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Cloning and functional expression of a novel marmoset cytochrome P450 2D enzyme, CYP2D30: comparison with the known marmoset CYP2D19

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Abstract

Using a primer set designed on the cDNA encoding the known marmoset cytochrome P450 2D19 (CYP2D19), a cDNA encoding a novel CYP2D enzyme (CYP2D30) was cloned from the liver of a female marmoset bred at Kyoto University (KYU). In addition, a cDNA encoding CYP2D19 was cloned from the liver of a female marmoset bred at Kagoshima University (KAU). CYP2D30 and CYP2D19 showed homologies of 93.6 and 93.4% in their nucleotide and amino acid sequences, respectively. Reverse transcription polymerase chain reaction (RT-PCR) and digestion with *Nde*I demonstrated that the KYU-marmoset liver contained mainly mRNA for CYP2D30, while the KAU-marmoset liver contained mainly mRNA for CYP2D19. Marmoset CYP2D30, like human CYP2D6, exhibited high debrisoquine (DB) 4-hydroxylase activity and relatively low DB 5-, 6-, 7- and 8-hydroxylase activities, whereas CYP2D19 lacked DB 4-hydroxylase but exhibited marked 5-, 6-, 7- and 8-hydroxylase activities. The two marmoset recombinant enzymes showed enantioselective bufuralol (BF) 1″-hydroxylase activities, similar to CYP2D6. BF 1″-hydroxylation by CYP2D30 exhibited product-enantioselectivity of (1″*R*-OH-BF $\ll$ 1″*S*-OH-BF), similar to that observed with human CYP2D6, whereas CYP2D19 showed a reversed selectivity of (1″*R*-OH-BF $\geq$ 1″*S*-OH-BF). BF 1″-hydroxylation in marmoset liver microsomes from both sources was inhibited by antibodies raised against rat CYP2D1 in a concentration-dependent manner. A known inhibitor of CYP2D6, quinidine, effectively inhibited the BF 1″-hydroxylation activities in liver microsomal fractions prepared from KYU- and KAU-marmosets. These results suggest that CYP2D19 and CYP2D30 proteins can be expressed as functional enzymes in marmoset livers, although it is unresolved whether both enzymes coexist in the same marmoset liver.

Keywords: CYP2D19; CYP2D30; CYP2D6; Marmoset, debrisoquine; Bufuralol

1. Introduction

In the fields of drug metabolism and toxicology, rodents such as mice, rats, guinea pigs and rabbits have been used as experimental models for the past 70 years, mainly due to their availability, low costs and genetic homogeneity. Metabolic profiles and/or toxicities of drugs are known to differ

Abbreviations: CYP, cytochrome P450; DB, debrisoquine; 4-OH-DB, 4-hydroxydebrisoquine; BF, bufuralol; 1″-OH-BF, 1″-hydroxybufuralol; KYU, Kyoto University; KAU, Kagoshima University; G-6-P, glucose-6-phosphate; RT-PCR, reverse transcriptase-polymerase chain reaction; HPLC, high-performance liquid chromatography

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between experimental animals and humans, i.e., there are species differences, such that it is difficult to accurately estimate the pharmacological activities or toxicities of drugs with the data obtained from experimental animals. With the recent developments of organ transplantation and gene engineering techniques, the metabolic fates of promising drug candidates can be predicted with data obtained from in vitro experiments using various human tissues or recombinant human enzymes expressed in heterogeneous or homogeneous expression systems [1].

However, the assessment of in vivo toxicity in experimental animals is still required for the development of new medicines. Therefore, species differences remain one of the big problems that need to be overcome. In this context, monkeys are thought to be valuable species as experimental animals for in vivo studies, since monkeys, like humans, are primates. However, cumulative experimental data have revealed that the profiles of drug metabolism in monkeys are often different from those in humans [2–4].

The marmoset *Callithrix jacchus* is one of the monkey species that is often used as an experimental model in drug metabolism studies [5–7]. Marmosets are categorized as New World monkeys and are easy to handle because of their small body size. Regarding cytochrome P450 (CYP) enzymes in the marmoset, three cDNAs encoding CYP1A2, CYP2D19 and CYP3A21 have been cloned to date [8,9], and CYP2E1 has also been suggested to exist in marmoset livers [10]. Over the past 10 years, we have been studying the relationship between the structure and function of various CYP2D enzymes such as human CYP2D6 [11–13] and rat CYP2D2 [14,15]. More recently, we have cloned a cDNA encoding CYP2D29 from the liver of the Japanese monkey [16]. Igarashi et al. [8] have also cloned a cDNA encoding CYP2D19, but, to date, its enzymatic functions have not been reported. It was therefore of interest for us to compare the catalytic activities of the Japanese monkey CYP2D29 with those of the marmoset CYP2D19. Accordingly, we tried to clone the cDNA of CYP2D19 from livers of marmosets that were bred in the Primate Research Institute, Kyoto University (KYU).

As a result, a cDNA encoding another CYP2D enzyme, CYP2D30, was unexpectedly cloned from KYU-marmoset livers, and a cDNA encoding CYP2D19 was also obtained from the liver of a female marmoset that was bred in the Faculty of Medicine, Kagoshima University (KAU). Here, we describe the cloning and catalytic properties of the CYP2D19 and CYP2D30 enzymes, each enzyme being heterologously expressed in yeast cells, and compared with the catalytic properties of recombinant human CYP2D6.

2. Materials and methods

2.1. Materials

Debrisoquine (DB) and 4-hydroxydebrisoquine (4-OH-DB) (as hemisulfates) were gifts from Roche Products Ltd. (Welwyn Garden City, UK). Authentic standards of 5-, 6-, 7- and 8-OH-DB were previously provided to one of us (SWE) by Professor R.L. Smith, Imperial College School of Medicine at St. Mary's, London, UK. Bufuralol (BF) enantiomers and their 1″-hydroxylated metabolites (1″-OH-BF) (as hydrochlorides) were kindly supplied by Dr. U. Meyer of Basel University (Basel, Switzerland). Quinidine hydrochloride was obtained from Wako Pure Chemical Inc. (Osaka, Japan). Glucose 6-phosphate (G-6-P), G-6-P dehydrogenase and NADPH were purchased from Oriental Yeast Co. (Tokyo, Japan). Zymolyase was obtained from Seikagaku Corp. (Tokyo, Japan). Female marmoset livers were supplied by the Primate Research Institute, KYU and the Faculty of Medicine, KAU. Human liver microsomes [three samples, two male and one female Caucasians, ages ranging from 38 to 65] were supplied by the Human and Animal Bridge Discussion Group (HAB), and have been characterized as extensive metabolizers with respect to CYP2D6, as reported previously [17]. Polyclonal antibodies raised against CYP2D1 (rabbit antiserum) were supplied by Professor Yoshihiko Funae, Osaka City University, Medical School, Osaka, Japan.

2.2. Isolation and sequencing of CYP2D30 and CYP2D19 cDNAs

Total RNA was isolated from individual marmoset livers using ISOGEN (Nippon Gene, Tokyo, Japan), and first-strand cDNA was synthesized using an RNA PCR kit (Ver. 2.1, Takara Biomedicals, Tokyo, Japan) according to the manufacturer's instructions. The cDNAs encoding marmoset CYP2D enzymes were amplified by polymerase chain reaction (PCR) from single stranded cDNA templates using the forward primers 5′-TCCAGGGGTGTCCAGAGGAG-3′ (nucleotide position −42 to −23) and 5′-GGAACTACCACATTGCTTTA-3′ (nucleotide position 1596–1615). These primers were designed based on the nucleotide sequences in the flanking regions of the marmoset CYP2D19 gene (GenBank Accession no. D29822).

Reaction mixtures (50 μL) contained 0.2 mM dNTPs, 1 mM $MgSO_4$, 1 U KOD-*plus* DNA polymerase (Toyobo, Osaka, Japan) and each oligonucleotide primer at 0.3 μM. The PCR using a TP2000 thermal cycler (Takara Biomedicals) consisted of 35 cycles with denaturation at 94 °C for 15 s, annealing at 50 °C for 30 s and extension at 68 °C for 90 s. The initial denaturation was at 94 °C for 2 min. Amplified products (1.5 kbp) were purified using a Prep-A-Gene DNA purification kit (Bio-Rad Laboratories, Richmond, CA) and sequenced in both the forward and reverse directions by the dideoxy chain termination method using a Thermo Sequenase fluorescent-labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Biosciences, Piscaraway, NJ) and Cy5-labeled primers designed based on the CYP2D19 nucleotide sequence (5′-GGAAGTCCACATGCAGCAGGTTGCC-3′, nucleotide position 178–202 and 5′-GGCCCAGCCCTCACGGTGTCT-

TTGC-3′, nucleotide position 1469–1493) on an ALF express DNA sequencer (Amersham Biosciences). Each CYP2D cDNA was further amplified using 5′-aaaagcttaaaaaa-ATGGGGCTGGATGCACTGGTGCCC-3′ (nucleotide position 1–24) and 5′-gggaagctt-GTTCTAG-CGGGGCACAGCACAAAGCTC-3′ (nucleotide position 1471–1497) as primers for PCR under the conditions as described above, where the underlined bases (*Hin*dIII sites) were introduced to facilitate subcloning into pGYRI. The six adenines just upstream of the ATG start codon were also added for efficient expression of the corresponding gene product in yeast cells.

The PCR products were digested with *Hin*dIII and were subsequently inserted into *Hin*dIII-digested pBluescript KS(+). The CYP2D plasmids were sequenced using appropriate 5′-Cy5-labeled primers as described above. Not only a cDNA encoding CYP2D19 from the KAU-marmoset liver but also another CYP2D cDNA from the KYU-marmoset liver was identified. The novel cDNA nucleotide and deduced amino acid sequences have been registered with GenBank (Assession no. AY082602), and named CYP2D30 by the Human CYP Allele Nomenclature Committee. Sequence analysis was conducted using a computer program, Genetyx v9.0 (GENETYX Software Development Co., Tokyo, Japan). The BLAST program of the Institute for Chemical Research, Kyoto University, was used to determine the homology of the deduced amino acid sequence of marmoset CYP2Ds to other CYP2D species.

2.3. Expression of CYP2D19 and CYP2D30 enzymes

The CYP2D19 and CYP2D30 plasmids were digested with *Hin*dIII, and the resultant fragments were subsequently inserted into pGYRI yeast expression vector. The expression vector, which contains a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) promoter and a yeast NADPH-CYP reductase cDNA, was used to transform the *Saccharomyces cerevisiae* AH-22 strain by the lithium acetate method [18]. The expression plasmids were sequenced to verify correct orientation with respect to the GAPDH promoter. Sequencing was performed using a 5′-Cy5-labelled primer (5′-CTCAAGGGA-GGATGTGTGG-GTGTGG-3′) designed on the basis of the nucleotide sequence upstream of the GAPDH promoter. Cultivation of the yeast cells was performed as described previously [16].

After centrifugation of the culture, the pellet was washed twice with buffer A containing 10 mM Tris–HCl (pH 7.5), 2 M D-sorbitol, 0.1 mM dithiothreitol, and 0.2 mM EDTA and digested with zymolyase in buffer A (0.5 mg/mL) for 2 h at 35 °C by gently shaking. The pellet obtained after centrifugation at 2000 × *g* for 10 min at 4 °C was washed again with buffer A before being resuspended in buffer B containing 10 mM Tris–HCl (pH 7.5), 0.65 M D-sorbitol, and 0.1 mM EDTA. After sonication of the suspension on ice 10 times (30 s each time), the microsomal fraction was prepared by centrifugation (105,000 × *g* for 60 min at 4 °C) and suspended in buffer C containing 0.1 M sodium phosphate (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol, and 20% glycerol. Recombinant CYP2D6 was expressed in yeast cells as reported elsewhere [12].

The protein concentrations were determined by the method of Lowry et al. [19] using bovine serum as a standard, and the CYP2D holoprotein content determined by reduced carbon monoxide-difference spectroscopy using 91 $mM^{-1}\,cm^{-1}$ as an absorption coefficient [20]. Appropriate portions of yeast cell microsomal fractions together with those from human, KYU- and KAU-marmoset livers were subjected to SDS-PAGE using a 10% slab gel, and CYP2D proteins were probed by Western blotting as described previously [16].

2.4. Identification of CYP2D19 and CYP2D30 mRNAs in marmoset livers

In order to identify mRNAs for CYP2D19 and CYP2D30 in the marmoset livers supplied by KAU and KYU, we performed reverse transcription (RT)-PCR analysis under the same conditions in which the cDNAs encoding CYP2D19 and CYP2D30 were identified as described above. The PCR products were digested with a restriction enzyme (*Nde*I) for 6 h at 37 °C and nucleotide fragments were visualized by ethidium bromide staining after electrophoresis on a 1.5% agarose slab-gel. CYP2D30 cDNA prepared from the KYU-marmoset liver was sensitive to the enzyme digestion, yielding two nucleotide fragments (about 1.1 kbp and 400 bp), but CYP2D19 cDNA prepared from the KAU-marmoset liver was resistant to digestion.

2.5. Assay of drug oxidation activities

DB hydroxylase activities were assayed as follows. Typical incubation mixture (final volume, 500 μL) contained G-6-P (5 μmol), $MgCl_2$ (10 μmol), DB (50 nmol, 100 μM), G-6-P dehydrogenase (0.5 IU), EDTA (50 nmol), in 50 mM potassium phosphate buffer (pH 7.4) in a brown conical glass tube with a glass stopper. After preincubation at 37 °C for 5 min, incubation was started by adding yeast microsomal fraction (10 pmol recombinant CYP), and continued for 2 min. Incubation was stopped by adding 50 μL of aqueous 60% $HClO_4$ and vigorously shaking. The mixture was then centrifuged at 3500 × *g* for 3 min at 4 °C, and an aliquot of the supernatant subjected to high-performance liquid chromatography (HPLC) under the conditions described below.

Enantiomeric BF 1″-hydroxylase activities were measured by HPLC as reported previously [17]. Kinetic analyses were performed using a BF enantiomer concentration range from 0.5 to 200 μM. Apparent Michaelis–Menten constants (K_m) and maximal velocities (V_{max}) were calculated using Eadie–Hofstee plots and least square analysis.

The HPLC conditions were: a Hitachi 655-12A liquid chromatograph equipped with a Hitachi L-75480 fluorescence detector, a Rheodyne model 7125 injector, and a Shimadzu C-R3A Chromatopac data processor; column-A, Phenomenex LUNA C18 (4.6 mm × 250 mm, Shimadzu) at 40 °C for DB hydroxylation; column-B, Chiralcel OD (4.6 mm × 250 mm, Daicel) at 25 °C for BF 1″-hydroxylation; mobile phase-A, 4 mM KH_2PO_4 (pH 2.5)/CH_3CN (93:7, by volume) at a flow rate of 1 mL/min for DB hydroxylation; mobile phase-B, *n*-hexane/ethanol/diethylamine (95:5:0.01, by volume) at a flow rate of 0.5 mL/min for BF 1″-hydroxylaton; detection, fluorescence 200/300 nm (excitation/emission) for DB hydroxylation and 252/302 nm for BF 1″-hydroxylation.

2.6. Inhibition studies

In antibody experiments, the marmoset liver microsomes in a reaction medium were preincubated at 25 °C for 30 min with varying amounts of polyclonal antibodies (rabbit antiserum) raised against CYP2D1. Then the substrate (BF enantiomer, 5 μM) and an NADPH-generating system were added to the reaction medium, and BF 1″-hydroxylation activity was assayed at 37 °C. For inhibitor experiments, quinidine (a final concentration of 1 μM) was added to the reaction medium containing various amounts of BF enantiomer (0.5–200 μM). The results were analyzed by Eadie–Hofstee plotting, and kinetic parameters were calculated.

3. Results

3.1. cDNA cloning

Total RNA was extracted from a fresh female marmoset liver supplied by KYU, and a full-length cDNA encoding CYP2D30 was isolated by PCR with oligonucleotide primers that were designed on the basis of the nucleotide sequence of CYP2D19 reported by Igarashi et al. [8]. The cDNA nucleotide and its deduced amino acid sequences are shown in Fig. 1. Fig. 2 shows a comparison of the amino acid sequences of CYP2D30, CYP2D19 and human CYP2D6. Table 1 summarizes identities of the nucleotide and amino acid sequences of these enzymes. CYP2D30 and CYP2D19 showed homologies of 93.6 and 93.4% in nucleotide and amino acid sequences, respectively. That is, there are 33 different amino acid residues between CYP2D30 and CYP2D19. Compared with human CYP2D6, on the other hand, CYP2D19 showed lower amino acid sequence homology (90.7%), while CYP2D30 revealed greater homology (94.6%). The novel CYP2D30 cDNA was also cloned from another female marmoset liver sample which was also supplied by KYU.

Table 1
Identities of the nucleotide and deduced amino acid sequences of three primate CYP2D enzymes

	CYP2D6	CYP2D19	CYP2D30
CYP2D6		91.6	96.2
CYP2D19	90.7		93.6
CYP2D30	94.6	93.4	

Upper-right values, percent identities of the nucleotide sequences; lower-left values, percent identities of the deduced amino acid sequences.

On the other hand, using the same primer set, a cDNA was also cloned from the liver of a female marmoset bred in KAU. The nucleotide sequence was found to be the same as that of CYP2D19 described by Igarashi et al. [8]. The cDNA encoding CYP2D30 was not obtained in repeated experiments using the KAU-marmoset liver.

3.2. Identification of mRNAs of CYP2D19 and CYP2D30 in marmoset livers

The difference of 96 nucleotides in the cDNAs encoding the two enzymes resulted in a different susceptibility to digestion by the restriction enzyme, *Nde*I. That is, the cDNA (1494 bp) of CYP2D30 contains the nucleotide sequence of 5′-CATATG-3′ from nucleotide position 1126 to 1131, resulting in the formation of two fragments of 1127 and 367 bp following digestion with *Nde*I, whereas the cDNA (1494 bp) of CYP2D19 has the nucleotide sequence 5′-CACATG-3′ at the corresponding region (Fig. 3A). Accordingly, the cDNA of CYP2D30 is sensitive to *Nde*I-digestion while the cDNA of CYP2D19 is resistant.

Fig. 3B shows the electrophoretic profile of the cDNA samples after *Nde*I-digestion. Digestion with the restriction enzyme of cDNAs consisting of the RT-PCR products obtained from the total RNA demonstrated that the cDNA amplified from the KYU-marmoset liver was digested into two nucleotide fragments of about 1.1 and 0.4 kbp. On the other hand, the cDNA derived from the KAU-marmoset liver did not yield apparent digestion products.

3.3. Expression of CYP2D19 and CYP2D30 in yeast cells

The cDNAs encoding CYP2D19 and CYP2D30, each of which was inserted into expression vector pGYRI, were transformed into yeast cells, and the catalytic properties of the resultant proteins compared. Microsomal fractions obtained from yeast cells expressing each of the CYP2D enzymes showed typical reduced carbon monoxide-difference spectra, exhibiting Soret peaks at 450 nm and no detectable P420 (spectra not shown). The calculated P450 holoprotein content was 4.3 and 11.1 pmol/mg protein for CYP2D19 and CYP2D30, respectively.

CYP2D proteins in the microsomal fractions of the marmoset livers from which each of the cDNAs had been cloned were analyzed by Western blotting with polyclonal antibodies raised against rat CYP2D1. As shown in Fig. 4,

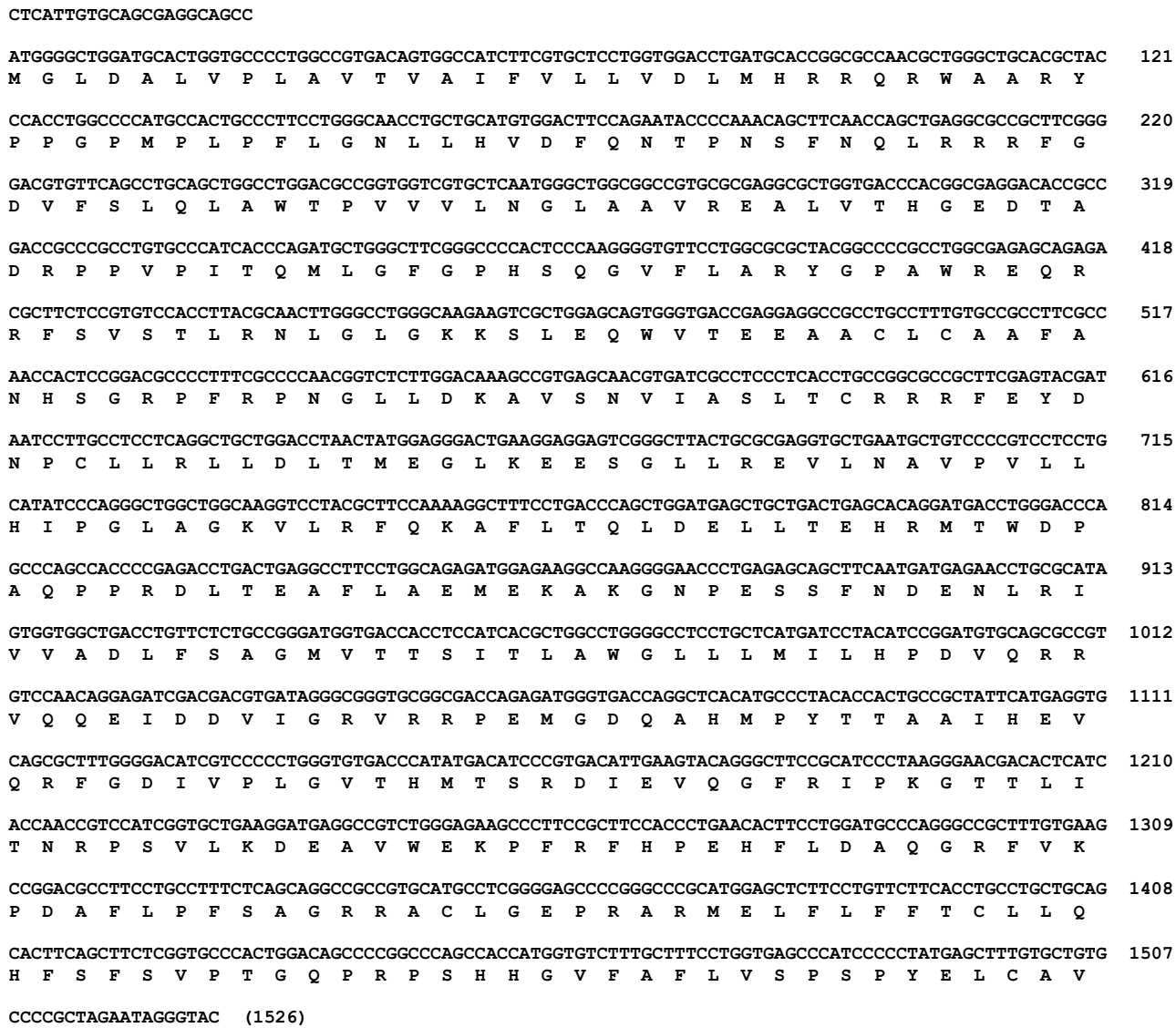

Fig. 1. Nucleotide and deduced amino acid sequences of marmoset CYP2D30. The numbers of the deduced amino acid and nucleotide sequences are shown on the left and right sides, respectively.

```
2D30      1 MGLDALVPLAVTVAIFVLLVDLMHRRQRWAARYPPGPMPLPFLGNLLHVDFQNTPNSFNQ 60
2D19      1 MGLDALVPLAVTVAIFVLLVDLMHRRQRWAARYPPGPMPLPGLGNLLHVDFQNTPNSFNQ 60
2D6       1 MGLEALVPLAVIVAIFLLLVDLMHRRQRWAARYPPGPLPLPGLGNLLHVDFQNTPYCFDQ 60
            *** ******* **** ******************** *** ************  * *

2D30     61 LRRRFGDVFSLQLAWTPVVVLNGLAAVREALVTHGEDTADRPPVPITQMLGFGPHSQGVF 120
2D19     61 LRRRFGDVFSLQLAWTPVVVLNGLEAVREALVTRGEDTADRPPVPITEMLGFGPHSQGLF 120
2D6      61 LRRRFGDVFSLQLAWTPVVVLNGLAAVREALVTHGEDTADRPPVPITQILGFGPRSQGVF 120
            ********************** ******** ************  ***** *** *

2D30    121 LARYGPAWREQRRFSVSTLRNLGLGKKSLEQWVTEEAACLCAAFANHSGRPFRPNGLLDK 180
2D19    121 LARYGPAWREQRRFSVSTLRNLGLGKKSLEQWVTEEATYLCAAFADHAGRPFRPNGLLDK 180
2D6     121 LARYGPAWREQRRFSVSTLRNLGLGKKSLEQWVTEEAACLCAAFANHSGRPFRPNGLLDK 180
            ************************************  ****** * ************

2D30    181 AVSNVIASLTCRRRFEYDNPCLLRLLDLTMEGLKEESGLLREVLNAVPVLLHIPGLAGKV 240
2D19    181 AVSNVIASLTCRRRFEYNDPCLLRLLDLTMEGLKEESGLLREVLNAIPVLLRIPGLAGKV 240
2D6     181 AVSNVIASLTCGRRFEYDDPRFLRLLDLAQEGLKEESGFLREVLNAVPVLLHIPALAGKV 240
            *********** *****  *  ******  ******** ******* **** ** *****

2D30    241 LRFQKAFLTQLDELLTEHRMTWDPAQPPRDLTEAFLAEMEKAKGNPESSFNDENLRIVVA 300
2D19    241 LRSQKAFLAQLDELLTEHRMTWDPAQPPRDLTEAFLAEMEKTKGNPESSFNDENLHLVVA 300
2D6     241 LRFQKAFLTQLDELLTEHRMTWDPAQPPRDLTEAFLAEMEKAKGNPESSFNDENLRIVVA 300
            ** ***** ******************************** *************  ***

2D30    301 DLFSAGMVTTSITLAWGLLLMILHPDVQRRVQQEIDDVIGRVRRPEMGDQAHMPYTTAAI 360
2D19    301 DLFSAGMVTTSITLAWGLLLMILHPDVQRRVQQEIDDVIGRVRRPEMGDQTYMPYTTAVI 360
2D6     301 DLFSAGMVTTSTTLAWGLLLMILHPDVQRRVQQEIDDVIGQVRRPEMGDQAHMPYTTAVI 360
            *********** ***************************** *********  ****** *

2D30    361 HEVQRFGDIVPLGVTHMTSRDIEVQGFRIPKGTTLITNRPSVLKDEAVWEKPFRFHPEHF 420
2D19    361 HEVQRFADIVPLGVTHMTSRDIEVQGFLIPKGTTLFTNLSSVLKDEANWEKPFRFHPEHF 420
2D6     361 HEVQRFGDIVPLGVTHMTSRDIEVQGFRIPKGTTLITNLSSVLKDEAVWEKPFRFHPEHF 420
            ****** ******************** ******* **  ******* ************

2D30    421 LDAQGRFVKPDAFLPFSAGRRACLGEPRARMELFLFFTCLLQHFSFSVPTGQPRPSHHGV 480
2D19    421 LDAQGRFVKPEAFLPFSAGRRACLGEPLARMELFLFFTCLLQRFSFSVPAGQPRPSPHGV 480
2D6     421 LDAQGHFVKPEAFLPFSAGRRACLGEPLARMELFLFFTSLLQHFSFSVPTGQPRPSHHGV 480
            ***** **** ***************** ********** *** ****** ****** ***

2D30    481 FAFLVSPSPYELCAVPR                                           497
2D19    481 FAFLVTPSPYELCAVPR                                           497
2D6     481 FAFLVSPSPYELCAVPR                                           497
            ***** ***********
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Fig. 2. Alignment of the amino cid sequences of CYP2D30, CYP2D19 and CYP2D6. CYP2D30 is from marmoset (GenBank Accession no. AY082602), CYP2D19 from marmoset (no. D29822), and CYP2D6 from human (no. M33388). (*) Amino acid residues conserved among the three CYP2D enzymes.

the microsomal fractions from the marmoset livers as well as those from transformed yeast cells exhibited a single protein band whose molecular weight was about 50 kDa. The content of CYP2D30 in the KYU-marmoset liver appears to be much lower than that of CYP2D19 in the KAU-marmoset liver.

Fig. 5 (upper panels) depicts the specific activities of the metabolite formation by the recombinant enzymes calculated from calibration curves of authentic DB metabolites. The profiles of DB metabolites formed by CYP2D6 and CYP2D30 were very similar, but the profile of CYP2D19 was unique. Remarkably, CYP2D19 did not form 4-OH-DB in detectable amounts, although substantial quantities of 5-, 6-, 7- and 8-OH-DB metabolites were produced. In contrast, CYP2D30 and CYP2D6 both produced substantial amounts of 4-OH-DB and generally smaller amounts of the aromatic hydroxylated metabolites.

Table 2 summarizes the apparent kinetic constants of DB hydroxylation by the recombinant enzymes. Apparent K_m values ranged from 4 to 17 μM and V_{max} values reflect the rates determined at a substrate concentration of 50 μM depicted in Fig. 5 (upper panels). No detectable aromatic DB 4-hydroxylase activity was seen with even at the highest DB concentration tested (200 μM).

3.4. BF 1″-hydroxylation by CYP2D19 and CYP2D30

When BF enantiomers (*R*-BF and *S*-BF, 100 μM each) were used as substrates, recombinant marmoset CYP2D19 and CYP2D30, as well as human CYP2D6, generated four

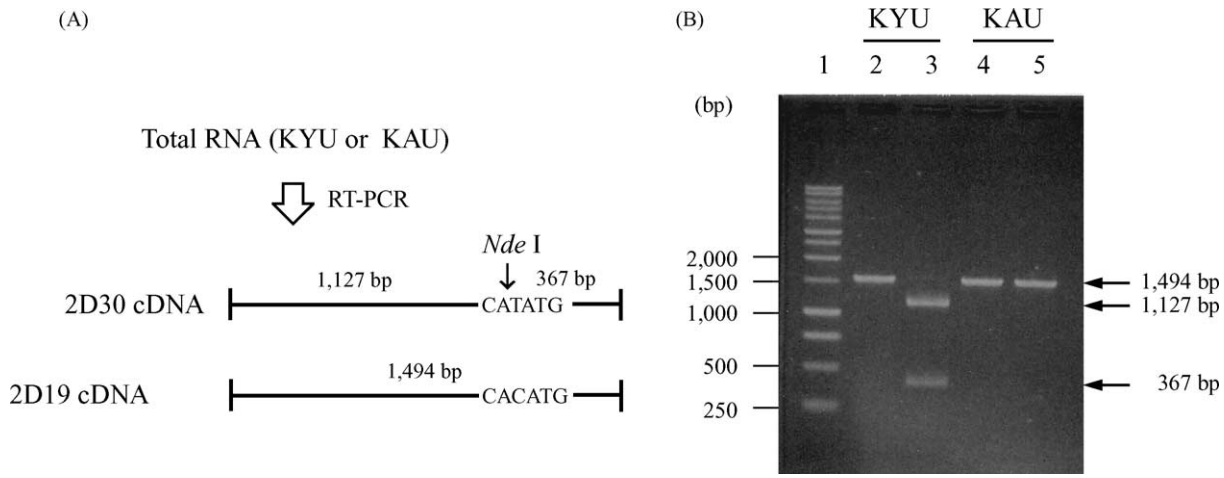

Fig. 3. Identification of CYP2D30 and CYP2D19 mRNAs in marmoset livers by digestion of cDNAs with restriction enzyme *Nde*I. Total RNA from marmoset livers was reverse transcribed, PCR amplified, digested with *Nde*I, and then separated by electrophoresis. (A) Diagram of the recognition site for the restriction enzyme *Nde*I in CYP2D30 cDNA. (B) Image of 1.5% agarose slab-gel of *Nde*I restriction digest of CYP2D cDNAs prepared from marmoset liver mRNA. Lane 1, 1 kb DNA ladder; lanes 2 and 4, reference nondigested PCR products; lanes 3 and 5, samples digested with *Nde*I to check for the presence of CYP2D30 and CYP2D19 mRNAs.

1″-hydroxylated BF products [1″*R*-OH-*R*-BF (A), 1″*S*-OH-*R*-BF (B), 1″*R*-OH-*S*-BF (C) and 1″*S*-OH-*S*-BF (D)] (Fig. 5 lower panels). CYP2D6 and CYP2D30 showed a similar substrate-enantioselectivity of [*R*-BF (A+B) > *S*-BF (C+D)], and a similar product-enantioselectivity [1″*R*-OH-BF (A+C) < 1″*S*-OH-BF (B+D)], although the total BF 1″-hydroxylase activity of CYP2D30 was much greater than that of CYP2D6 (Fig. 5 left and right panels). On the other hand, although CYP2D19 showed the same substrate-enantioselectivity [*R*-BF (A+B) > *S*-BF (C+D)] as that of the other two enzymes, it did not exhibit a clear product-enantioselectivity [1″*R*-OH-BF (A+C) = 1″*S*-OH-BF (B+D)] (Fig. 5 middle panel).

Kinetics of BF 1″-hydroxylation demonstrated clear differences between recombinant CYP2D19 and CYP2D30 (Table 3). Apparent K_m values of *R*-BF 1″-hydroxylation (A and B) by CYP2D30 were almost double those of *S*-BF 1″-hydroxylation (C and D), whereas apparent K_m values of CYP2D19 for *S*-BF 1″-hydroxylation (C and D) were about double those of *R*-BF 1″-hydroxylation (A and B). In contrast, the K_m values of *R*-BF and *S*-BF 1″-hydroxylation by CYP2D6 were similar to each other. The profile of V_{max} values paralleled that of the substrate-enantioselectivity

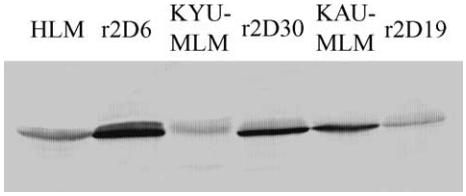

Fig. 4. Western blot analysis of microsomes prepared from human and marmoset livers, and from yeast cells expressing cDNAs of CYP2D6, CYP2D30 and CYP2D19. Fifty μg of each microsomal protein sample was applied to each well, and resolved by SDS-PAGE. The proteins were then transblotted to a PVDF membrane and immunochemically probed with polyclonal antibodies raised against rat CYP2D1. HLM and MLM represent human liver microsomes and marmoset liver microsomes, respectively.

data generated at a single substrate concentration, as described earlier.

3.5. Inhibitory effect of antibody and quinidine on marmoset liver microsomal BF 1″-hydroxylation

In antibody-inhibition experiments, assays were performed at a substrate concentration of 5 μM. Anti-rat CYP2D1 antibody inhibited BF 1″-hydroxylation by microsomal fractions from KYU- and KAU-marmoset livers in a

Table 2
Kinetic parameters for DB 4-hydroxylation by recombinant CYP2D6, CYP2D19 and CYP2D30

	K_m (μM)	V_{max} (nmol/min/nmol CYP)
4-OH-DB		
rCYP2D6	9.1	7.0
rCYP2D19	ND	ND
rCYP2D30	7.2	10.8
5-OH-DB		
rCYP2D6	8.1	0.4
rCYP2D19	7.7	3.1
rCYP2D30	7.1	0.6
6-OH-DB		
rCYP2D6	6.0	0.5
rCYP2D19	7.1	7.4
rCYP2D30	10.0	0.8
7-OH-DB		
rCYP2D6	10.6	1.0
rCYP2D19	4.2	5.7
rCYP2D30	7.2	1.6
8-OH-DB		
rCYP2D6	10.1	0.6
rCYP2D19	13.4	3.1
rCYP2D30	17.2	1.2

Each value represents the mean of two independent determinations. ND: not detectable.

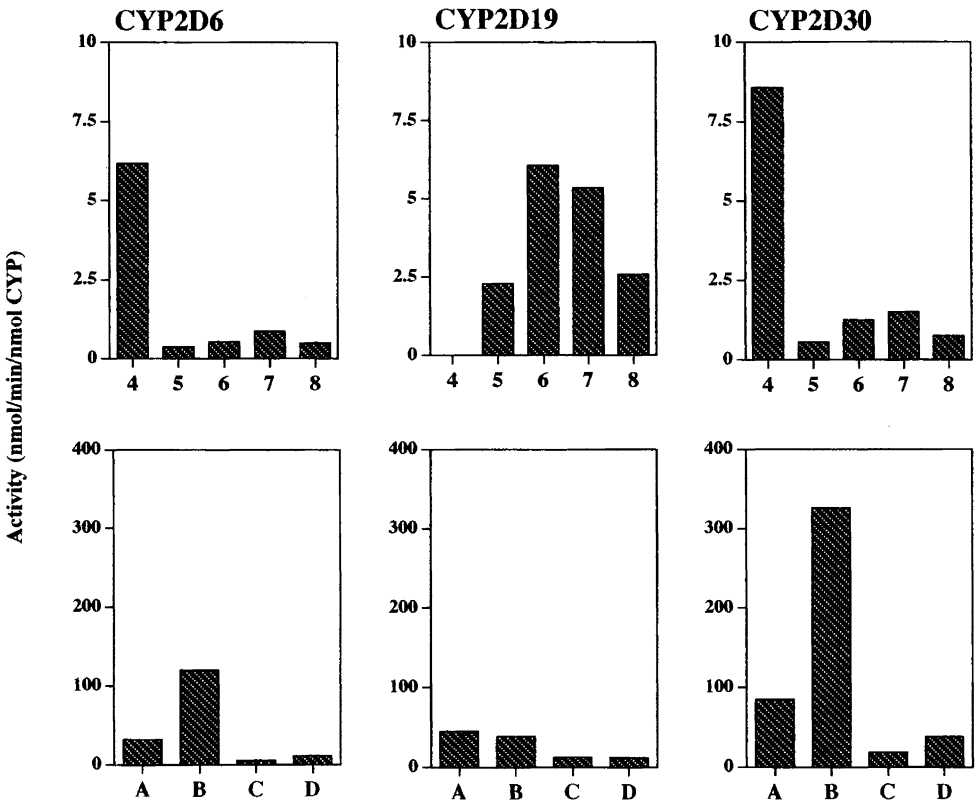

Fig. 5. Comparison of DB hydroxylation (upper panels) and BF 1″-hydroxylation (lower panels) by recombinant marmoset CYP2D19, CYP2D30 and human CYP2D6. The substrate concentrations used were 50 and 100 μM for DB and BF enantiomers, respectively. Numbers under the upper panels depict the positions of the hydroxylated carbon atom of DB. Alphabets under the lower panels show four BF 1″-hydroxylated metabolites: (A) 1″*R*-OH-*R*-BF; (B) 1″*S*-OH-*R*-BF: (C) 1″*R*-OH-*S*-BF; (D) 1″*S*-OH-*S*-BF. Each value represents the mean of two independent measurements.

Table 3
Kinetic parameters for enantiomeric BF 1″-hydroxylation by recombinant CYP2D6, CYP2D19 and CYP2D30

	K_m (μM)	V_{max} (nmol/min/nmol CYP)
(A) 1″*R*-OH-*R*-BF		
rCYP2D6	5.6	31
rCYP2D19	2.5	53
rCYP2D30	7.7	114
KYU-Ms	1.4 (K_{m1})	0.26 (V_{max1})[a]
	15.7 (K_{m2})	0.24 (V_{max2})[a]
(B) 1″*S*-OH-*R*-BF		
rCYP2D6	5.2	125
rCYP2D19	2.4	44
rCYP2D30	8.2	435
KYU-Ms	1.5 (K_{m1})	0.24 (V_{max1})[a]
	7.8 (K_{m2})	0.07 (V_{max2})[a]
(C) 1″*R*-OH-*S*-BF		
rCYP2D6	4.1	6
rCYP2D19	5.2	13
rCYP2D30	3.8	21
KYU-Ms	1.0 (K_{m1})	0.11 (V_{max1})[a]
	16.4 (K_{m2})	0.08 (V_{max2})[a]
(D) 1″*S*-OH-*S*-BF		
rCYP2D6	4.1	13
rCYP2D19	4.6	11
rCYP2D30	4.0	44
KYU-Ms	0.8 (K_{m1})	0.15 (V_{max1})[a]
	22.4 (K_{m2})	0.14 (V_{max2})[a]

Each value represents the mean of two independent determinations.
[a] nmol/(min mg) protein.

concentration-dependent manner (Fig. 6). However, the extent of the inhibition by the antibody seemed to be higher in KYU-marmoset liver microsomes.

To obtain further information on enantiomeric BF 1″-hydroxylation by KYU-marmoset liver microsomes, inhibition kinetics with quinidine, a known inhibitor of human CYP2D6, was performed and the data analyzed by Eadie–Hofstee plots (Fig. 7). The kinetic parameters are listed in Table 3. The formation of 1″-OH-BF diastereomers showed a biphasic kinetic pattern. Quinidine completely inhibited the formation of all 1″-OH-BF diastereomers at low substrate concentrations (0.5–10 μM). Similar results were obtained for KAU-marmoset liver microsomes (data not shown).

4. Discussion

Recently, marmoset monkeys have been utilized in drug metabolism and toxicology studies because of their ease of handling due to their relatively small body size. Igarashi et al. [8] reported the nucleotide sequence of a marmoset cDNA encoding CYP2D19; however, its catalytic properties have not been characterized. We have also been studying the properties of various CYP2D enzymes in primates [3,11], and recently succeeded in cloning a cDNA encoding CYP2D29 from the Japanese monkey [16]. In order to

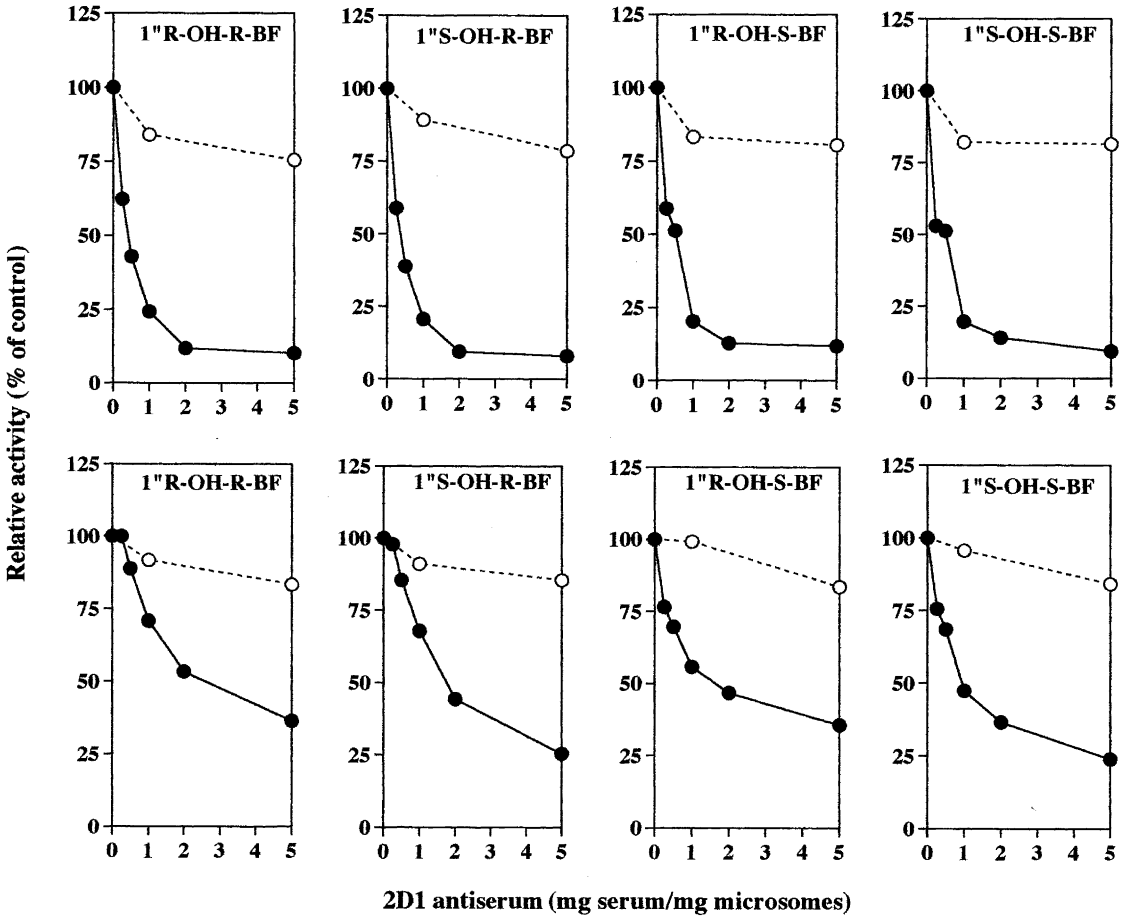

Fig. 6. Inhibitory effects of antibodies raised against CYP2D1 on BF 1″-hydroxylation by microsomal fractions prepared from KYU (upper panels) and KAU (lower panels) livers. Marmoset liver microsomal fractions were preincubated with various amounts of rabbit antiserum raised against rat CYP2D1 at 25 °C for 30 min, and then BF 1″-hydroxylase activities were assayed. Serum obtained from a preimmune-rabbit was added as a control. Closed circles, antiserum added; open circles, preimmune serum added. Each point represents the mean of duplicate determinations.

compare the catalytic properties of CYP2D enzymes of Japanese monkeys and marmosets, in the present study we attempted to clone the cDNA encoding CYP2D19 from marmoset livers using the primer sets employed by Igarashi et al. [8].

Unexpectedly, a cDNA encoding a novel CYP2D enzyme, CYP2D30, was cloned from marmoset livers supplied from the Primate Research Institute of KYU. The deduced amino acid sequence of CYP2D30 showed 94% homology to that of CYP2D19. Repeated attempts to clone a CYP2D19 cDNA from marmoset livers provided by KYU were unsuccessful. Interestingly, however, using the same primer set, we succeeded in cloning a CYP2D19 cDNA from total RNA isolated from a marmoset liver that was supplied from a different source, KAU. The nucleotide sequence of this cloned cDNA from the KAU-marmoset liver was identical to the cDNA encoding CYP2D19 reported by Igarashi et al. [8].

The question arises whether KYU- and KAU-marmosets express only mRNA encoding CYP2D30 and CYP2D19, respectively. By analysis of the cDNA nucleotide sequences, we found that the cDNA encoding CYP2D30, but not CYP2D19, contained a unique *Nde*I-sensitive site. Digestion of the cDNAs with *Nde*I revealed that the KYU-marmoset livers contained mainly mRNA for CYP2D30, while the KAU-marmoset liver contained mainly mRNA for CYP2D19. Analysis of the genomic DNA would reveal whether genes for both *CYP2D19* and *CYP2D30* (and other *CYP2D* genes) coexist in marmoset bred in KYU and KAU.

To the best of our knowledge, this is the first report describing the catalytic properties of marmoset CYP2D19 and CYP2D30 enzymes. The deduced amino acid sequences revealed a total of 33 amino acid residues differences between CYP2D19 and CYP2D30, amounting to the two proteins being 93.4% identical. When the catalytic properties of the enzymes were compared with respect to the hydroxylation of DB, a classical probe substrate of CYP2D6, some remarkable differences were observed between the two marmoset CYP2D enzymes.

CYP2D30, which as described above is 94.6% identical to CYP2D6, hydroxylated DB primarily at the alicyclic 4-position, and also generated modest amounts of the aromatic 5-, 6-, 7-, and 8-monohydroxylated metabolites, almost identical to that observed with human CYP2D6.

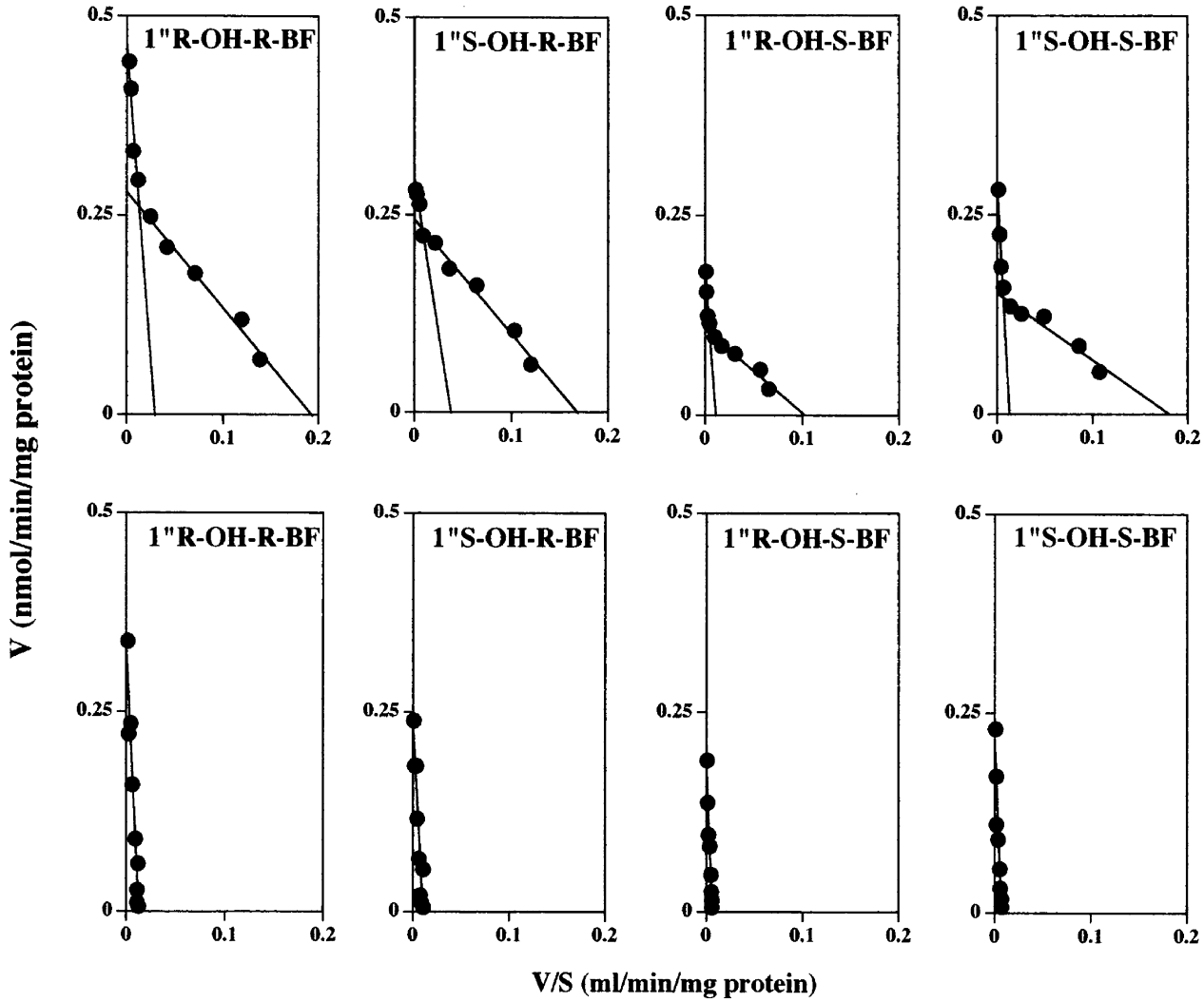

Fig. 7. Inhibitory effects of quinidine on BF 1″-hydroxylation by a liver microsomal fraction prepared from KYU-marmoset liver. Quinidine (a final concentration of 1 μM) was added to the reaction mixture containing KYU-marmoset liver microsomes and NADPH-generating system and varying amounts of the substrate (1–200 μM). Results were analyzed by Eadie–Hofstee plots, and kinetic parameters are listed in Table 3. Upper panels, control without quinidine; lower panels, quinidine added. Each point represents the mean of duplicate determinations.

In contrast, CYP2D19 showed no detectable DB 4-hydroxylase activity, but substantial amounts of the four aromatic hydroxylated products. Hence, it can be postulated that some of the amino acid differences between CYP2D19 and CYP2D30, when compared to CYP2D6, are determinants of the CYP2D enzyme's capacity to 4-OH-DB.

With regards to BF 1″-hydroxylation, a clear difference was observed in product enantioselectivity between the two marmoset CYP2D enzymes. On the basis of V_{max} values, CYP2D30 showed (1″*R*-OH-BF≪1″*S*-OH-BF) product-enantioselectivity, which was similar to that also observed with human CYP2D6, whereas CYP2D19 showed a modest reversed selectivity (1″*R*-OH-BF ≥ 1″*S*-OH-BF). Thus, some additional catalytic properties appear to be determined by the amino acid differences between the two marmoset CYP2D enzymes.

The questions also arise, which of CYP2D19 or CYP2D30 is the major CYP2D enzyme in marmoset livers, and what are their respective functional roles? Western blot analysis indicated that a greater level of immuno-detectable protein, recognized by anti-rat CYP2D1 antibodies, was found in the KAU-marmoset liver compared with that present in the KYU-marmoset liver (Fig. 4). However, more specific antibodies, e.g., peptide specific antibodies, are needed to know their precise contents in KYU- and KAU-marmoset livers.

To further characterize the catalytic properties of CYP2D30, kinetic analyses of BF 1″-hydroxylation by KYU-marmoset liver microsomes in the presence and absence of quinidine, a potent inhibitor of human CYP2D6 [21,22]. BF 1″-hydroxylation was shown to be biphasic in these microsomes, and that the *low*-K_m phase enzyme was inhibited by quinidine.

In preliminary experiments using BF racemate (100 μM), we had confirmed that BF 1″-hydroxylation by recombinant CYP2D19 and CYP2D30 was effectively inhibited by quinidine (1 μM) (unpublished data). Moreover, anti-rat CYP2D1 antibodies suppressed BF 1″-hydroxylation by

approximately 60 and 90% in liver microsomes from KYU- and KAU-marmosets, respectively. These findings and results suggest that CYP2D19 in KAU-marmoset livers and CYP2D30 in KYU-marmoset livers might function as CYP2D-like enzymes. Further detailed studies on the genomic DNA will be necessary to fully understand the expression and regulation of the two CYP2D enzymes in marmoset livers.

In summary, a cDNA encoding the novel CYP2D30 was cloned from the marmoset liver supplied by KYU, whereas a cDNA encoding the known CYP2D19 was cloned from the marmoset liver from KAU. RT-PCR analyses and digestion with *Nde*I demonstrated that the KYU-marmoset liver contained mainly mRNA for CYP2D30 while the KAU-marmoset liver contained mainly mRNA for CYP2D19. The two recombinant enzymes showed different profiles in the hydroxylation of DB. CYP2D19 mainly showed DB 6- and 7-hydroxylase activities followed by 5- and 8-hydroxylase activities but without 4-hydroxylase activity, whereas CYP2D6 and CYP2D30 mainly exhibited DB 4-hydroxylase activities. The two marmoset enzymes showed BF 1″-hydroxylase activities, similar to human CYP2D6, but the product-enantioselectivity in BF 1″-hydroxylation was different between CYP2D30 and CYP2D19. BF 1″-hydroxylation in marmoset liver microsomal fractions from KYU and KAU was effectively inhibited by antibodies and quinidine, both of which were specific for CYP2D6-like enzymes. These results suggest that not only CYP2D19 protein but also CYP2D30 protein is expressed in marmoset livers, although it is still not known whether these enzymes coexist in the same marmoset livers. It is feasible that CYP2D30 in the KYU-marmoset liver as well as CYP2D19 in the KAU-marmoset liver functions as CYP2D6-like enzymes.

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References

[1] Donato MT, Castell JV. Strategies and molecular probes to investigate the role of cytochrome P450 in drug metabolism: focus on in vitro studies. Clin Pharmacokinet 2003;42:153–78.

[2] Sharer JE, Shipley LA, Vandenbranden MR, Binkley SN, Wrighton SA. Comparisons of phase I and phase II in vitro hepatic enzyme activities of human, dog, rhesus monkey, and cynomolgus monkey. Drug Metab Dispos 1995;23:1231–41.

[3] Narimatsu S, Gotoh M, Masubuchi Y, Horie T, Ohmori S, Kitada M, et al. Stereoselectivity in bunitrolol 4-hydroxylation in liver microsomes from marmosets and Japanese monkeys. Biol Pharm Bull 1996;19:1429–33.

[4] Amato G, Longo V, Mazzaccaro A, Gervasi PG. Chlorzoxazone 6-hydroxylase and *p*-nitrophenol hydroxylase as the most suitable activities for assaying cytochrome P450 2E1 in cynomolgus monkey liver. Drug Metab Dispos 1998;26:483–9.

[5] Lake BG, Evans JG, Gray TJ, Korosi SA, North CJ. Comparative studies on nafenopin-induced hepatic peroxisome proliferation in the rat, Syrian hamster, guinea pig, and marmoset. Toxicol Appl Pharmacol 1989;99:148–60.

[6] Edwards RJ, Murray BP, Murray S, Schulz T, Neubert D, Gant TW, et al. Contribution of CYP1A1 and CYP1A2 to the activation of heterocyclic amines in monkeys and human. Carcinogenesis 1994;15:829–36.

[7] Wiltshire HR, Sutton BM, Heeps G, Betty AM, Angus DW, Harris SR, et al. Metabolism of the calcium antagonist, mibefradil (POSICOR, marmoset, cynomolgus monkey and man. Xenobiotica 1997;27:557–71.

[8] Igarashi T, Sakuma T, Isogai M, Nagata R, Kamataki T. Marmoset liver cytochrome P450s: study for expression and molecular cloning of their cDNAs. Arch Biochem Biophys 1997;339:85–91.

[9] Sakuma T, Igarashi T, Hieda M, Ohgiya S, Isogai M, Ninomiya S, et al. Marmoset CYP1A2: primary structure and constitutive expression in livers. Carcinogenesis 1997;18:1985–91.

[10] Schulz TG, Thiel R, Davies DS, Edwards RJ. Identification of CYP2E1 in marmoset monkey. Biochim Biophys Acta 1998;1382:287–94.

[11] Narimatsu S, Kato R, Horie T, Ono S, Tsutsui M, Yabusaki Y, et al. Enantioselectivity of bunitrolol 4-hydroxylation is reversed by the change of an amino acid residue from valine to methionine at position 374 of cytochrome P450-2D6. Chirality 1999;11:1–9.

[12] Tsuzuki D, Takemi C, Yamamoto S, Tamagake K, Imaoka S, Funae Y, et al. Functional evaluation of cytochrome P450 2D6 with $Gly^{42}Arg$ substitution expressed in *Saccharomyces cerevisiae*. Pharmacogenetics 2001;11:709–18.

[13] Tsuzuki D, Hichiya H, Okuda Y, Yamamoto S, Tamagake K, Shinoda S, et al. Alteration in catalytic properties of human CYP2D6 caused by substitution of glycine-42 with arginine, lysine and glutamic acid. Drug Metab Pharmacokin 2003;18:79–85.

[14] Suzuki T, Narimatsu S, Fujita S, Masubuchi Y, Umeda S, Imaoka S, et al. Purification and characterization of a cytochrome P-450 isozyme catalyzing bunitrolol 4-hydroxylation in liver microsomes of male rats. Drug Metab Dispos 1992;20:367–73.

[15] Masubuchi Y, Narimatsu S, Hosakawa S, Suzuki T. Role of the CYP2D subfamily in metabolism-dependent propranolol binding to liver microsomal protein in rats. Biochem Pharmacol 1994;48:1891–8.

[16] Hichiya H, Takemi C, Tsuzuki D, Yamamoto S, Asaoka K, Suzuki S, et al. Complementary DNA cloning and characterization of cytochrome P450 2D29 from Japanese monkey liver. Biochem Pharmacol 2002;64:1101–10.

[17] Narimatsu S, Takemi C, Tsuzuki D, Kataoka H, Yamamoto S, Shimada N, et al. Stereoselective metabolism of bufuralol racemate and enantiomers in human liver microsomes. J Pharmacol Exp Ther 2002;303:172–8.

[18] Wan J, Imaoka S, Chow T, Hiroi T, Yabusaki Y, Funae Y. Expression and four rat CYP2D isoforms in *Saccharomyces cerevisiae* and their catalytic specificity. Arch Biochem Biophys 1997;348:383–90.

[19] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265–75.

[20] Omura T, Sato R. The carbon monoxide-binding pigment of liver, icrosomes. I. Evidence for its hemoprotein nature. J Biol Chem 1964;239:2370–8.

[21] Halpert JR, Guengerich FP, Bend JR, Correia MA. Selective inhibitors of cytochromes P450. Toxicol Appl Pharmacol 1994;125:163–75.

[22] Sai Y, Dai R, Yang TJ, Krausz KW, Gonzalez FJ, Gelboin HV, et al. Assessment of specificity of eight chemical inhibitors using cDNA-expressed cytochromes P450. Xenobiotica 2000;30:327–43.