Chemico-Biological Interactions 189 (2011) 146–152

Contents lists available at ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

Regio- and stereoselective oxidation of propranolol enantiomers by human CYP2D6, cynomolgus monkey CYP2D17 and marmoset CYP2D19

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ARTICLE INFO

Article history:
Received 16 September 2010
Received in revised form
22 November 2010
Accepted 8 December 2010
Available online 22 December 2010

Keywords:
Propranolol enantiomer
CYP2D6
CYP2D17
CYP2D19
Regioselectivity
Stereoselectivity

ABSTRACT

Toxic and pharmacokinetic profiles of drug candidates are evaluated in vivo often using monkeys as experimental animals, and the data obtained are extrapolated to humans. Well understanding physiological properties, including drug-metabolizing enzymes, of monkeys should increase the accuracy of the extrapolation. The present study was performed to compare regio- and stereoselectivity in the oxidation of propranolol (PL), a chiral substrate, by cytochrome P450 2D (CYP2D) enzymes among humans, cynomolgus monkeys and marmosets. Complimentary DNAs encoding human CYP2D6, cynomolgus monkey CYP2D17 and marmoset CYP2D19 were cloned, and their proteins expressed in a yeast cell expression system. The regio- and stereoselective oxidation of PL enantiomers by yeast cell microsomal fractions were compared. In terms of efficiency of expression in the system, the holo-proteins ranked CYP2D6 ≒ CYP2D17 ≫ CYP2D19. This may be caused by the bulky side chain of the amino acid residue at position 119 (leucine for CYP2D19 vs. valine for CYP2D6 and CYP2D17), which can disturb the incorporation of the heme moiety into the active-site cavity. PL enantiomers were oxidized by all of the enzymes mainly into 4-hydroxyproranolol (4-OH-PL), followed by 5-OH-PL and N-desisopropylpropranolol (NDP). In the kinetic analysis, apparent K_m values were commonly in the μM range and substrate enantioselectivity of *R*-PL < *S*-PL was observed in both K_m and V_{max} values for the formation of the three metabolites from PL enantiomers. The activity to produce NDP tended to be higher for the monkey enzymes, particularly CYP2D17, than for the human enzyme. These results indicate that in the oxidation of PL enantiomers by CYP2D enzymes, stereoselectivity is similar but regioselectivity is different between humans and monkeys.

1. Introduction

Since various human tissues and recombinant enzymes have become available, the metabolic profiles of drug candidates can be predicted fairly accurately in experiments in vitro. However, toxic and pharmacokinetic profiles of drug candidates should still be evaluated in vivo using experimental animals and the data obtained extrapolated to humans. In this context, monkeys such as cynomolgus monkeys, rhesus monkeys and marmosets are superior to non-primates such as rodents, rabbits, and dogs as experimental animal species. Cynomolgus monkeys are rather big (5–10 kg in body weight) as compared with marmosets (200–300 g), which affects handling and feeding. Though cynomolgus monkeys and rhesus monkeys have been used extensively in research into drug metabolism and toxicology, relatively little data has been obtained from marmosets.

Drug metabolism is divided into phase I reactions consisting of oxidation, reduction and hydrolysis and phase II reactions consisting of various kinds of conjugation. The oxidation catalyzed by cytochrome P450s (CYPs) makes up about 80% of phase I reactions [1]. CYPs compose a superfamily of hemethiolate enzymes, and over 10,000 CYPs from animals, birds, fish, plants, microor-

Abbreviations: CYP, cytochrome P450; PL, propranolol; X-OH-PL, X-hydroxypropranolol; NDP, N-desisopropylpropranolol; 4-OH-BTL, 4-hydroxybunitrolol; PVDF, polyvinylidene difluoride; PCR, polymerase chain reaction; SRS, substrate recognition site.

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doi:10.1016/j.cbi.2010.12.014

ganisms etc. are known to exist [2]. Major isoenzymes of CYP1, 2 and 3 are responsible for drug metabolism in humans, namely CYP1A1/2, -2A6, -2B6, 2C8, -2C9, -2C19, -2D6, -2E1 and -3A4/5 [3]. CYP2D6 is clinically important because it contributes as the major enzyme to the oxidation of 15% of clinically prescribed medicines [4], though it accounts for only about 2% of all hepatic CYPs [5]. CYP2D6 shows extensive genetic polymorphism, and some 80 allelic variants have been reported to date [6], resulting in variation in drug-response phenotypes such as poor, intermediate, extensive and super-extensive metabolizers [4]. Cynomolgus monkeys and marmosets also have many CYPs [2] including CYP2D enzymes; CYP2D17 for cynomolgus monkeys [7] and CYP2D19 [8] and CYP2D30 [9] for marmosets.

Propranolol (PL) is a classical adrenoceptor blocking agent used clinically to treat arrhythmia and hypertension. PL has an asymmetric carbon atom in its side-chain, yielding the enantiomers *R*-PL and *S*-PL. Though *S*-PL has much more pharmacological activity as a β-blocker than *R*-PL [10], PL is given as a racemate. PL undergoes extensive metabolism in humans as shown in Fig. 1. For example, it is oxidized at the aromatic 4- and 5-positions mainly by CYP2D6 yielding 4-hydroxypropranolol (4-OH-PL) and 5-OH-PL, respectively, whereas the oxidation of the PL side-chain is mainly catalyzed by CYP1A2 giving N-desisopropylpropranolol (NDP) [11]. The oxidative metabolites as well as the parental compound are the substrates for UDP-glucuronosyltransferases and sulfotransferases [12,13]. PL is thus a useful substrate to study species differences in the regio- and stereoselective metabolism by CYP and conjugation enzymes.

Recently, we examined the oxidation of PL enantiomers by microsomal fractions from cynomolgus monkey and marmoset livers, and compared it with that by a human liver microsomal fraction [14]. As a result, we obtained experimental evidence that CYP2D enzymes are involved not only in the ring hydroxylation at the 4- and 5-positions but also in the side-chain N-desisopropylation in the monkey liver microsomal fractions [14]. In the present study, we expressed cynomolgus monkey CYP2D17 and marmoset CYP2D19 as well as human CYP2D6 in yeast cells, and compared the oxidation of PL enantiomers by yeast cell microsomal fractions among monkeys and humans.

2. Materials and methods

2.1. Materials

PL enantiomers as hydrochlorides were obtained from Sigma–Aldrich (St. Louis, MO); 4-OH-PL and 5-OH-PL as hydrochlorides from C/D/N Isotopes Inc. (Quebec, Canada); NDP as a hydrochloride from AstraZenaca (Cheshire, England); and 4-hydroxybunitrolol (4-OH-BTL) as a hydrochloride from Nippon Boehringer Ingelheim Co. (Hyogo, Japan). The RNeasy Mini kit, QIA shredder, and MiniElute Gel Extraction kit were purchased from Qiagen (Heiden, Germany). The RNA PCR kit v3.0, DNA ligation kit v2.1, Taq DNA polymerase, calf intestinal alkaline phosphatase and HindIII were from Takara Bio (Ohtsu, Japan); pGEM-T vector and T4 DNA ligase from Promega (Madison, WI); KOD-plus DNA polymerase from Toyobo (Osaka, Japan); Quantum Prep Plasmid Miniprep kit and polyvinylidene difluoride (PVDF) membrane from BioRad (Hercules, CA); and BigDye terminator cycle sequencing reaction kit v3.1 from Applied Biosystems (Foster City, CA). Horse radish peroxidase-conjugated anti-rabbit IgG was obtained from ICN Pharmaceuticals Inc. (Costa Mesa, CA). Enhanced chemiluminescence-plus reagents were from GE Healthcare Bio-Sciences Inc. (Little Chalfont, UK). Livers from adult male common marmosets were supplied by Professor Atsuro Miyata of Kagoshima University (Kagoshima, Japan). Livers from adult male cynomolgus monkeys were from Ina Research Co. Ltd. (Ina, Japan). Microsomal fractions from cynomolgus monkey and marmoset livers were prepared according to published methods [15]. Pooled human liver microsomal fractions were obtained from BD Biosciences (San Jose, CA).

Table 1
Primers used for the amplification of CYP2D17 cDNA.

Primer	Sequence	T_m (°C)	Length (bp)
CYP2D17-F1	ATGGAGCTAGATGCACTGGTGCCCCTGGC	69.0	29
CYP2D17-R1	CTAGCGGGGCACAGCACAAAGCTCATAGG	69.0	29
CYP2D17-F2	AAGCTT*AAAAAA*ATGGAGCTAGATGCACTG	59.2	30
CYP2D17-R2	AAGCTTTCTAGCGGGGCACAGCACA	63.9	25

Underlined and italic letters indicate restriction enzyme sites and the Kozak sequence, respectively.

2.2. Cloning of cDNA encoding CYP2D17

Total RNA was extracted from cynomolgus monkey liver using the RNeasy minikit and QIA shredder according to the manufacturer's instructions. The total RNA was reverse-transcribed to cDNA using the RNA PCR kit v3.0. The full-length cDNA encoding CYP2D17 was amplified by PCR from single-strand cDNA templates using CYP2D17-F1 and -R1 as primers (Table 1). These primers were designed based on the nucleotide sequence in the flanking regions of CYP2D17 cDNA (GenBank accession number Q29488). The PCR reaction mixture contained 1× PCR buffer, 0.2 mM dNTPs, each primer at 0.2 μM, 1.5 mM $MgSO_4$, and 1 U of KOD plus DNA polymerase in a final volume of 50 μL. The PCR consisted of 30 cycles with denaturation at 94 °C for 15 s, annealing at 65 °C for 30 s and extension at 68 °C for 100 s. The PCR product was isolated and purified by agarose electrophoresis, and the 5′- and 3′-ends of the coding region were sequenced in both the forward and reverse directions. The full-length cDNA obtained was modified by amplification with CYP2D17-F2 and -R2 as primers (Table 1). The PCR was performed with a similar reaction medium to that as described above though the annealing temperature was 58 °C. The PCR product was isolated and purified by agarose electrophoresis. The PCR product was introduced into the pGEM-T vector after A-tailing and sequenced in both the forward and reverse directions. A DNA fragment corresponding to CYP2D17 was cut from the pGEM-T vector with HindIII, and subcloned into pGYR1 digested with the same restriction enzyme. The insert of the plasmid was sequenced to verify the correct orientation with respect to the promoter for pGYR1. Construction of the expression plasmids containing each of CYP2D6 and CYP2D19 was described previously [9].

2.3. Expression of CYP2D enzymes

Saccharomyces cerevisiae AH22 was transformed with pGYR1 containing each of the CYP2D cDNAs by the lithium acetate method, and the culture of yeast transformants thus obtained was performed as described previously [16]. Microsomal fractions were prepared from yeast cells expressing the CYP2D enzymes by methods reported previously [16]. The fractions were diluted to a protein concentration of 5.0 mg/mL with 100 mM potassium phosphate buffer (pH 7.4) containing 0.4% Emulgen 911 and 20% glycerol. Total holo-CYP content was measured spectrophotometrically by the method of Omura and Sato [17] using 91 $mM^{-1}\,cm^{-1}$ as the absorption coefficient. Appropriate portions of the yeast cell microsomal fractions together with microsomal fractions from human, cynomolgus monkey and marmoset livers were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a 10% slab gel. Following the electrophoresis, proteins on the gel were electroblotted to a PVDF membrane, and analyzed by Western