Regioselectivity and Enantioselectivity of Metoprolol Oxidation by Two Variants of cDNA-Expressed P4502D6

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Purpose. The oxidative metabolism of metoprolol was investigated in two human lymphoblastoma cell-lines transfected with variants of cDNA for cytochrome P4502D6.

Methods. The regioselective and enantioselective features of the oxidations of deuterium-labeled pseudoracemic metoprolol were characterized by GC/MS analysis of the substrate and products.

Results. There were significant differences between the two P4502D6 variants in the formation kinetics of O-demethylmetoprolol and α-hydroxymetoprolol. The h2D6-Val microsomes highly favored the formation of the O-demethylmetoprolol regioisomer 6.3:1 and 2.8:1, respectively from (R)-metoprolol-d_0 and (S)-metoprolol-d_2, while the corresponding ratios for h2D6v2 microsomes were much lower. For both variants, O-demethylmetoprolol formation favored the (R)-substrate 1.5 to 2-fold, while α-hydroxymetoprolol formation was non-enantioselective. Similar Km values of metoprolol oxidation, 10-20 μM, were observed for the two microsomal preparations.

Conclusions. The regioselectivity, enantioselectivity, and Km values for the h2D6-Val microsomes resemble those observed for the native P4502D6 in human liver microsomes, whereas the h2D6v2 microsomes deviated remarkably in regioselectivity.

KEY WORDS: cytochrome P4502D6; h2D6v2; h2D6-Val; enzyme kinetics; metoprolol.

INTRODUCTION

Metabolic profiling of new drug candidates using microsomes containing individual cDNA-expressed human cytochrome P450 enzymes has become a wide spread supplement to traditional studies with animal or human liver microsomes (1). Several clonal systems have been developed to express P4502D6, an isoform known to metabolize more than 30 therapeutic agents. Although the cDNA's encoding for the P4502D6 were derived from human livers possessing extensive P4502D6 activity, the quantity and qualities of P4502D6 in the donor livers are not well documented. Recent findings suggest allelic variants of functional P4502D can differ substantially in their catalytic behavior (2,3). Therefore, the cDNA sequence and catalytic characteristics of the P4502D6 enzymes expressed in recombinant cell-lines must be carefully evaluated to determine their applicability.

Previously, we reported that oxidations of pseudoracemic metoprolol by a commercially available cDNA-expressed enzyme designated h2D6v2[1] (Gentest Corp.) showed an unexpectedly low regioselectivity between the oxidative products, ODM and HM (4), (see fig. 1.) The observed ODM/HM ratio of 1.3:1, was more than 3-fold lower than the metabolite ratios observed for purified human liver P4502D6 (5), human liver microsomes (6), and microsomes of a yeast cDNA-expression system of human P4502D6 (2). Ellis *et al.*, suggested that the P4502D6 expressed in the h2D6v2 and yeast (C. Wolf cDNA) systems were $M_{374}V$ variants, based on their respective cDNA sequences. Recently, the Gentest Corporation has developed a new cell-line, h2D6-Val, that expresses an $A_{1120}G$ variant of the cDNA used for the h2D6v2 cell-line (7,8). As the result of this point mutation, the h2D6-Val cell-line should produce a P4502D6-Val_{374} instead of the P4502D6-Met_{374} expressed in the h2D6v2 cell-line. The P4502D6-Val enzyme may share catalytic characteristics similar to the P4502D6 in human livers, and therefore it may serve as a better model for metabolic profiling than the P4502D6-Met expressed by the h2D6v2 cell-line. We describe here a comparison of the kinetics of ODM and HM formation catalyzed by the h2D6v2 and h2D6-Val microsomes.

MATERIALS AND METHODS

Chemicals

(2R)-Metoprolol-d_0 tartrate (96% e.e.) and (2S)-metoprolol-d_2 tartrate (97% $^2H_2$, 94% e.e.) were obtained as previously reported (9). The (2S)-metoprolol-d_2 used in for this study has the deuterium labels located at the 2',6'-ortho positions of the aromatic ring, adjacent to the oxypropanolamine side chain. Pseudoracemic metoprolol was prepared by dissolving an equimolar mixture of (2R)-metoprolol-d_0 and (2S)-metoprolol-d_2 tartrates in distilled water and then diluting to a 1 mM metoprolol concentration. O-Demethylmetoprolol and α-hydroxymetoprolol hydroxybenzoates were obtained from AB Hassle (Molndal, Sweden). Triphosgene was obtained from Fluka Chemie AG (Buchs, Switzerland). BSTFA was obtained Pierce Chemical Co. (Rockford, Illinois). NADP monosodium salt, D-glucose-6-phosphate monosodium salt, D-glucose-6-phosphate dehydrogenase (Type XV) were purchased from the Sigma Chemical Co. (St. Louis, Missouri). All other chemicals and solvents used were of the highest quality available and were used without further purification.

Microsomes

Suspensions of microsomes (10 mg protein/mL) that

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ABBREVIATIONS: P4502D6, cytochrome P4502D6 enzyme; HM, α-hydroxymetoprolol; ODM, O-demethylmetoprolol; h2D6v2, AHH-1 cell-line infected with the pEBVHistk vector containing cDNA for human P4502D6-Met_{374}; h2D6-Val, AHH-1 cell-line infected with the pRedHyHo vector containing cDNA for human P4502D6-Val_{374}, also known as CYP2D6-Val; GC/MS, gas chromatography/mass spectrometry; e.e., enantiomeric excess.

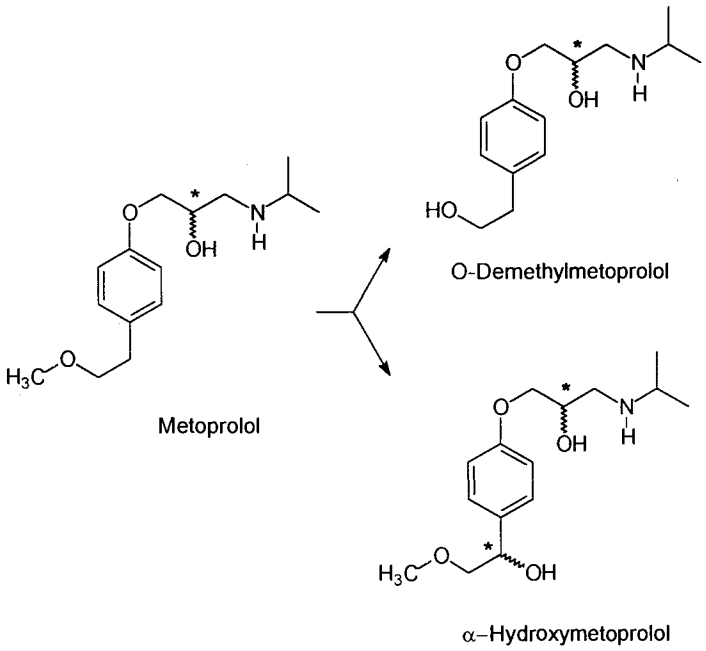

Fig. 1. Routes of metabolism for metoprolol.

were isolated from human AHH-1 cells transfected with (M150b lots 38 or M117r lot 2) and without (Gentest M101a lot 24 or M101b lot 4) a functional human CYP2D6 gene were obtained as a gift from the Gentest Corporation (Woburn, Massachusetts).

Microsomal Incubations

Each incubate contained a pseudoracemic mixture of (R)-metoprolol-d_0 and (S)-metoprolol-d_2 and a NADPH-generating system in 1 mL of 0.2 M potassium phosphate buffer (pH 7.4). The NADPH-generating system consisted of 1 mM $NADP^+$, 20 mM glucose-6-phosphate, 1.3 mM magnesium chloride, and one unit of glucose-6-phosphate dehydrogenase. The mixtures were pre-incubated before the addition of 0.5 mg of microsomal protein (Gentest M105b lot 38 or M117r lot 2). After 30 min at 37 °C, the metabolic reactions were terminated by the addition of 300 μL of 0.5 M tirbasic sodium phosphate (pH 12). Four sets of incubations at pseudoracemic metoprolol concentrations of 7, 10.4, 20, and 60 μM were used to determine the kinetic parameters for the O-demethylation and α-hydroxylation. Control incuba-

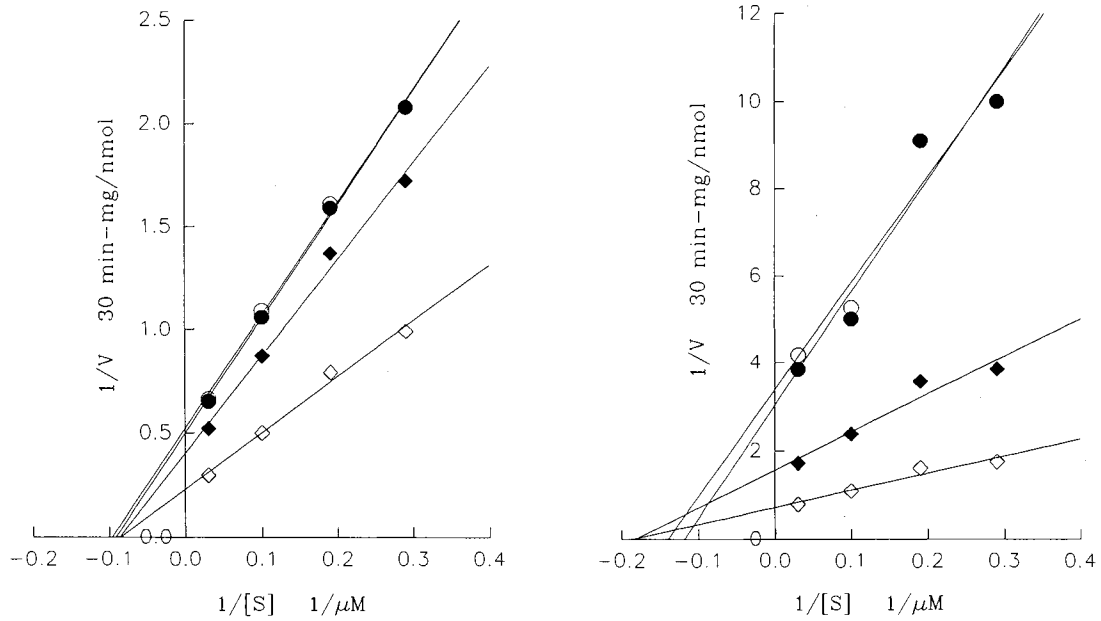

Fig. 2. Left: Lineweaver-Burk plot of metoprolol product data (h2D6v2 lot 38). (◇) (R)-ODM, (◆) (S)-ODM, (○) (R)-HM and (●) (S)-HM. Right: Lineweaver-Burk plot of metoprolol product data (h2D6-Val lot 2). (◇) (R)-ODM, (◆) (S)-ODM, (○) (R)-HM and (●) (S)-HM.

Table I. Apparent Michaelis-Menten Constants for O-Demethylation and α-Hydroxylation of (R)- and (S)-Metoprolol by h2D6v2, h2D6-Val and Human Liver Microsomes

Microsomes	(R)-metoprolol K_m (μM)	(R)-metoprolol V_{max} (nmol) 30 min · mg	(R)-metoprolol V_{max}/K_m (ml) 30 min · mg	(S)-metoprolol K_m (μM)	(S)-metoprolol V_{max} (nmol) 30 min · mg	(S)-metoprolol V_{max}/K_m (ml) 30 min · mg	R/S ratio (R)-(V_{max}/K_m) / (S)-(V_{max}/K_m)
O-Demethylmetoprolol:							
h2D6v2 mean ± S.D.[a]	15 ± 3	5.0 ± 0.8	0.34 ± 0.05	15 ± 3	2.9 ± 0.4	0.19 ± 0.03	1.8 ± 0.4
h2D6-Val mean ± S.D.[a]	7 ± 1	1.5 ± 0.2	0.22 ± 0.02	7 ± 1	0.72 ± 0.09	0.099 ± 0.008	2.2 ± 0.2
HLM P4502D6[b] mean ± S.D.	17 ± 7	5.0 ± 2.6	0.30 ± 0.13	16 ± 10	3.2 ± 1.5	0.18 ± 0.08	1.6 ± 0.1
α-Hydroxymetoprolol:							
h2D6v2 mean ± S.D.[a]	14 ± 2	2.2 ± 0.2	0.16 ± 0.02	13 ± 2	2.2 ± 0.2	0.17 ± 0.02	0.9 ± 0.2
h2D6-Val mean ± S.D.[a]	10 ± 2	0.35 ± 0.05	0.035 ± 0.004	10 ± 2	0.36 ± 0.08	0.036 ± 0.001	1.0 ± 0.1
HLM P4502D6[b] mean ± S.D.	22 ± 7	1.1 ± 0.6	0.049 ± 0.017	26 ± 8	1.4 ± 0.7	0.052 ± 0.018	1.0 ± 0.1

[a] The mean and standard deviations were obtained from four replicate sets of data.
[b] Data adapted from Kim *et al.* (6), representing the mean of four human livers: HL101, 107, 108, and 111.

tions were performed by using microsomes from cells without the cDNA 2D6 inserts (Gentest M101a lot 24 or M101b lot 4). The amounts of ODM and HM in the microsomal incubates were quantitated by a GC/MS method reported by Gyllenhaal, *et al.* (10) by using propranolol (1 μg) as the internal standard. The method was extended to quantify the enantiomers, (R)-metoprolol-d_0 and (S)-metoprolol-d_2. Derivatization was performed subsequently with a triphosgene solution in toluene (2 M) and then with N,O-bis(trimethylsilyl)trifluoroacetamide.

GC-MS Analysis of Metoprolol Metabolites

Chromatographic resolution of the analytes were achieved with a 30 m × 0.25 mm medium polarity fused silica DB-5 column (J&W Scientific, Folsom, California) with a column head pressure of 7 psi. The column temperature was programmed at 200 °C for the first min, followed by an increase to 280 °C at a rate of 10 °C/min, and then held at that temperature for 6 min. The injector port and transfer line were kept at 250 °C and 280 °C, respectively. The derivatives of metoprolol, ODM, propranolol and HM were eluted at 11.4, 12.1, 12.3 and 12.4 min, respectively.

The Hewlett Packard 5972A mass analyzer, was programmed to detect with high resolution selected ion masses generated by an ionizing current of 70 eV. The intensities of the following characteristic fragments were monitored in two groups: group 1, *m/z* 293.1 and *m/z* 295.1 for the oxazolidinone derivatives of (R)-metoprolol-d_0 and (S)-metoprolol-d_2, respectively; group 2, *m/z* 285.1 for propranolol, *m/z* 336.1 for (R)-metoprolol-d_0 metabolites and *m/z* 338.1 for (S)-metoprolol-d_2 metabolites. The selected ions were scanned for a dwell time of 100 msec for group 1 and 60 msec for group 2.

The standard curves were constructed with pseudoracemic metoprolol and non-deuterated ODM and αH. The peak area ratios (analyte/propranolol) *versus* analyte concentration were linear ($r^2>0.98$) over the range of 3.5-30 nmol/mL metoprolol, 0.1-5.0 nmol/mL of ODM, and 0.05-2.5 nmol/mL of HM. The peak areas for the deuterated analyte were corrected for the M+2 isotopic contribution from the non-deuterated analyte (5.87% of non-deuterated analyte

Table II. Product Selectivities in the Oxidation of Pseudoracemic Metoprolol by h2D6v2, h2D6-Val, or Human Liver Microsomes as Expressed by the Ratio of V_{max}/K_m for the O-Demethylation (ODM) and α-Hydroxylation (HM) of (R)- and (S)-Metoprolol

Microsomes Lot I.D.	(R)-metoprolol ODM/HM	(S)-metoprolol ODM/HM	(R)-ODM + (S)-ODM / (R)-HM + (S)-HM	(R)-ODM + (R)-HM / (S)-ODM + (S)-HM
h2D6v2 mean ± S.D.[a]	2.1 ± 0.4	1.1 ± 0.3	1.6 ± 0.4	1.4 ± 0.4
h2D6-Val mean ± S.D.[a]	6.3 ± 0.9	2.8 ± 0.2	4.6 ± 0.8	1.9 ± 0.3
HLM P4502D6[b] mean ± S.D.	6.0 ± 1.4	3.5 ± 0.9	4.7 ± 1.1	1.5 ± 0.1

[a] The mean and standard deviations were obtained from four replicate sets of data.
[b] Data adapted from Kim *et al.* (6), representing the mean of four human livers: HL101, 107, 108, and 111.

peak area). Inter-day coefficients of variation were 7% (−4% bias) and 5% (+2% bias) for quality control samples (n=4) containing 0.50 and 0.25 nmol/mL ODM and HM, respectively.

RESULTS AND DISCUSSION

The h2D6v2 and h2D6-Val microsomes catalyzed the formation of ODM and HM from metoprolol. With each clone, the loss of (R)-metoprolol-d_0 and (S)-metoprolol-d_2 was entirely accounted for by ODM and HM formation in both quantity and stereoselectivity. The mass balance control data were in agreement with the results of previous studies that showed formation of ODM and HM are the predominent pathway in microsomal oxidation of metoprolol (11).

Estimates of Michaelis-Menten parameters, Km and Vmax, were obtained by Lineweaver-Burk analyses (fig. 2). The sum of the clearances (Vmax/Km) for the formation of metabolites in each system listed in Table I indicates that the h2D6-Val microsomal preparation was 45% as active as the h2D6v2 preparation. This lower activity was expected because the expression of P450 in the h2D6-Val system is an order of magnitude less than for the h2D6v2 cell-line (8). Kinetic parameters for the h2D6v2 system reported here are indistinguishable from those obtained from lots evaluated more than two years prior to this study, indicating good stability of the cell-line (4). Both recombinant P4502D6 have similar Km values and similar enantiomeric preferences for the formation of ODM and HM from pseudoracemic metoprolol. However, unlike h2D6v2 microsomes, the h2D6-Val microsomes display a strong regioselective preference for the O-demethylation of metoprolol, particularly with the (R)-substrate. The ODM/HM product ratios are presented in the first three columns of Table II. The ratios for the h2D6-Val microsomes are approximately 3-fold higher than for the h2D6v2 microsomes and compare favorably to the data from human liver microsomes at low substrate concentrations (*i.e.*, within the low-Km region where P4502D6 activity predominates). The substrate enantioselectivity data listed in the last column of Table II suggests that the microsomes share a similar preference for the (R)-metoprolol. The microsomal h2D6-Val is clearly the more appropriate model for metoprolol oxidation because its Km values, regioselectivities and stereoselectivities are similar to that of purified P4502D6, the native enzyme in human liver microsomes, and the microsomal yeast P4502D6-Val.

In conclusion, our study of microsomal h2D6v2 and h2D6-Val underscore the need to understand the molecular and functional characteristics of the recombinant P450 systems. Although the kinetic differences between the P4502D6-Met_{374} and -Val enzymes are modest, the study of rat P4502D1-Phe_{380} and -Ile variants by Matsunga et al. (3) suggests that other point mutations could have even more dramatic effects on enzyme activity. Until there is a greater understanding of the variety and population distribution of functional variants in human liver (12, 13) and well described recombinant systems, we urge caution in complete reliance on recombinant systems for metabolic profiling of new chemical entities.

NOTE ADDED IN PROOF

Crespi et al., *Pharmacogenetics* 5:234–243 (1995), report additional dissimilarities in substrate and product selectivities between the h2D6-Val and h2D6v2 microsomes.

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