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POTENT INHIBITION OF YEAST-EXPRESSED CYP2D6 BY DIHYDROQUINIDINE, QUINIDINE, AND ITS METABOLITES

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Abstract—The inhibitory effects of dihydroquinidine, quinidine and several quinidine metabolites on cytochrome P450 2D6 (CYP2D6) activity were examined. CYP2D6 heterologously expressed in yeast cells *O*-demethylated dextromethorphan with a mean K_m of 5.4 μM and a V_{max} of 0.47 nmol/min/nmol. Quinidine and dihydroquinidine both potently inhibited CYP2D6 metabolic activity (mean K_i = 0.027 and 0.013 μM, respectively) in yeast microsomes and in human liver microsomes. The metabolites, 3-hydroxyquinidine, *O*-desmethylquinidine and quinidine *N*-oxide also inhibited CYP2D6, but their K_i values (0.43 to 2.3 μM) were one to two orders of magnitude weaker than the values for quinidine and dihydroquinidine. There was a trend towards an inverse relationship between K_i and lipophilicity (r = –0.90, N = 5, P = 0.07), as determined by the retention-time parameter k' using reverse-phase HPLC. Thus, although the metabolites of quinidine have the capacity to inhibit CYP2D6 activity, quinidine and the impurity dihydroquinidine are the important inhibitors of CYP2D6.

Key words: cytochrome P450 2D6; metabolic inhibition; quinidine; dihydroquinidine; quinidine metabolites

CYP2D6§ is responsible in humans for the polymorphic oxidation of many clinically used drugs [1, 2]. It is well established that the anti-arrhythmic cinchona alkaloid, quinidine, is a potent inhibitor of CYP2D6 [3] although it does not appear to be metabolized by this isoenzyme [4, 5]. The inhibition by quinidine is sufficient to imitate an artifactual poor-metabolizer phenocopy in subjects who are phenotypically extensive metabolizers [6, 7].

Quinidine is metabolized extensively by the liver; renal excretion of unchanged drug accounting for only 10–20% of the dose. The oxidation products that have been identified are 3-hydroxyquinidine, *O*-desmethylquinidine, quinidine *N*-oxide, 2-oxoquinidinone, quinidine-10,11-dihydrodiol and quinidine-10,11-dihydrodiol *N*-oxide [8, 9]. Quinidine 3-hydroxylation and *N*-oxygenation are catalysed by CYP3A4 [10, 11]. 3-Hydroxyquinidine, *O*-desmethylquinidine and quinidine *N*-oxide possess anti-arrhythmic activity in animals, but quantification of this activity in humans is difficult to ascertain [8]. Commercially available quinidine contains dihydroquinidine, an impurity that accounts usually for less than 10% but can be as high as 30% of the dose [8, 12]. In humans, dihydroquinidine has anti-arrhythmic and pharmacokinetic properties similar to that of quinidine [8]. The possibility that the metabolites of quinidine may inhibit CYP2D6, thereby contributing to impaired drug clearance seen after quinidine administration in humans, has been suggested by Ayesh *et al.* [7], who compared the inhibitory effects of quinidine and its diastereoisomer quinine *in vivo*. The aim of the present study was to determine whether the quinidine metabolites 3-hydroxyquinidine, *O*-desmethylquinidine and quinidine *N*-oxide, and the quinidine impurity dihydroquinidine, can inhibit CYP2D6, using an experimental system in which the isoenzyme was expressed in yeast. Previous characterization of the substrate specificity of this system has shown that recombinant CYP2D6 can metabolize the classical CYP2D6 substrates sparteine and metoprolol, but not tolbutamide and *S*-mephenytoin, substrates for the CYP2C subfamily, nor *p*-nitrophenol, a CYP2E1 substrate [13]. In the present study, another classical CYP2D6 substrate, dextromethorphan, was used to examine the activity of recombinant CYP2D6.

MATERIALS AND METHODS

Materials

Dihydroquinidine and quinidine that was free of dihydroquinidine (97% quinidine by HPLC, with 3% quinine as an impurity) were obtained from the Aldrich Chemical Co. (Milwaukee, WI). Quinine, dextromethorphan, tolbutamide, *p*-nitrophenol and erythromycin were obtained from the Sigma Chemical Co. (St. Louis, MO). 3-Hydroxyquinidine was a gift from Dr. Kim Brosen, Odense University (Denmark) and *O*-desmethylquinidine was a gift from Professor Grant Wilkinson, Vanderbilt University (U.S.A.). Quinidine *N*-oxide was synthesized using the *m*-chloroperbenzoic acid method [14], and its structure was confirmed by NMR. Dextrorphan and levallorphan (as tartrate salts) were obtained from Hoffmann-La Roche (Basel, Switzerland). Glucose-6-phosphate dehydrogenase and the disodium salts of glucose-6-phosphate and NADP were purchased from Boehringer Mannheim (Germany). All other chemicals were of analytical grade.

Expression of CYP2D6 in yeast

CYP2D6 was expressed in yeast as described previously [13]. Yeast microsomes were prepared as de-

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§ Abbreviations: CYP, cytochrome P450; and k', HPLC retention factor.

scribed by Ching *et al.* [15] and resuspended in 0.1 M potassium phosphate buffer, pH 7.4, containing 1 mM EDTA and 20% (v/v) glycerol. Ten batches of CYP2D6 yeast microsomes were prepared from AH22 yeast cells transformed with the CYP2D6 expression plasmid pELT1 (AH22::pELT1), and the P450 content ranged from 40 to 270 pmol CYP2D6/mg of microsomal protein. Control yeast microsomes prepared from AH22 cells transformed with the control vector pMA91 (AH22::pMA91) showed no detectable absorption peak at around 450 nm.

Microsomal incubations

Solutions of dextromethorphan and the various test substances were prepared in 1.15% (w/v) KCl. The incubation mixture (0.5 mL reaction volume) was comprised of 0.05 mL of yeast microsomal suspension, 0.15 mL of 1.15% (w/v) KCl, 0.1 mL of 0.2 M potassium phosphate buffer (pH 7.4) and 0.1 mL of potassium phosphate buffer containing the NADPH-generating system (2 μmol glucose-6-phosphate, 0.2 μmol NADP, 0.2 U of glucose-6-phosphate dehydrogenase and 1 μmol $MgCl_2$). After incubating the mixture at 37° for 3 min, the reaction was commenced by the addition of 0.1 mL KCl containing either drug or drug plus inhibitor. It was then allowed to proceed for 15 min in a shaking water bath. Reactions were stopped by the addition of 15 μL of 60% (v/v) perchloric acid. Preliminary experiments showed that the production of dextrorphan was linear over the first 20 min of incubation and linear up to 2 mg/mL of yeast microsomal protein added.

Utilization of the original substrate was less than 10% at the end of the reaction [16]. To estimate the K_m and V_{max} values of dextromethorphan *O*-demethylation, eight concentrations (0.5 to 50.0 μM) of dextromethorphan were used. To determine the K_i values, seven concentrations of each inhibitor were used: quinidine and dihydroquinidine (0.005 to 0.25 μM) and 3-hydroxyquinidine, quinidine *N*-oxide and *O*-desmethylquinidine (0.1 to 40 μM).

The IC_{50} values for quinidine and dihydroquinidine were determined at a dextromethorphan concentration of 3 μM, i.e. at a substrate concentration approximating K_m so that the IC_{50} would equal 2 K_i for a competitive inhibitor [16]. The effects of phenacetin (up to 100 μM), and tolbutamide, erythromycin and *p*-nitrophenol (up to 500 μM) on dextromethorphan metabolism were also examined.

Human liver microsomes

A sample of normal liver was taken from an adult patient at the time of hepatic resection for liver carcinoma. Liver was homogenized and centrifuged at 9000 *g* to pellet cell debris. The supernatant was centrifuged at 100,000 *g* for 90 min, and the microsomal pellet was resuspended in 0.25 M potassium phosphate buffer, pH 7.4, containing 30% (v/v) glycerol. Preliminary experiments showed that the production of dextrorphan was linear over the first 30 min of incubation and linear up to 1.5 mg/mL of microsomal protein added. Human liver microsomal incubations were conducted in a fashion similar to that of yeast microsomal incubations except that the time of incubation was 10 min, and the final concentration of microsomal protein in the incubation was 0.2 mg/mL. K_m and V_{max} values were determined as described above for dextromethorphan *O*-demethylation, and K_i values were determined only for quinidine and dihydroquinidine inhibition.

Dextrorphan assay

Following centrifugation of the acid-precipitated sample, 400 μL of the supernatant was mixed with 40 μL of internal standard (levallorphan, 15 μM) and 20–50 μL was injected directly into the HPLC. The HPLC system was comprised of a model 6000A pump (Waters Assoc.), a model 232 auto-injector (Gilson, France), a Novapak C18 4 μm, 3.9 × 150 mm stainless steel column, a model RF551 fluorescence detector (Shimadzu, Japan, excitation 270 nm, emission 312 nm) and a Maxima data acquisition system (Waters Assoc.). The mobile phase was acetonitrile:water (20:80) with 1% (v/v) triethylamine adjusted to pH 3.5 with orthophosphoric acid, and the flow rate was 1 mL/min. The retention times of dextrorphan, levallorphan and dextromethorphan were 3.0, 5.0 and 17.0 min, respectively, and no interference was seen from quinidine-related compounds under these conditions. Calibration curves were constructed over the dextrorphan concentration range of 0.125 to 1.00 μM, the coefficient of variation of the assay being less than 1% at 0.50 μM.

Determination of k′ *values*

The retention factors (k') of quinidine, dihydroquinidine, 3-hydroxyquinidine, quinidine *N*-oxide and *O*-desmethylquinidine were determined using reverse-phase HPLC [17]. Chromatographic conditions were the same as those described above except that the mobile phase consisted of methanol:0.02 M phosphate buffer, pH 7.4 (55:45), and a model 481 UV detector set at 254 nm (Waters Assoc.).

Data analysis

The (v) vs [S] data (as determined by the rate of appearance of dextrorphan) were fitted by the Henri–Michaelis–Menten equation [16] using Minim 1.6 (RD Purves, University of Otago, New Zealand), an iterative non-linear least squares regression program, using initial estimates of K_m and V_{max}. Lineweaver–Burk plots were constructed for each inhibitor, and inhibition constants (K_i) were determined from plots of K_m/V_{max} vs [I] [16]. Data are expressed as means ± SD.

RESULTS

The mean K_m for the *O*-demethylation of dextromethorphan by microsomes from yeast expressing CYP2D6 was 5.4 μM and V_{max} was 0.47 nmol/min/nmol (Table 1). When V_{max} was expressed as nmol/hr/mg mi-

Table 1. K_m and V_{max} values for dextromethorphan *O*-demethylation in yeast and human liver microsomes

	K_m (μM)	V_{max}
Yeast microsomes*	5.4 ± 1.6	0.47 ± 0.10
Human liver microsomes†	5.5 ± 0.4	5.9 ± 0.2

* K_m and V_{max} values (means ± SD) were determined 4 times for each of the 6 inhibitors studied. V_{max} is expressed in nmol/min/nmol CYP2D6.

†K_m and V_{max} values (means ± SD) were determined 4 times in one liver. V_{max} is expressed in nmol/hr/mg protein.

Table 2. Retention factor (k') as determined by reverse-phase HPLC, and K_i values for inhibition of dextromethorphan O-demethylation by yeast-expressed CYP2D6 and human liver microsomes

Compound	K_i (μM) Yeast microsomes*	k'	IC_{50} (μM) Yeast microsomes†	K_i (μM) Human liver microsomes
Quinidine	0.026, 0.028	34.6	0.061 ± 0.011	0.028
Dihydroquinidine	0.010, 0.017	51.6	0.038 ± 0.004	0.013
3-Hydroxyquinidine	2.3, 2.3	6.08		
Quinidine N-oxide	0.41, 0.46	6.33		
O-Desmethylquinidine	1.2, 1.4	8.90		
Quinine	1.8, 2.8			

* Duplicate determinations.
† N = 4 determinations. Values are means ± SD.

crosomal protein the value was 3.0 ± 1.9. Dextromethorphan O-demethylation was not detectable in control AH22::pMA91 microsomes. The K_m for O-demethylation of dextromethorphan by human liver microsomes was 5.5 μM and V_{max} was 5.9 nmol/hr/mg (Table 1).

Both quinidine and dihydroquinidine were potent inhibitors of O-demethylation (Table 2). At a concentration of 3 μM dextromethorphan, the IC_{50} for dihydroquinidine (0.038 μM) was significantly less than that for quinidine (0.061 μM. Fig. 1, P = 0.01, paired t-test). Inhibition appeared to be competitive from inspection of the Lineweaver–Burk plots (Fig. 2). The K_i values for quinidine and dihydroquinidine inhibition of dextromethorphan O-demethylation by human liver microsomes (Table 2) were identical to the values obtained for recombinant CYP2D6.

3-Hydroxyquinidine, O-desmethylquinidine, and quinine were two orders of magnitude weaker as inhibitors of CYP2D6 (Table 2), whilst quinidine N-oxide was about one order of magnitude weaker. The effect of these compounds on dextromethorphan O-demethylation suggested that inhibition was competitive (data not shown). There was a negative correlation between k' (Table 2) and K_i values (Spearman rank correlation coefficient r = −0.90, P = 0.07). Phenacetin (1–100 μM) and tolbutamide, p-nitrophenol and erythromycin (1–500 μM) did not inhibit the O-demethylation of dextromethorphan.

DISCUSSION

Recombinant CYP2D6 expressed in yeast was capable of O-demethylating dextromethorphan, and the K_m of this reaction was identical to the K_m of the reaction in human liver microsomes (Table 1). These values are similar to those reported by others (2.3 to 7.6 μM) for human liver microsomes [18–21]. The mean value of V_{max} in yeast microsomes was 0.47 nmol/min/nmol (i.e. 0.028 nmol/hr/pmol of CYP2D6). The V_{max} of dextromethorphan O-demethylation in human liver microsomes was 5.9 nmol/hr/mg protein (Table 1), which is comparable to previous studies [18–20]. This equates to approximately 1.1 nmol/hr/pmol of CYP2D6, assuming that the content of CYP2D6 in our human liver microsomes is similar (~5.2 pmol CYP2D6/mg protein) to that in a panel of human liver microsomes recently reported by Shimada *et al.* [22]. Thus, on a pmol of CYP2D6 basis, the V_{max} of dextromethorphan O-demethylation by cDNA-expressed CYP2D6 in yeast microsomes is approximately one-fortieth that of native CYP2D6 in human liver microsomes.

The K_i values for inhibition by quinidine of dextromethorphan metabolism in yeast (0.027 μM) and human liver microsomes (0.028 μM) compare with the values of 0.015 to 0.04 μM in human liver microsomes reported by others [3, 19, 20]. The new finding in this study is that the potential quinidine contaminant dihydroquinidine has similar potency to quinidine as an inhibitor of CYP2D6. In addition, we confirmed an earlier suggestion by Ayesh *et al.* [7] that quinidine metabolites may interact with CYP2D6. The inverse relationship between K_i and k' suggests that the more lipophilic the compound, the greater its propensity to inhibit CYP2D6. Quinidine, dihydroquinidine and the metabolites of quinidine all appeared to inhibit CYP2D6 competitively, and other cinchona alkaloids such as cinchonine and cinchonidine also appear to be competitive inhibitors of CYP2D6 [3]. Few other studies have examined drug metabolites as possible inhibitors of CYP2D6. 4-Hydroxy-debrisoquine, the oxidation product of debrisoquine, was shown recently to inhibit dextromethorphan O-demethylation competitively in perfused rat liver and human liver microsomes, although its potency was low (K_i = 600 μM, [21]). Fluoxetine and its N-demethylated metabolite norfluoxetine were shown to be potent inhibitors (K_i values ~0.2 to 0.6 μM) of CYP2D6 in human liver microsomes [23–25].

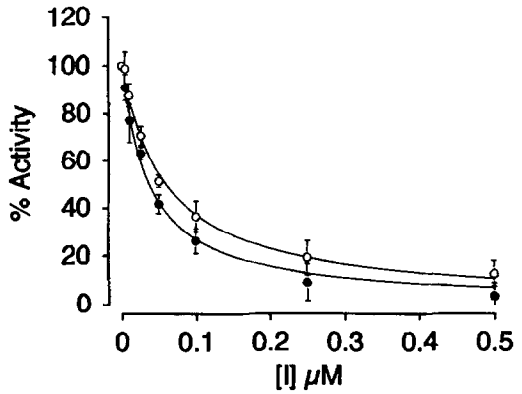

Fig. 1. Decrease in percent activity (means ± SD, N = 4) of dextromethorphan (3 μM) O-demethylation by yeast-expressed CYP2D6 in the presence of quinidine (○, IC_{50} = 0.061 μM) and dihydroquinidine (●, IC_{50} = 0.038 μM).

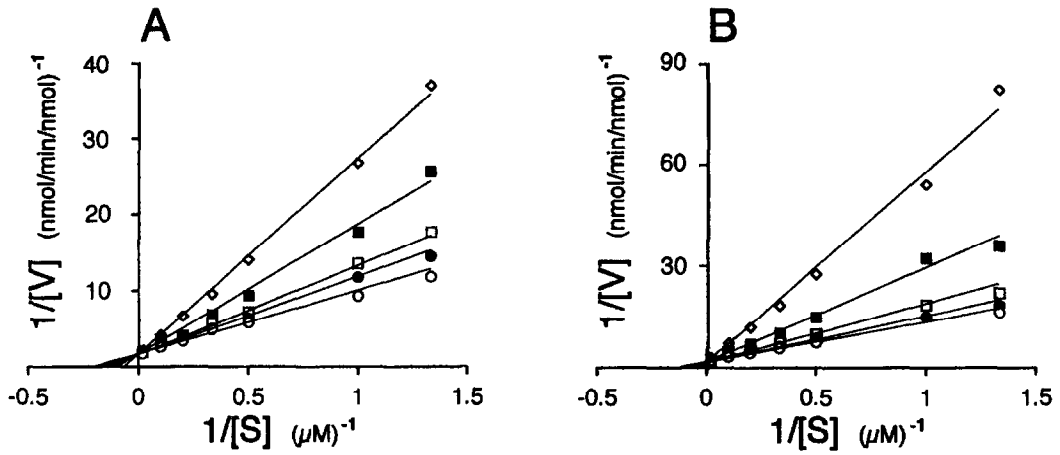

Fig. 2. Lineweaver–Burk plots of quinidine (A) and dihydroquinidine (B) inhibition of dextromethorphan *O*-demethylation by yeast-expressed CYP2D6. The inhibitor concentrations shown are: no inhibitor (○), 0.005 μM (●), 0.01 μM (□), 0.025 μM (■) and 0.05 μM (◇).

Our data suggest that when quinidine is administered to humans, the contaminant dihydroquinidine may contribute significantly to the inhibition of CYP2D6. Thompson *et al.* [12] measured free unbound plasma concentrations of quinidine, dihydroquinidine, and quinidine metabolites in patients with torsade de pointes, and in control subjects taking quinidine. Unbound concentration ratios of dihydroquinidine to quinidine were 0.05 to 0.1, for 3-hydroxyquinidine to quinidine they were 0.5 to 4.0, and for quinidine *N*-oxide to quinidine they were 0.1 to 0.5. However, given that the K_i values of the metabolites are one to two orders of magnitude greater than those of quinidine and dihydroquinidine, the latter should make the greatest contribution to inhibition of CYP2D6 *in vivo*.

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