. The assays were determined as mentioned above.

cDNA-expressed P450 incubations

In this experiment, microsomes from human B-lymphoblastoid cell lines expressing CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C9 (CYP2C9-Arg), CYP2C19, CYP2D6 (CYP2D6-Val), CYP2E1, and CYP3A4 were used. Control microsomes were from human B-lymphoblastoid cells which were not transfected. The incubation mixture was of the same composition as mentioned above, except for a 1.0 mg ml⁻¹ of microsomal protein concentration. The mexiletine concentration was 20 µM. The mixture was incubated at 37°C for 60 min after a 2 min pre-incubation.

Kinetic analyses

The kinetic studies were performed using microsomes from three different human livers, cDNA-expressed CYP1A2 and cDNA-expressed CYP2D6. In determining kinetic parameters, the mexiletine concentration ranged from 5–100 µM. Eadie-Hofstee plots were constructed for determination of the presence of a mono- or biphasic model. Kinetic parameters were evaluated by non-linear regression analysis of untransformed data.

Ethoxyresorufin O-deethylase activity

The activity of ethoxyresorufin O-deethylase in human liver microsomes was determined fluorometrically as described previously [22] with slight modifications. The incubation mixture (0.5 ml total volume) contained 100 mM Tris-HCl buffer (pH 7.4), 1.6 mg ml⁻¹ bovine serum albumin, 0.25 mM NADPH and 0.2 mg ml⁻¹ microsomal protein. The reaction was initiated by the addition of 2.5 µl of 0.4 mM 7-ethoxyresorufin (final concentration, 2 µM) dissolved in dimethyl sulphoxide following a 3 min pre-incubation at 37° C. After the incubation for 15 min at 37° C, the reaction was terminated by addition of 2 ml of ice-cold methanol. The incubation mixtures were centrifuged at 1000 g for 10 min and the concentration of the formed resorufin in the supernatant was determined fluorometrically (excitation; 574 nm, emission; 596 nm) with F-4000 fluorescence spectrophotometer (Hitachi, Tokyo, Japan).

Bufuralol 1'-hydroxylase activity

The activity of bufuralol 1'-hydroxylase in human liver microsomes was determined as described previously [21] with slight modifications. The incubation mixture (0.2 ml total volume) contained 50 mM potassium phosphate buffer (pH 7.4), an NADPH-generating system, 0.2 mg ml⁻¹ microsomal protein and 1 µM bufuralol as a substrate. The reaction was initiated by the addition of the NADPH-generating system following a 2 min pre-incubation at 37° C. After incubation for 10 min at 37° C, the reaction was terminated by addition of 20 µl of 70% perchloric acid. The incubation mixtures were centrifuged at 10 000 g for 5 min and a 20 µl portion of supernatant was subjected to h.p.l.c. H.p.l.c. equipment was the same as described above. An analytical column was Capcell Pak C18 UG120 (4.6 × 250 mm, 4 µm, Shiseido, Tokyo, Japan) and the formed 1'-hydroxybufuralol was detected fluorometrically (excitation; 252 nm, emission; 302 nm). The mobile phase was 20% acetonitrile containing 1 mM perchloric acid. The flow rate was 1.0 ml min⁻¹ and the column temperature was 35° C.

Contributions of CYP1A2 and CYP2D6 to mexiletine hydroxylase activities in human liver microsomes

The percentage contributions of CYP1A2 and CYP2D6 to mexiletine *p*- and 2-hydroxylase activities (at a 200 µM mexiletine concentration) were estimated as described previously [23] by application of the relative activity factor (RAF) proposed by Crespi [24]. This approach makes the assumption that any effects on the rate of metabolism are independent of substrate, *i.e.*, the rank order of rates of metabolism is the same for a particular cDNA-expressed CYP isoform and the same CYP isoform present in human liver microsomes, and any factor which affects the rate of metabolism for one substrate also does so equally for other substrates [24]. The RAF of CYP1A2 (*i.e.*, RAF_{CYP1A2}) was determined as the ratio of the activity of ethoxyresorufin O-deethylation (2 µM), a specific metabolic reaction mediated by CYP1A2 [22], in human liver microsomes to

that with recombinant CYP1A2. The RAF of CYP2D6 (*i.e.*, RAF_{CYP2D6}) was determined as the ratio of the activity of bufuralol 1'-hydroxylation (1 µM), a specific metabolic reaction mediated by CYP2D6 [21], in human liver microsomes to that with recombinant CYP2D6. It has been reported that CYP1A2 also catalyze bufuralol 1'-hydroxylation with the higher *K_m* value than that of CYP2D6 [25]. However, it was confirmed that microsomes expressing CYP1A2 exhibited no measurable bufuralol 1'-hydroxylation at a 1 µM bufuralol concentration in this study. We also confirmed that microsomes expressing CYP2D6 exhibit no measurable ethoxyresorufin O-deethylation at a 2 µM ethoxyresorufin concentration. Using RAF, mexiletine hydroxylation by CYP1A2 and CYP2D6 in human liver microsomes (MXL_{CYP1A2} and MXL_{CYP2D6}, respectively) are expressed as follows:

$$\text{MXL}_{\text{CYP1A2}} = \text{MXL}_{\text{rec-CYP1A2}} \times \text{RAF}_{\text{CYP1A2}} \quad (1)$$

$$\text{MXL}_{\text{CYP2D6}} = \text{MXL}_{\text{rec-CYP2D6}} \times \text{RAF}_{\text{CYP2D6}} \quad (2)$$

where MXL_{rec-CYP1A2} and MXL_{rec-CYP2D6} are the mexiletine hydroxylation by recombinant CYP1A2 and CYP2D6, respectively. The contributions of CYP1A2 and CYP2D6 to the overall activity by human liver microsomes (MXL_{HL}) is estimated as follows:

$$\begin{aligned} \text{Contribution of CYP1A2 (\%)} &= (\text{MXL}_{\text{CYP1A2}} / \text{MXL}_{\text{HL}}) \\ &\times 100 \end{aligned} \quad (3)$$

$$\begin{aligned} \text{Contribution of CYP2D6 (\%)} &= (\text{MXL}_{\text{CYP2D6}} / \text{MXL}_{\text{HL}}) \\ &\times 100 \end{aligned} \quad (4)$$

Statistical analysis

Correlations between mexiletine hydroxylase activities and bufuralol 1'-hydroxylase activity or ethoxyresorufin O-deethylase activity in microsomes from nine human livers were determined by Pearson's product moment method. Results are expressed as the mean ± s.d.

Results

Mexiletine *p*- and 2-hydroxylase activities in human liver microsomes

With 5–200 µM mexiletine concentration, the formation of *p*- and 2-hydroxymexiletine increased linearly with incubation time up to 60 min in the presence of 0.5 mg ml⁻¹ microsomal protein. Unless specified, an incubation time of 30 min was employed to ensure initial rate conditions for the formation of *p*- and 2-hydroxymexiletine.

Figure 2 depicts typical Eadie-Hofstee plots of mexiletine *p*- and 2-hydroxylase activities in human liver microsomes. Both plots of mexiletine *p*- and 2-hydroxylase activities were linear. As shown in Table 1, apparent Michaelis-Menten constants (*K_{mapp}*) for mexiletine *p*- and 2-hydroxylase activities were 15.8 ± 2.7 µM and 22.5 ± 12.4 µM, respectively. Maximal velocities (*V_{max}*) for mexiletine *p*- and 2-hydroxylase activities were 41.3 ± 19.1 fmol min⁻¹ pmol⁻¹ P450 and 71.1 ± 33.2 fmol min⁻¹ pmol⁻¹ P450, respectively.

Figure 2 Typical Eadie-Hofstee plots of mexiletine *p*-hydroxylase activity (○) and 2-hydroxylase activity (●) in human liver microsomes. Liver microsomes from a subject (HL 2) were incubated with 5–100 μM of mexiletine.

Inhibition analysis

As shown in Figure 3, the CYP-isoform specific xenobiotic probes were screened for inhibitory effects on mexiletine hydroxylase activities at a 20 μM mexiletine concentration ($\sim K_{\text{mapp}}$). Quinidine, a specific inhibitor of CYP2D6, essentially abolished the *p*- and 2-hydroxylase activities of mexiletine (both $\text{IC}_{50} = 0.2 \mu\text{M}$). Furaflavine, a specific inhibitor of CYP1A2, also efficiently inhibited *p*- and 2-hydroxylase activities of mexiletine ($\text{IC}_{50} = 1.4$ and $1.9 \mu\text{M}$, respectively). In addition, ethoxyresorufin, a substrate of CYP1A2, efficiently inhibited *p*- and 2-hydroxylase activities of mexiletine ($\text{IC}_{50} = 0.9$ and $1.5 \mu\text{M}$, respectively).

Bufuralol, a substrate of CYP2D6, weakly inhibited the activity ($\text{IC}_{50} = 27.2$ and $32.1 \mu\text{M}$, respectively).

Correlation between mexiletine hydroxylase activities and ethoxyresorufin O-deethylase activity or bufuralol 1'-hydroxylase activity in human liver microsomes

Mexiletine *p*- and 2-hydroxylase activities in microsomes from nine human livers were determined at 20 μM of mexiletine. Ethoxyresorufin O-deethylase activity and bufuralol 1'-hydroxylase activities in these human liver microsomes were determined as a probe of CYP1A2 and CYP2D6 activity, respectively. As shown in Figure 4, bufuralol 1'-hydroxylase activity significantly correlated with mexiletine *p*-hydroxylase activity ($r = 0.907$, $P < 0.001$), and with 2-hydroxylase activity ($r = 0.886$, $P < 0.01$). Ethoxyresorufin O-deethylase activity significantly correlated with mexiletine *p*-hydroxylase activity ($r = 0.700$, $P < 0.05$), but not with 2-hydroxylase activity ($r = 0.543$). In addition, mexiletine *p*-hydroxylase activity in these human liver microsomes significantly correlated with mexiletine 2-hydroxylase activity ($r = 0.957$, $P < 0.001$, data not shown). Ethoxyresorufin O-deethylase activity did not correlate with bufuralol 1'-hydroxylase activities ($r = 0.606$, data not shown).

Mexiletine hydroxylase activities in microsomes of B-lymphoblastoid cells expressing human cytochrome P450s

The capabilities of microsomes of B-lymphoblastoid cells expressing human CYPs to perform a biotransformation of mexiletine were determined (Table 2). cDNA-expressed

Table 1 Kinetic parameters of mexiletine *p*- and 2-hydroxylase activities in human liver microsomes.

Subject number	K_{mapp} (μM)	<i>p</i> -hydroxylase activity		K_{mapp} (μM)	2-hydroxylase activity	
		V_{max} (fmol min^{-1} pmol^{-1} P450)	$V_{\text{max}}/K_{\text{m}}$ (nl min^{-1} pmol^{-1} P450)		V_{max} (fmol min^{-1} pmol^{-1} P450)	$V_{\text{max}}/K_{\text{m}}$ (nl min^{-1} pmol^{-1} P450)
HL1	18.4	52.1	2.8	36.8	99.6	2.7
HL2	13.0	52.5	4.0	14.4	79.0	5.5
HL3	16.0	19.2	1.2	16.3	34.7	2.1
Mean $\pm$ s.d.	15.8 ± 2.7	41.3 ± 19.1	2.7 ± 1.4	22.5 ± 12.4	71.1 ± 33.2	3.4 ± 1.8

Figure 3 Effects of CYP inhibitors on mexiletine a) *p*-hydroxylase activity and b) 2-hydroxylase activity in human liver microsomes. The concentration of mexiletine was 20 μM . The ranges of concentration of furaflavine (Δ), ethoxyresorufin ($\blacktriangle$) and quinidine ($\bullet$) were 0.25–10 μM , and that of bufuralol ($\circ$) was 1–100 μM . Each data point represents the mean of duplicate determinations (HL 2).

Figure 4 Correlations between mexiletine hydroxylase activities and bufuralol 1'-hydroxylase activity or ethoxyresorufin O-deethylase activity in microsomes from nine human livers. The concentrations of mexiletine, ethoxyresorufin and bufuralol were 20, 2 and 1 μM , respectively. a) Bufoalol 1'-hydroxylase activity and mexiletine *p*-hydroxylase activity; b) bufoalol 1'-hydroxylase activity and mexiletine 2-hydroxylase activity; c) ethoxyresorufin O-deethylase activity and mexiletine *p*-hydroxylase activity; d) ethoxyresorufin O-deethylase activity and mexiletine 2-hydroxylase activity.

CYP2D6 exhibited the highest mexiletine hydroxylase activities in isoforms determined in this study (1058, 1196 $\text{fmol min}^{-1} \text{pmol}^{-1} \text{P450}$ for *p*- and 2-hydroxylase activities of mexiletine, respectively); CYP1A2 exhibited low activities (2.5, 3.6 $\text{fmol min}^{-1} \text{pmol}^{-1} \text{P450}$ for *p*- and 2-hydroxylase activities of mexiletine, respectively).

Table 2 Mexiletine *p*- and 2-hydroxylase activities in microsomes from human B-lymphoblastoid cells expressing CYP isoforms.

Isoforms	<i>p</i> -hydroxylase activity ($\text{fmol min}^{-1} \text{pmol}^{-1} \text{P450}$)	2-hydroxylase activity ($\text{fmol min}^{-1} \text{pmol}^{-1} \text{P450}$)
Control	ND*	ND†
CYP1A1	ND	ND
CYP1A2	2.5	3.6
CYP2A6	ND	ND
CYP2B6	ND	ND
CYP2C9	ND	ND
CYP2C19	ND	ND
CYP2D6	1058	1196
CYP2E1	ND	ND
CYP3A4	ND	ND

*ND, Not detected (<0.5 pmol formed).

†ND, Not detected (<5 pmol formed).

Incubations with cDNA-expressed CYP1A1, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2E1 and CYP3A4 did not produce detectable levels of *p*- and 2-hydroxymexiletine (*i.e.* <0.5 pmol, 5 pmol formed, respectively).

Kinetic analyses

Kinetic analyses were performed using the microsomes from cDNA-expressed CYP1A2 or CYP2D6 (Table 3). The K_m values for mexiletine *p*-hydroxylase activity in CYP1A2 or CYP2D6 cDNA expressed microsomes were 13.9 and

Table 3 Kinetic parameters of mexiletine *p*- and 2-hydroxylase activities in microsomes of human B-lymphoblastoid cells expressing CYP1A2 or CYP2D6.

Isoforms	K_m (μM)	V_{max} (fmol min^{-1} $\text{pmol}^{-1} \text{P450}$)	V_{max}/K_m (nl min^{-1} $\text{pmol}^{-1} \text{P450}$)
<i>p</i> -hydroxylase activity			
CYP1A2	13.9	4.7	0.3
CYP2D6	22.6	2249	100
2-hydroxylase activity			
CYP1A2	15.2	6.1	0.4
CYP2D6	22.1	2492	113

22.6 μM , respectively. The K_m values for mexiletine 2-hydroxylase activity in these expressed microsomes were 15.2 and 22.1 μM , respectively. These K_m values were similar to that obtained from human liver microsomes (Table 1). The $V_{\max}$ values for mexiletine *p*-hydroxylase activity in CYP1A2 or CYP2D6 cDNA expressed microsomes were 4.7 and 2149 $\text{fmol min}^{-1} \text{pmol}^{-1}$ P450, respectively. The $V_{\max}$ values for mexiletine 2-hydroxylase activity in these expressed microsomes were 6.1 and 2492 $\text{fmol min}^{-1} \text{pmol}^{-1}$ P450, respectively. The intrinsic clearance (CL_{int}) for mexiletine *p*-hydroxylase activity in CYP1A2 or CYP2D6 cDNA expressed microsomes were 0.3 and 100 $\text{nl min}^{-1} \text{pmol}^{-1}$ P450, respectively. The CL_{int} for mexiletine 2-hydroxylase activity in these expressed microsomes were 0.4 and 113 $\text{nl min}^{-1} \text{pmol}^{-1}$ P450, respectively.

Contributions of CYP1A2 and CYP2D6 to mexiletine hydroxylase activities in human liver microsomes

As shown in Table 4, the contributions of CYP1A2 and CYP2D6 to mexiletine *p*- and 2-hydroxylase activities in microsomes from five different human livers were estimated. Ethoxyresorufin O-deethylase activities in five different human liver microsomes ranged from 23.5 to 135.0 $\text{fmol min}^{-1} \text{pmol}^{-1}$ P450, and in recombinant CYP1A2 was 152.7 $\text{fmol min}^{-1} \text{pmol}^{-1}$ P450. Thus, $\text{RAF}_{\text{CYP1A2}}$ was estimated to range from 0.154 to 0.884. Bufuralol 1'-hydroxylase activities in microsomes from the five different human livers ranged from 13.0 to 24.1 $\text{fmol min}^{-1} \text{pmol}^{-1}$ P450, and in recombinant CYP2D6 was 1858 $\text{fmol min}^{-1} \text{pmol}^{-1}$ P450. Thus, $\text{RAF}_{\text{CYP2D6}}$ was estimated to range 0.007 to 0.013. $\text{MXL}_{\text{rec-CYP1A2}}$ and $\text{MXL}_{\text{rec-CYP2D6}}$ for *p*-hydroxylation was 8.8 and 1557 $\text{fmol min}^{-1} \text{pmol}^{-1}$ P450, respectively. $\text{MXL}_{\text{rec-CYP1A2}}$ and $\text{MXL}_{\text{rec-CYP2D6}}$ for 2-hydroxylation was 15.6 and 2155 $\text{fmol min}^{-1} \text{pmol}^{-1}$ P450, respectively. Therefore, the activities by CYP1A2 and CYP2D6 in human liver microsomes were estimated by using equations (1) and (2) described above. The contributions of CYP1A2 and CYP2D6 to human microsomal mexiletine *p*-hydroxylase activities were 7.9–29.0% and 75.8–98.1%, respectively. The contributions of CYP1A2 and CYP2D6 to human microsomal mexiletine 2-hydroxylase activities were 6.5–29.4% and 59.6–70.8%, respectively.

Discussion

Mexiletine has been reported to be hydroxylated by CYP2D6 in human liver microsomes [6, 7]. However, the involvement of CYP1A2 in mexiletine hydroxylase activity is suggested by some observations. Firstly, mexiletine reduces theophylline clearance by inhibiting *N*-demethylation of theophylline which is catalyzed by CYP1A2 [14]. Secondly, cigarette smoking induces 2-hydroxylation of mexiletine [26]. Cigarette smoking has been reported to induce CYP1A2 in humans [27]. Finally, phenacetin O-deethylation catalyzed by cDNA-expressed CYP1A2 was competitively inhibited by mexiletine in our previous study [28]. Accordingly, the role of CYP1A2 in mexiletine hydroxylation in human liver microsomes was investigated in this study.

It has been reported that quinidine is a potent inhibitor of CYP2D6 with a K_i value of 0.025 μM [20], and bufuralol is a substrate of CYP2D6 with a K_m value of 18 μM [25]. In this study, mexiletine hydroxylase activities in human liver microsomes were more strongly inhibited by quinidine than bufuralol. Furaflavone is a potent inhibitor of CYP1A2 with a K_i value of 3 μM [19]. Ethoxyresorufin is a substrate of CYP1A2 with a K_m value of 0.24 μM [29]. Mexiletine hydroxylase activities in human liver microsomes were similarly inhibited by furaflavone and ethoxyresorufin (Figure 3). We confirmed that bufuralol 1'-hydroxylase activity in microsomes expressed CYP2D6 was not affected by 10 μM ethoxyresorufin or 10 μM furaflavone (data not shown). Taking these data together, it was conceivable that mexiletine hydroxylase activities in human liver microsomes are inhibited not only by an inhibitor and a substrate of CYP2D6 but also by those of CYP1A2.

In microsomes of B-lymphoblastoid cells expressing human CYPs, CYP1A2 exhibited 423- and 332-fold lower mexiletine *p*- and 2-hydroxylase activities than CYP2D6, respectively at a 20 μM mexiletine concentration. In kinetic analyses, the K_m values for mexiletine *p*- and 2-hydroxylase activities of CYP1A2 were slightly lower than those of CYP2D6. However, the $V_{\max}$ values for *p*- and 2-hydroxylase activities of CYP1A2 exhibited 479- and 409-fold lower than that of CYP2D6, respectively. Therefore, it was suggested that the differences of the activities were dependent on the $V_{\max}$ values and not the K_m values.

Table 4 Contribution of CYP1A2 and CYP2D6 to mexiletine *p*- and 2-hydroxylase activities in human liver microsomes.

Number	RAF		p-hydroxylase activity			2-hydroxylase activity		
	CYP1A2	CYP2D6	MXL_{HL}	$\text{MXL}_{\text{CYP1A2}}$ (Contribution)*	$\text{MXL}_{\text{CYP2D6}}$	MXL_{HL}	$\text{MXL}_{\text{CYP1A2}}$ (Contribution)*	$\text{MXL}_{\text{CYP2D6}}$
				($\text{fmol min}^{-1} \text{pmol}^{-1}$ P450) (%)			($\text{fmol min}^{-1} \text{pmol}^{-1}$ P450) (%)	
HL3	0.884	0.013	26.7	7.7 (29.0)	20.2 (75.8)	46.9	13.8 (29.4)	28.0 (59.6)
HL4	0.465	0.013	24.1	4.1 (16.9)	19.7 (81.8)	45.4	7.3 (16.0)	27.3 (60.1)
HL5	0.252	0.007	14.0	2.2 (15.8)	10.9 (77.7)	21.2	3.9 (18.6)	15.0 (70.8)
HL6	0.299	0.013	20.9	2.6 (12.5)	19.6 (93.8)	44.9	4.7 (10.4)	27.1 (60.4)
HL7	0.154	0.011	17.1	1.3 (7.9)	16.8 (98.1)	37.0	2.4 (6.5)	23.2 (62.8)

*The contributions of CYP1A2 and CYP2D6 to human microsomal mexiletine hydroxylation were calculated by using equations (3) and (4) as described in Methods.

In general, when several enzymes whose K_m value differed from each other are responsible for certain activity, Eadie-Hofstee plots of the activity in microsomes are biphasic. In this study, it was clarified that two enzymes, CYP1A2 and CYP2D6, are responsible for mexiletine *p*- and 2-hydroxylase activities. However, Eadie-Hofstee plots of mexiletine *p*- and 2-hydroxylase activities in human liver microsomes were linear. The reason for these results might be due to the similar K_m values of CYP1A2 and CYP2D6 for mexiletine hydroxylase activities.

Crespi [24] proposed a concept of RAF to extrapolate the data obtained from cDNA-expressed CYPs to those from human liver microsomes. The validity of this assumption has not been rigorously tested, but for most enzymes an appropriate set of test compound is available [24]. We applied the RAF to estimate the relative contributions of CYP1A2 and CYP2D6 to mexiletine hydroxylase activities in human liver microsomes. Ethoxyresorufin *O*-deethylase activity and bufuralol 1'-hydroxylase activity were selected for CYP1A2 and CYP2D6 marker activities, respectively. Ethoxyresorufin *O*-deethylase activity is catalyzed by CYP1A1 and CYP1A2 in humans. It has been reported that the affinity of CYP1A1 for ethoxyresorufin is higher than that of CYP1A2 [30]. However, Ethoxyresorufin *O*-deethylase activity could be marker activity of CYP1A2, since CYP1A1 is generally lacked in human livers [31]. Bufuralol 1'-hydroxylase activity has been known as a marker activity of CYP2D6. Recently, it has been reported that CYP1A2 also catalyze bufuralol 1'-hydroxylation [25]. However, the K_m value of CYP1A2 (0.21 mM) was reported to be higher than that of CYP2D6 (18 μ M). Therefore, it was considered that the contribution of CYP1A2 for bufuralol 1'-hydroxylation in human liver microsomes was negligible at a 1 μ M substrate concentration. We confirmed that microsomes expressing CYP1A2 and CYP2D6 exhibited no measurable bufuralol 1'-hydroxylation and ethoxyresorufin *O*-deethylation, respectively, in this experimental condition as described in Methods. Accordingly, these activities determined in this study are suitable for the estimation of the contributions of CYP1A2 and CYP2D6 to mexiletine hydroxylation.

The estimated contributions of CYP1A2 and CYP2D6 to mexiletine hydroxylase activities at a 200 μ M mexiletine concentration were about 7–30% and 60–98%, respectively. The intrinsic clearances for mexiletine *p*- and 2-hydroxylase activities by CYP2D6 were 333- and 283-fold higher than those by CYP1A2, respectively (Table 3). In human liver microsomes, it has been reported that the contents of CYP1A2 and CYP2D6 protein are $12.7 \pm 6.2\%$ and $1.5 \pm 1.3\%$ of total CYP, respectively [32], indicating *ca.* 10-fold higher CYP1A2 content than CYP2D6 content. Accordingly, it is estimated that the contribution percentage of CYP1A2 to mexiletine hydroxylation is about 3%. However, CYP1A2 content might be >100-fold larger than CYP2D6 content in some human livers, because of the large interindividual differences in CYP1A2 and CYP2D6 expression levels. In deed, microsomes with 100-fold larger CYP1A2 content than CYP2D6 content also exist in commercially available sources [33]. In these microsomes, the contribution percentage of CYP1A2 to mexiletine hydroxylation is estimated to be >30%. The

contribution percentage estimated by RAF approach of 7–30% of CYP1A2 to mexiletine hydroxylation in the current study was not far from the estimation by intrinsic clearance and protein content. Total contribution of CYP1A2 and CYP2D6 did not reach 100% in some microsomes and was over 100% in other microsomes. It might be limitations of the estimation of RAF since the involvement of enzymes other than CYP1A2 and CYP2D6 are not considered by our results. Another possible reason for the limitation might be the differences of lipid constitution, expression levels of NADPH-cytochrome *c* reductase and/or cytochrome *b*₅ in human liver and cDNA-expression systems [24]. The results obtained by estimation using RAF coincided with the reports that suggested the main enzyme responsible for mexiletine hydroxylation is CYP2D6. The therapeutic range of mexiletine is 4–11 μ M in plasma [2]. Therefore, the contributions of CYP1A2 and CYP2D6 to mexiletine hydroxylase activities at a low mexiletine concentration (20 μ M) were also estimated in this study. Consequently, the percentage of contributions of CYP1A2 and CYP2D6 did not change from the results at 200 μ M (data not shown). This is the first report of the involvement of CYP1A2 in mexiletine *p*- and 2-hydroxylase activities in human liver microsomes.

Theophylline is metabolized to 1X, 3X and 13U in humans. It was reported that the primary enzyme involved in these metabolic pathways is CYP1A2 [12, 13]. Robson *et al.* [34] reported that the mean K_m values for 1X, 3X and 13U formation in human liver microsomes were 0.545 mM, 0.630 mM and 0.788 mM, respectively. In addition, it was also reported that the K_m values for these three activities in cDNA-expressed human CYP1A2 were 0.38 mM, 1.09 mM and 0.23 mM, respectively [35]. The affinity of CYP1A2 to theophylline has been indicated to be relatively low in comparison with other substrates for CYP1A2 such as ethoxyresorufin and phenacetin. In addition, our data suggest that mexiletine is a substrate for CYP1A2 with higher affinity than theophylline.

In conclusion, the present study using human liver microsomes and recombinant human CYPs suggested that mexiletine *p*- and 2-hydroxylation is catalyzed mainly, but not exclusively, by CYP2D6 and partially by CYP1A2. Although the contribution of CYP1A2 to mexiletine hydroxylation was relatively low, the affinity of CYP1A2 for mexiletine was clarified to be higher than that for theophylline. Therefore, it is indicated that the interaction of mexiletine with theophylline is mediated by CYP1A2. Thus, when some CYP isoforms are involved in certain drug metabolism, it is important to investigate the contribution of each isoform in human liver microsomes and the affinity of the isoform(s), even if the contribution is low.

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