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Cloning of a cDNA encoding a novel marmoset CYP2C enzyme, expression in yeast cells and characterization of its enzymatic functions

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CYP, cytochrome P450
TB, tolbutamide
PT, paclitaxel
DF, diclofenac
S-MP, S-mephenytoin
G-6-P, glucose 6-phosphate

ABSTRACT

We cloned a cDNA encoding a novel CYP2C enzyme, called P450 M-2C, from a marmoset liver. The deduced amino acid sequence showed high identities to those of human CYP2C8 (87%), CYP2C9 (78%) and CYP2C19 (77%). The P450 M-2C enzyme expressed in yeast cells catalyzed *p*-methylhydroxylation of only tolbutamide among four substrates tested, paclitaxel as a CYP2C8 substrate, diclofenac and tolbutamide as CYP2C9 substrates and S-mephenytoin as a CYP2C19 substrate. *p*-Methylhydroxylation of tolbutamide by marmoset liver microsomes showed monophasic kinetics, and the apparent K_m value (1.2 mM) for the substrate was similar to that of the recombinant P450 M-2C (1.8 mM). Although all of the recombinant human CYP2C8, CYP2C9 and CYP2C19 expressed in yeast cells catalyzed tolbutamide *p*-methylhydroxylation, the kinetic profile of CYP2C8 was most similar to that of P450 M-2C. Tolbutamide oxidation by the marmoset liver microsomes and the recombinant P450 M-2C was inhibited most effectively by quercetin, a CYP2C8 inhibitor, followed by omeprazole, a CYP2C19 inhibitor, whereas sulfaphenazole, a CYP2C9 inhibitor, was less potent under the conditions used. These results indicate that P450 M-2C is the major tolbutamide *p*-methylhydroxylase in the marmoset liver.

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1. Introduction

Several kinds of monkeys such as rhesus monkeys, crab-eating monkeys, Japanese monkeys and marmoset monkeys, have been employed as one of experimental animals in research on drug metabolism and toxicity. The old-world monkeys, including rhesus monkeys, crab-eating monkeys and Japanese monkeys ranging through Africa, Europe and Asia, have disadvantages such as body sizes too big for easy handling and poor fertility. In contrast, marmoset monkeys, belonging to the new-world monkeys ranging through Central and South America are thought to be promising candidates for experimental animals, because of their small size, easy handling and breeding.

Cytochrome P450 (CYP) is a key enzyme for oxidative drug metabolism in mammals including humans and monkeys. CYP constitutes a superfamily and four CYP subfamilies, namely, CYP1, −2, −3 and −4, are mainly responsible for drug metabolism in humans [1–3]. Although CYP enzymes have been extensively characterized in humans and the old-world monkeys, relatively little information is available about the properties of CYP enzymes in marmoset monkeys.

Previous studies provided experimental evidence supporting the notion that pretreatment with chemical compounds such as phenobarbital [4], 3-methylcholanthrene, polychlorinated biphenyl [5], 2,3,7,8-tetrachlorodibenzo-*p*-dioxin [6,7] or isoniazide [8] induced CYP isoenzymes in marmosets. Using targeted anti-peptide antibodies, Schulz et al. [9] suggested the possible expression of CYP1A1, CYP1A2, CYP2A, CYP2B, CYP2C, CYP2E1 and CYP3A21 enzymes in marmosets. Moreover, the research group of Kamataki isolated cDNA clones encoding CYP1A2 [5], CYP2D19 and CYP3A21 [10] from marmoset livers, and characterized the enzymatic properties of CYP1A2 expressed in high-red yeast cells [5]. Recently, we also cloned cDNAs encoding CYP1A2 [11], CYP2D19 and CYP2D30 [12] from fresh marmoset livers, and expressed the proteins in yeast cells to examine their enzymatic functions. However, for prompt utilization of marmoset monkeys as experimental animals in the study of drug metabolism and toxicity, functional characterization of other drug-metabolzing enzymes in this species would be required. In the present study, we have cloned a cDNA encoding a novel CYP2C enzyme from marmoset liver, expressed the protein in yeast cells, and characterized its enzymatic functions.

2. Materials and methods

2.1. Materials

Peclitaxel (PT), tolbutamide (TB), quercetin, sulfaphenazole and omeprazole were purchased from Sigma Chemical Co. (St. Louis, MO); 6α-hydroxypaclitaxel was from Calbiochem (San Diego, CA), docetaxel trihydrate was from Toronto Research Chemicals Inc. (North York, Ontario, Canada); diclofenac (DF), *N*-phenylanthranilic acid, glucose 6-phosphate (G-6-P) dehydrogenase (from yeast) and NADPH were from Wako Pure Chemicals Co. (Osaka, Japan); 4′-hydroxydiclofenac, *S*-mephenytoin (S-MP) and 4′-hydroxymephenytoin were from Daiichi Chemical Co. (Tokyo, Japan); phenobarbital and chlorpropamide were from Tokyo Kasei Kogyo Co. (Tokyo, Japan). *p*-Methylhydroxytolbutamide was supplied from Dr. Takahiko Baba. Pooled human liver microsomes from donors (12 Caucasians and 1 Hispanic, 13 males, 4–62 years old; 9 females, 40–74 years old) were purchased from BD Biosciences Discovery Labware (Bedford, MA). Other chemical reagents or solvents used were of the highest quality commercially available.

2.2. Cloning of cDNA encoding a marmoset CYP2C enzyme

Total RNA was extracted from an adult female marmoset liver (2 years old, supplied from Kagoshima University) using an RNeasy mini kit (Qiagen, Hilden, Germany), and first-strand DNA was synthesized using an RNA PCR kit (Version 3.0, Takara Bio, Ohtsu, Japan) according to the manufacturer's instructions. A full length cDNA encoding a marmoset CYP enzyme was amplified by polymerase chain reaction (PCR) using the forward primer 5′-GTAAGAAGAGAAGTCTTCAATG-3′ and the reverse primer 5′-ATACAAGTGTTACCGAGTATGA-3′. These primers were designed based on the nucleotide sequence in the flanking regions of the crab-eating monkey CYP2C20 cDNA (GenBank accession no. S53046). The reaction mixtures (50 μl) contained 0.2 mM dNTPs, 1 mM $MgSO_4$, 1 U of KOD-plus DNA polymerase (Toyobo, Osaka, Japan) and each oligonucleotide primer at 0.5 μM. PCR consisted of 35 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s and extension at 68 °C for 100 s. The initial denaturation was performed at 94 °C for 120 s. The amplified product (1.5 kbp) was purified with a MinElute gel extraction kit (Qiagen), and the 5′- and 3′-ends of the coding region were sequenced in both the forward and reverse directions using ABI BigDye terminator cycle sequencing reaction kit v3.1 (Applied Biosystems, Piscaway, NJ).

The full-length cDNA thus obtained was modified by PCR amplification with 5′-AAGCTTAAAAAAATGGATCCTTTTGT-GGTCC-3′ and 5′-AAGCTTCAGACAGGAATGAAGCAGATC-TG-3′ as primers under the conditions described above. *Hin*dIII sites (marked with solid lines) were introduced to the 5′-end of the start codon and the 3′-end of the stop codon to facilitate subcloning into the yeast expression vector (pGYR1). A Kozak sequence (marked in italics) was also introduced just upstream of the start codon to achieve high expression of the protein in yeast cells. The PCR products were ligated into pGEM-T (Promega, Madison, WI) using the TA cloning system, and the insert was sequenced in both the forward and reverse directions. The DNA fragment encoding a marmoset CYP2C (tentatively called P450 M-2C) was cut out with *Hin*dIII from the cloned pGEM-T and was subsequently subcloned into pGYR1 digested with the same 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 CYP2C8, CYP2C9 and CYP2C19 cDNAs was described previously [13].

2.3. Expression of CYP2C enzymes

Saccharomyces cerevisiae AH22 was transformed with pGYR1 containing each of CYP cDNAs by the lithium acetate method, and the cultivation of yeast transformants thus obtained was performed as described [14]. A microsomal fraction was

prepared from yeast cells by the method previously reported [15].

2.4. Assays of M-2C holo- and apoproteins

The microsomal fraction prepared as above was diluted to a protein concentration of 10 mg/ml with 100 mM potassium phosphate buffer (pH 7.4) containing 20% (v/v) glycerol, and the total holo-CYP content was measured spectrophotometrically according to the method of Omura and Sato [16] using 91 mM^{-1} cm^{-1} as the absorption coefficient.

Marmoset liver microsomes were also prepared according to a published method [17]. Appropriate portions of the microsomal fractions of yeast cells, marmoset livers and pooled human livers were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a 10% slab gel. Following the electrophosresis, proteins on the gel were electroblotted to a polyvinylidene fluoride membrane, and were analyzed by Western blotting according to the method of Guengerich et al. [18] using rabbit anti-human CYP2C19 polyclonal antibody as a primary antibody (Daiichi Chemical Co.) and peroxidase-goat-anti-rabbit IgG (H + L) as a secondary antibody (Daiichi Chemical Co.).

2.5. Enzyme assay

PT 6α-hydroxylase activity in microsomal fractions from yeast cells expressing P450 M-2C or CYP2C8 was determined by the method of Soyama et al. [19] with a slight modification. Briefly, an ice-cold reaction mixture (500 μl) in a conical glass tube (10 ml) with a stopper contained 5 mM G-6-P, 1 IU of G-6-P dehydrogenase, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM NADPH and PT (2.5, 5 and 10 μM). After preincubation at 37 °C for 5 min, the reaction was started by adding the microsomal fraction (20 pmol CYP) and was performed at 37 °C for 10 min. After the reaction was stopped by adding 3 ml of ethyl acetate and vortex mixing, 10 nmol of docetaxel was added as an internal standard, and the mixture was shaken at room temperature for 10 min. The mixture was then centrifuged at 1200 × *g* for 15 min, and 2 ml of the organic layer was taken, and evaporated *in vacuo*. The residue was dissolved in 200 μl of methanol/water (1:1, v/v), and an aliquot (10 μl) was subjected to HPLC under the conditions described below.

DF 4′-hydroxylase activity in microsomal fractions from yeast cells expressing M-2C or CYP2C9 was determined by the method of Schmitz et al. [20] with a slight modification. Briefly, a reaction mixture containing the same components described above except for the substrate (5, 20 or 100 μM DF instead of PT) was preincubated at 37 °C for 5 min, and the reaction was started by adding the microsomal fraction (20 pmol CYP) and was stopped 5 min later by adding 20 μl of 2 M phosphoric acid and vortexing. Then, 3 ml of *t*-butylmethylether and 0.8 nmol of *N*-phenylanthranilic acid as an internal standard were added, shaken vigorously, and centrifuged at 1200 × *g* for 15 min. The organic layer (2 ml) was taken, and evaporated *in vacuo*, and the residue was dissolved in 200 μl of methanol/water (1:1, v/v). An aliquot (10 μl) was subjected to HPLC under the conditions described below.

TB *p*-methylhydroxylase activities in microsomal fractions from yeast cells expressing P450 M-2C, CYP2C8, CYP2C9 or CYP2C19 and in pooled human liver microsomes were determined by the method of Komatsu et al. [21] with a slight modification. Briefly, a reaction mixture containing the same components described for PT 6α-hydroxylation except for the substrate (0.25, 1 or 2.5 μM TB instead of PT) was preincubated at 37 °C for 5 min, and the reaction was started by adding the microsomal fraction (20 pmol of recombinant CYP or 1 mg of human liver microsomes) and was stopped 10 min later for the recombinant enzymes and 40 min later for the human liver microsomes by adding 3 ml of ethyl acetate and vortexing. Then, 1.5 μg of chlorpropamide was added as an internal standard, and the mixture was shaken vigorously, and centrifuged at 1200 × *g* for 15 min. The organic layer (2 ml) was taken, and evaporated *in vacuo*, and the residue was dissolved in 200 μl of methanol/water (1:1, v/v). An aliquot (10 μl) was subjected to HPLC under the conditions described below.

S-MP 4′-hydroxylase activity in microsomal fractions from yeast cells expressing P450 M-2C or CYP2C19 was determined by the method of Nakajima et al. [22] with a slight modification. Briefly, a reaction mixture containing the same components described for PT 6α-hydroxylation except for the substrate (10, 50 or 200 μM S-MP instead of PT) was preincubated at 37 °C for 5 min, and the reaction was started by adding the microsomal fraction (20 pmol CYP) and was stopped 5 min later by adding 3 ml of dichloromethane and vortexing. Then 4 nmol of phenobarbital was added as an internal standard, and the mixture was shaken vigorously, and centrifuged at 1200 × *g* for 15 min. The organic layer (2 ml) was taken, and evaporated *in vacuo*, and the residue was dissolved in 200 μl of CH_3OH/water (1:1, v/v). An aliquot (10 μl) was subjected to HPLC under the conditions described below.

The HPLC conditions were: a Hitachi 655A-12 liquid chromatograph equipped with an L-5000 LC controller, a 655A variable wavelength UV monitor, a Rheodyne model 7125 injector and a Shimadzu C-R3A Chromatopac data processor; column, Inertsil ODS 80A (4.6 mm × 150 mm, GL Science Co., Tokyo, Japan) at 40 °C; mobile phase, water/CH_3CN/CH_3OH (52:34:14, v/v) at a flow rate of 1.2 ml/min for PT 6α-hydroxylation (detection, 230 nm), 30 mM potassium phosphate buffer (pH 6.5)/CH_3CN/CH_3OH (64:16:20, v/v) at a flow rate of 1.2 ml/min for DF 4′-hydroxylation (detection, 280 nm), 20 mM potassium phosphate buffer (pH 4)/CH_3CN/CH_3OH (77:6:17, v/v) at a flow rate of 1.0 ml/min for S-MP 4′-hydroxylation (detection, 204 nm), and 0.05% phosphoric acid/CH_3CN (72:28, v/v) at a flow rate of 1.0 ml/min for TB *p*-methylhydroxylation (detection, 230 nm). For each enzyme assay, calibration curves were made by adding various amounts of synthetic metabolites to ice-cold reaction mixtures containing the same components described above. Intra- and inter-day variation coefficients did not exceed 10% in any assay.

2.6. Kinetic and inhibition studies

Kinetic studies for TB *p*-methylhydroxylation were performed using substrate concentration ranges of 0.1–10 mM for P450 M-2C, 0.1–7.5 mM for CYP2C8, 0.025–2.5 mM for CYP2C9 and human liver microsomes, 0.05–5 mM for CYP2C19, and 0.01–5 mM for marmoset liver microsomes. Apparent Michaelis–Menten constants (K_m) and maximal velocities (V_{max}) were

```
   1  ATG GAT CCT TTT GTG GTC CTG TTG CTC TGT CTC TCT TTT TTG CTT CTC TTT TCA CTC TGG   60
   1  Met Asp Pro Phe Val Val Leu Leu Leu Cys Leu Ser Phe Leu Leu Leu Phe Ser Leu Trp   20

  61  AGA CAG AGC TCT GGG AGA GGG AAG CTC CCT CCT GGC CCC ACT CCT CTT CCT ATT ATT GGA   120
  21  Arg Gln Ser Ser Gly Arg Gly Lys Leu Pro Pro Gly Pro Thr Pro Leu Pro Ile Ile Gly   40

 121  AAC ATC CTA CAG ATA AGT GTT AAG GAC ATC GGC AAA TCT TTC AGC AAT CTC TCA AAA GTC   180
  41  Asn Ile Leu Gln Ile Ser Val Lys Asp Ile Gly Lys Ser Phe Ser Asn Leu Ser Lys Val   60

 181  TAT GGT CCT CTG TTC ACC GTG TAT TTT GGC ACG AAG CCC GTA GTG GTG TTG CAC GGA TAT   240
  61  Tyr Gly Pro Leu Phe Thr Val Tyr Phe Gly Thr Lys Pro Val Val Val Leu His Gly Tyr   80

 241  GAG GCA GTA AAG GAA GCC CTG ATT GAT AAT GGA GAG GAG TTT TCT GGA AGA AGC ATT TTC   300
  81  Glu Ala Val Lys Glu Ala Leu Ile Asp Asn Gly Glu Glu Phe Ser Gly Arg Ser Ile Phe   100

 301  CCA GTA TCT CAA AGA ACT TCT AAA GAT CTT GGA ATC ATT TCC AGC AAT GGA AAG AGA TGG   360
 101  Pro Val Ser Gln Arg Thr Ser Lys Asp Leu Gly Ile Ile Ser Ser Asn Gly Lys Arg Trp   120

 361  AAG GAG ATC CGG CGT TTC TCC CTT ACA ACA TTG CGG AAT TTT GGG ATG GGG AAG AGG AGC   420
 121  Lys Glu Ile Arg Arg Phe Ser Leu Thr Thr Leu Arg Asn Phe Gly Met Gly Lys Arg Ser   140

 421  ATT GAG GAC CGT GTT CAA CAA GAA GCC CGC TGC CTT GTG GAG GAG TTG AGA AAA ACC AAG   480
 141  Ile Glu Asp Arg Val Gln Gln Glu Ala Arg Cys Leu Val Glu Glu Leu Arg Lys Thr Lys   160

 481  GCC TCA CCC TGT GAT CCC ACT TTC ATC CTG GGC TGT GCT CCC TGC AAT GTG ATC TGC TCC   540
 161  Ala Ser Pro Cys Asp Pro Thr Phe Ile Leu Gly Cys Ala Pro Cys Asn Val Ile Cys Ser   180

 541  GTT GTT TTC CAG AAT CGA TTT GAT TAT AAA GAT GAA AAT TTT CTC ACC CTG ATG AAA AGG   600
 181  Val Val Phe Gln Asn Arg Phe Asp Tyr Lys Asp Glu Asn Phe Leu Thr Leu Met Lys Arg   200

 601  TTC AAT GAA AAC TTC AAG ATT CTG AGC TCT CCA TGG ATC CAG TTC TGC AAT AAT TTC CCT   660
 201  Phe Asn Glu Asn Phe Lys Ile Leu Ser Ser Pro Trp Ile Gln Phe Cys Asn Asn Phe Pro   220

 661  CTC CTC ATG GAT TAT TTC CCA GGA CCT CAC AAC AAA TTG TTT AAA AAT GTT GCT CTT ACA   720
 221  Leu Leu Met Asp Tyr Phe Pro Gly Pro His Asn Lys Leu Phe Lys Asn Val Ala Leu Thr   240

 721  AAA AGC TAT ATT TGG GAG AAA ATA AAA GAA CAC CAA GCA TCA CTG GAT GTT AAC AAT CCT   780
 241  Lys Ser Tyr Ile Trp Glu Lys Ile Lys Glu His Gln Ala Ser Leu Asp Val Asn Asn Pro   260

 781  CGG GAC TTT ATC GAT TGC TTT CTG ATC AAA ATG CAG CAG GAA AAG GAC AAC CAA GAG TCT   840
 261  Arg Asp Phe Ile Asp Cys Phe Leu Ile Lys Met Gln Gln Glu Lys Asp Asn Gln Glu Ser   280

 841  GAA TTC ACT ATT GAA AGC TTG GTT GGC ACT GTA GCT GAT CTA TTT GTT GCT GGA ACA GAG   900
 281  Glu Phe Thr Ile Glu Ser Leu Val Gly Thr Val Ala Asp Leu Phe Val Ala Gly Thr Glu   300

 901  ACA ACA AGC ACC ACT CTG AGA TAT GGA CTC CTA CTC CTG CTG AAG CAC CCA GAG GTC ACA   960
 301  Thr Thr Ser Thr Thr Leu Arg Tyr Gly Leu Leu Leu Leu Leu Lys His Pro Glu Val Thr   320

 961  GCT AAA GTC CAG GAA GAG ATT GAT CAT GTA ATT GGC AGA CAC AGG AGC CCC TGC ATG CAG  1020
 321  Ala Lys Val Gln Glu Glu Ile Asp His Val Ile Gly Arg His Arg Ser Pro Cys Met Gln   340

1021  GAT AGG AGC CAC ATG CCT TAT ACA GAT GCT GTC ATG CAC GAG ATC CAG AGA TAC ATT GAC  1080
 341  Asp Arg Ser His Met Pro Tyr Thr Asp Ala Val Met His Glu Ile Gln Arg Tyr Ile Asp   360

1081  CTT GTC CCC ACC AGT GTG CCC CAT GCA GTG ACC ACT GAC ATT AAG TTC AGA AAT TAC CTC  1140
 361  Leu Val Pro Thr Ser Val Pro His Ala Val Thr Thr Asp Ile Lys Phe Arg Asn Tyr Leu   380

1141  ATC CCC AAG GGC ACA GCC ATA ATG ACA TCA CTG ACT TCA GTG CTG CAC AGT GAC AAA GAA  1200
 381  Ile Pro Lys Gly Thr Ala Ile Met Thr Ser Leu Thr Ser Val Leu His Ser Asp Lys Glu   400

1201  TTT CCC AAT CCA AAG ACC TTT GAC CCT GGC CAC TTT CTG GAT AAA AAT GGC AAC TTT AAG  1260
 401  Phe Pro Asn Pro Lys Thr Phe Asp Pro Gly His Phe Leu Asp Lys Asn Gly Asn Phe Lys   420

1261  AAA AGT GAC CAC TTC ATG CCT TTC TCA GCA GGG AAA CGA ATT TGT GCT GGA GAG GGA CTC  1320
 421  Lys Ser Asp His Phe Met Pro Phe Ser Ala Gly Lys Arg Ile Cys Ala Gly Glu Gly Leu   440

1321  GCC CGC ATG GAG ATA TTT TTA TTC CTA ACC ACA ATT TTA CAG AAC TTT AAT CTG AAA TCT  1380
 441  Ala Arg Met Glu Ile Phe Leu Phe Leu Thr Thr Ile Leu Gln Asn Phe Asn Leu Lys Ser   460

1381  GTT GGC GAT ATA AAG AAC CTC AAT ACT ACT TCA GCT AGC AAA TCA ATT GTT TCT TTG CCA  1440
 461  Val Gly Asp Ile Lys Asn Leu Asn Thr Thr Ser Ala Ser Lys Ser Ile Val Ser Leu Pro   480

1441  CCC CCG TAC CAG ATC TGC TTC ATT CCT GTC TGA  1473
 481  Pro Pro Tyr Gln Ile Cys Phe Ile Pro Val End
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Fig. 1 – Nucleotide and deduced amino acid sequences of marmoset P450 M-2C. The numbers of the amino acids and nucleotides are shown in upper and lower lines, respectively.

```
M-2C        1  MDPFVVLLLCLSFLLLFSLWRQSSGRGKLPPGPTPLPIIGNILQISVKDIGKSFSNLSKV  60
CYP2C8      1  MEPFVVLVLCLSFMLLFSLWRQSCRRRKLPPGPTPLPIIGNMLQIDVKDICKSFTNFSKV  60
CYP2C9      1  MDSLVVLVLCLSCLLLLSLWRQSSGRGKLPPGPTPLPVIGNILQIGIKDISKSLTNLSKV  60
CYP2C19    61  MDPFVVLVLCLSCLLLLSIWRQSSGRGKLPPGPTPLPVIGNILQIDIKDVSKSLTNLSKI  60
               *   *** ****  ** * ****  * ********** *** ***  **  **  * **

                                                          SRS-1
M-2C       61  YGPLFTVYFGTKPVVVLHGYEAVKEALIDNGEEFSGRSIFPVSQRTSKDLGIISSNGKRW  120
CYP2C8     61  YGPVFTVYFGMNPIVVFHGYEAVKEALIDNGEEFSGRGNSPISQRITKGLGIISSNGKRW  120
CYP2C9     61  YGPVFTLYFGLKPIVVLHGYEAVKEALIDLGEEFSGRGIFPLAERANRGFGIVFSNGKKW  120
CYP2C19    61  YGPVFTLYFGLERMVVLHGYEVVKEALIDLGEEFSGRGHFPLAERANRGFGIVFSNGKRW  120
               *** ** ***    ** **** ******* *******   *   *     **  **** *

M-2C      121  KEIRRFSLTTLRNFGMGKRSIEDRVQQEARCLVEELRKTKASPCDPTFILGCAPCNVICS  180
CYP2C8    121  KEIRRFSLTNLRNFGMGKRSIEDRVQEEAHCLVEELRKTKASPCDPTFILGCAPCNVICS  180
CYP2C9    121  KEIRRFSLMTLRNFGMGKRSIEDRVQEEARCLVEELRKTKASPCDPTFILGCAPCNVICS  180
CYP2C19   121  KEIRRFSLMTLRNFGMGKRSIEDRVQEEARCLVEELRKTKASPCDPTFILGCAPCNVICS  180
               ********  ***************** ** ****************************

                          SRS-2                                SRS-3
M-2C      181  VVFQNRFDYKDENFLTLMKRFNENFKILSSPWIQFCNNFPLLMDYFPGPHNKLFKNVALT  240
CYP2C8    181  VVFQKRFDYKDQNFLTLMKRFNENFRILNSPWIQVCNNFPLLIDCFPGTHNKVLKNVALT  240
CYP2C9    181  IIFHKRFDYKDQQFLNLMEKLNENIKILSSPWIQICNNFSPIIDYFPGTHNKLLKNVAFM  240
CYP2C19   181  IIFQKRFDYKDQQFLNLMEKLNENIRIVSTPWIQICNNFPTIIDYFPGTHNKLLKNLAFM  240
                 *  ******  ** **   ***  *   **** ****    * *** ***  ** *

                                                          SRS-4
M-2C      241  KSYIWEKIKEHQASLDVNNPRDFIDCFLIKMQQEKDNQESEFTIESLVGTVADLFVAGTE  300
CYP2C8    241  RSYIREKVKEHQASLDVNNPRDFMDCFLIKMEQEKDNQKSEFNIENLVGTVADLFVAGTE  300
CYP2C9    241  KSYILEKVKEHQESMDMNNPQDFIDCFLMKMEKEKHNQPSEFTIESLENTAVDLFGAGTE  300
CYP2C19   241  ESDILEKVKEHQESMDINNPRDFIDCFLIKMEKEKQNQQSEFTIENLVITAADLLGAGTE  300
                * * ** **** * * *** ** **** **  ** ** *** ** *  *  **  ****

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M-2C      301  TTSTTLRYGLLLLLKHPEVTAKVQEEIDHVIGRHRSPCMQDRSHMPYTDAVMHEIQRYID  360
CYP2C8    301  TTSTTLRYGLLLLLKHPEVTAKVQEEIDHVIGRHRSPCMQDRSHMPYTDAVVHEIQRYSD  360
CYP2C9    301  TTSTTLRYALLLLLKHPEVTAKVQEEIERVIGRNRSPCMQDRSHMPYTDAVVHEVQRYID  360
CYP2C19   301  TTSTTLRYALLLLLKHPEVTAKVQEEIERVIGRNRSPCMQDRGHMPYTDAVVHEVQRYID  360
               ******** ******************  **** ******** ******** ** *** *

               SRS-5
M-2C      361  LVPTSVPHAVTTDIKFRNYLIPKGTAIMTSLTSVLHSDKEFPNPKTFDPGHFLDKNGNFK  420
CYP2C8    361  LVPTGVPHAVTTDTKFRNYLIPKGTTIMALLTSVLHDDKEFPNPNIFDPGHFLDKNGNFK  420
CYP2C9    361  LLPTSLPHAVTCDIKFRNYLIPKGTTILISLTSVLHDNKEFPNPEMFDPHHFLDEGGNFK  420
CYP2C19   361  LIPTSLPHAVTCDVKFRNYLIPKGTTILTSLTSVLHDNKEFPNPEMFDPRHFLDEGGNFK  420
               * **  ***** * ********** *   ******  ******  *** ****  ****

                                                          SRS-6
M-2C      421  KSDHFMPFSAGKRICAGEGLARMEIFLFLTTILQNFNLKSVGDIKNLNTTSASKSIVSLP  480
CYP2C8    421  KSDYFMPFSAGKRICAGEGLARMELFLFLTTILQNFNLKSVDDLKNLNTTAVTKGIVSLP  480
CYP2C9    421  KSKYFMPFSAGKRICVGEALAGMELFLFLTSILQNFNLKSLVDPKNLDTTPVVNGFASVP  480
CYP2C19   421  KSNYFMPFSAGKRICVGEGLARMELFLFLTFILQNFNLKSLIDPKDLDTTPVVNGFASVP  480
               **  ********** ** ** ** ***** *********  * * * **       * *

M-2C      481  PPYQICFIPV  491
CYP2C8    481  PSYQICFIPV  491
CYP2C9    481  PFYQLCFIPV  491
CYP2C19   481  PFYQLCFIPV  491
               * ** *****
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Fig. 2 – Multiple alignment of the amino acid sequences of P450 M-2C, human CYP2C8, CYP2C9 and CYP2C19. *Amino acid residues conserved among the four CYP2C enzymes. Six substrate recognition sites (SRSs) are shown with lines.

analyzed on the basis of Michaelis–Menten plots or Eadie–Hofstee plots using Prism Version 4 (Graphpad Software, San Diego, CA). Inhibition experiments using quercetin (10, 50 and 200 μM), sulfaphenazole (20, 50 and 200 μM) and omeprazole (50, 200 and 500 μM) and substrate (TB) concentrations of 0.1 and 1 mM were performed for marmoset liver microsomes and yeast cell microsomes expressing P450 M-2C. Each inhibitor was dissolved in a mixture of methanol/dimethylsulfoxide (1:1, v/v) and the final concentration of the organic solvent in the reaction mixture was less than 1%. Control experiments were run with the vehicle only instead of the inhibitors. IC_{50} values were analyzed using Prism. Protein concentrations were measured by the method of Lowry et al. [23] using bovine plasma albumin as a standard.

2.7. *Molecular modeling*

The homology model of P450 M-2C was constructed by Swiss-Model (http://swissmodel.expasy.org/) using the crystallo-

Table 1 – Identities of the nucleotide and deduced amino acid sequences of eight CYP2C enzymes in primates

	M-2C	CYP2C8	CYP2C9	CYP2C19	CYP2C20	CYP2C43	CYP2C74	CYP2C75
M-2C		92.7	84.5	83.8	93.1	83.3	93.1	83.6
CYP2C8	87.1		84.7	84.9	95.6	83.8	95.5	84.5
CYP2C9	78.4	77.6		94.8	84.5	95.2	84.5	95.9
CYP2C19	77.1	77.8	91.4		84.7	93.8	84.6	94.8
CYP2C20	88.8	91.6	78.2	78.6		83.2	99.6	84.3
CYP2C43	76.7	77.1	92.2	90.0	77.1		83.1	95.1
CYP2C74	89.0	91.6	78.2	78.4	99.4	76.9		84.2
CYP2C75	76.5	76.7	93.9	92.0	77.1	93.5	76.9	

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

graphic data of CYP2C8 (1PQ2) obtained from Protein Data Bank (http://www.rcsb.org/pdb/) and the primary amino acid sequence of P450 M-2C determined in this work. Hydrogen atoms were further added for the P450 M-2C homology model using the Biopolymer module of Insight II software package (Molecular Simulations Inc., San Diego, CA). Six peptides of P450 M-2C (Arg-97 to Asn-116, Met-198 to Ser-209, Phe-234 to Leu-239, Gly-289 to Ser-303, Ile-359 to His-368, and Thr-469 to Ser-478) were extracted as substrate recognition sites (SRSs). The active-site cavities of CYP2C8 and P450 M-2C were made manually above the sixth ligand of heme at 1.0 Å resolution using a homemade CG program working on Windows PC. The amino acid residues at the active sites of CYP2C8 and P450 M-2C were drawn using RasMol Version 2.6-ucb-1.0 as described elsewhere [24].

3. Results

3.1. *Sequence analysis*

As shown in Fig. 1, the cloned cDNA consisted of 1473 base pairs starting with an initiation codon ATG and ending with a termination codon TGA. Fig. 2 depicts a comparison of deduced amino acid sequences of P450 M-2C, human CYP2C8, CYP2C9 and CYP2C19. The nucleotide and amino acid sequences are compared with those of human and monkey P450s belonging to the CYP2C subfamily in Table 1. The nucleotide sequence of the cDNA encoding marmoset P450 M-2C showed 92.7, 84.5, 83.8, 93.1, 83.3, 93.1 and 83.6% identities to human CYP2C8 (GenBank accession no. NM-000770), CYP2C9 (NM-000771), CYP2C19 (NM-000769), crab-eating mon-

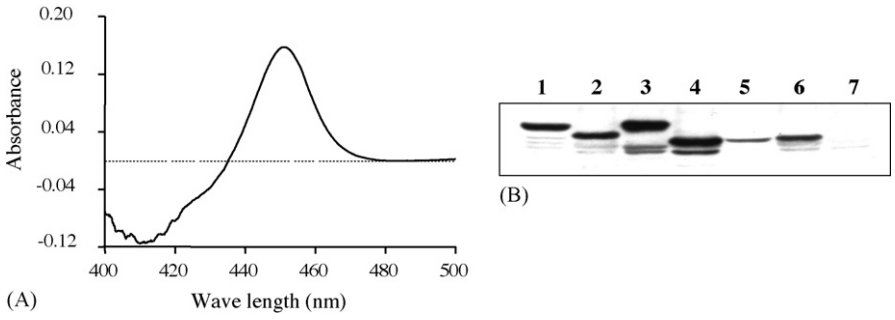

Fig. 3 – A reduced CO-difference spectrum of yeast cell microsomes expressing marmoset P450 M-2C (A) and Western blot analysis of microsomal fractions from human and marmoset livers and of yeast cells expressing P450 M-2C and human CYP2C enzymes (B). (A) The protein concentration used was 10 mg/ml. (B) Lane 1, human liver microsomes; lane 2, yeast cell microsomes expressing human CYP2C8; lane 3, yeast cell microsomes expressing human CYP2C9; lane 4, yeast cell microsomes expressing human CYP2C19; lane 5, yeast cell microsomes expressing marmoset P450 M-2C; lane 6, marmoset liver microsomes; lane 7, mock. The amounts of microsomal proteins used were 30 μg for human and marmoset livers and 15 μg for yeast cells expressing P450 M-2C and human CYP2C enzymes.

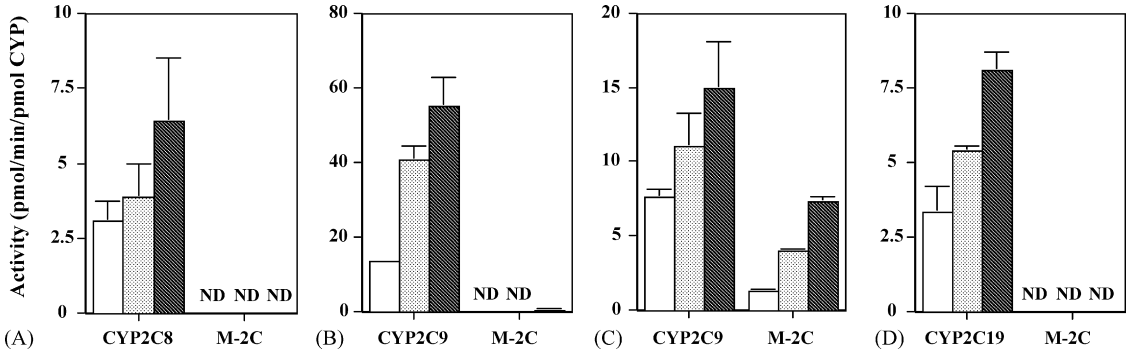

Fig. 4 – Comparison of various drug oxidation activities between P450 M-2C and human CYP2C enzymes. (A) PT (2.5, 5 and 10 μM) 6α-hydroxylation, (B) DF (5, 20 and 100 μM) 4′-hydroxylation, (C) TB (0.25, 1 and 2.5 mM) *p*-methylhydroxylation and (D) S-MP (10, 50 and 200 μM) 4′-hydroxylation. Open, dotted and hatched columns show the lowest, intermediate and highest concentrations, respectively. Each value represents the mean ± S.D. of three independent determinations. ND, not detectable.

key CYP2C20 (S53046), rhesus monkey CYP2C43 (AB212264), CYP2C74 (AY635462) and CYP2C75 (AY635463), respectively. The deduced amino acid sequence of P450 M-2C was highly identical to those of human CYP2C8 (87.1% identity), crab-eating monkey CYP2C20 (88.8%) and rhesus monkey CYP2C74 (89.0%).

3.2. *Expression of marmoset P450-M-2C protein in yeast cells*

The microsomal fraction was prepared from yeast cells expressing P450 M-2C, and the content of the recombinant holoenzyme was determined by reduced CO-difference spectroscopy (Fig. 3, left panel). The spectrum showed a Soret peak at 450 nm and a negligible level of peak at 420 nm. The content of P450 M-2C was calculated to be 133 pmol/mg protein (the mean value of two independent determinations). In Western blot analysis using polyclonal antibodies raised against human CYP2C19 (Fig. 3, right panel), microsomal fractions from yeast cells expressing P450 M-2C (lane no. 5) and from the marmoset liver (lane no. 6) exhibited a single protein band with a molecular weight similar to that of recombinant CYP2C19 (lane no. 4). In contrast, the pooled human liver microsomal fraction (lane no. 1) showed a major protein band whose molecular weight was similar to that of recombinant CYP2C9 (lane no. 3) and additional three faint protein bands, two of which exhibited similar mobilities to recombinant CYP2C8 (lane no. 2) and CYP2C19 (lane no. 4).

3.3. *Drug oxidation activities*

The recombinant P450 M-2C did not show any detectable oxidation activities towards PT or S-MP under the conditions used (Fig. 4A and D). A slight activity was observed for DF 4′-hydroxylation by P450 M-2C at the highest substrate concentration used (100 μM) (Fig. 4B). P450 M-2C exerted considerable TB *p*-methylhydroxylase activities, which were 20–50% those of CYP2C9 at substrate concentrations from 0.25 to 2.5 mM (Fig. 4C). Based on these results, we performed kinetic studies for TB *p*-methylhydroxylation by P450 M-2C and compared the results with those of human CYP2C8, CYP2C9 and CYP2C19.

TB *p*-methylhydroxylation by four recombinant CYP enzymes showed monophasic kinetics in Michaelis–Menten plots (data not shown). The kinetic parameters obtained are summarized in Table 2. The recombinant CYP enzymes could be divided into two groups, *i.e.*, high-K_m group (P450 M-2C and CYP2C8) and low-K_m group (CYP2C9 and CYP2C19).

TB *p*-methylhydroxylation by microsomal fractions from human and marmoset livers was analyzed by Eadie–Hofstee plots (data not shown). In human liver, microsomal TB

Table 2 – Kinetic parameters for TB *p*-methylhydroxylation by microsomal fractions from yeast cells expressing marmoset and human CYP enzymes and from human and marmoset livers

Enzyme source	K_m (μM)	V_{max}	V_{max}/K_m
Recombinant enzyme[a]			
P450 M-2C	1780	11.8	0.0066
CYP2C8	1520	2.5	0.0017
CYP2C9	335	16.2	0.048
CYP2C19	649	32.4	0.050
Liver microsomal fraction[b]			
HLM	318 (K_{m1})	185 (V_{max1})	0.582 (V_{max1}/K_{m1})
	72.7 (K_{m2})	246 (V_{max2})	3.38 (V_{max2}/K_{m2})
MLM	1170	470	0.402

[a] V_{max}, pmol/min/pmol CYP; V_{max}/K_m, μl/min/pmol CYP.
[b] V_{max}, pmol/min/mg protein; V_{max}/K_m, μl/min/mg protein. HLM, human liver microsomes; MLM, marmoset liver microsomes. Each value represents the mean of two independent determinations.

Table 3 – The IC_{50} values for inhibitors of TB *p*-methylhydroxylation by mormoset liver microsomes and recombinant P450 M-2C

Inhibitor	Marmoset liver microsomes		Recombinant P450 M-2C	
	0.1 mM[a]	1 mM[a]	1 mM[a]	2 mM[a]
Quercetin	51.4	16.1	105	61.2
Sulfaphenazole	>200	>200	>200	>200
Omeprazole	146	247	328	274

[a] Substrate concentration. IC_{50} values are expressed as μM.

p-methylhydroxylation showing biphasic kinetics, the higher K_m value (K_{m1}, 320 μM) was close to that of CYP2C9 (340 μM), while the lower K_m value (K_{m2}, 70 μM) was much smaller than any K_m values of the recombinant CYP enzymes examined (Table 2). This indicates that together with CYP2C9, another CYP enzyme having a lower K_m value is also involved in TB *p*-methylhydroxylation by the pooled human liver microsomal fractions employed. On the other hand, in marmoset liver microsomal TB oxidation showing monophasic kinetics (data not shown), the K_m value (1.2 mM) was close to that of P450 M-2C (1.8 mM) (Table 2), suggesting that P450 M-2C is the major TB *p*-methylhydroxylase in the mormoset liver.

3.4. *Inhibition studies*

The effects of three kinds of inhibitors, quercetin as a CYP2C8 inhibitor [25], sulfaphenazole as a CYP2C9 inhibitor [26] and omeprazole as a CYP2C19 inhibitor [27], on TB *p*-methylhydroxylation by microsomal fractions from mormoset liver (Fig. 5, upper panels) and yeast cells expressing P450 M-2C (Fig. 5, lower panels) were examined using two substrate concentrations of 0.1 and 1 mM. Quercetin (Fig. 5A and D) and omeprazole (Fig. 5C and F) similarly inhibited the TB oxidation activity of marmoset liver microsomes and recombinant P450 M-2C in a concentration-dependent manner. The potency of sulfaphenazole was lower than those of the other inhibitors (Fig. 5B and E). Table 3 lists the IC_{50} values for the inhibitors. The potencies of the inhibitors were ranked as quercetin > omeprazole > sulfaphenazole for both marmoset liver microsomes and recombinant P450 M-2C.

4. Discussion

In the present study, we have cloned a cDNA encoding a novel CYP enzyme from the fresh liver of an adult female marmoset. The deduced amino acid sequence exhibited high identities to human CYP2C8 (87%), crab-eating monkey CYP2C20 (89%) and rhesus monkey CYP2C74 (89%). The nucleotide and amino acid sequences were registered to GenBank (accession no. AB242600). Dr. David Nelson, University of Tennessee Menphis, recommended us to call this CYP "marmoset CYP2C8" (his personal communication). In this paper, however, we tentatively called the enzyme P450 M-2C, standing for the Marmoset CYP2C enzyme to avoid confusion with human CYP2C8.

According to the list of P450 families and subfamilies of Dr. Nelson's home page (http://drnelson.utmem.edu/P450.stats.all.2005.htm), four monkey cDNA sequences encoding CYP2C enzymes had been registered as of January 8, 2005: crab-eating monkey CYP2C20 (S53046), rhesus monkey CYP2C43 (AB212264), CYP2C74 (AY635462) and CYP2C75 (AY635463). The functions of these monkey CYP enzymes have not been studied in detail, except for CYP2C43.

Matsunaga et al. [28] cloned a cDNA encoding CYP2C43 and characterized the enzymatic properties of CYP2C43 protein expressed in yeast cells. They reported that the recombinant CYP2C43 catalyzed S-MP 4′-hydroxylation but not TB *p*-methylhydroxylation under the conditions they employed. Interestingly, marmoset P450 M-2C showed the reverse substrate specificity, *i.e.*, it catalyzed TB *p*-methylhydroxylation but not S-MP 4′-hydroxylation.

P450 M-2C showed considerable oxidation activity only for TB among the four substrates of human CYP2C enzymes examined. Although all of the human CYP2C enzymes (CYP2C8, CYP2C9 and CYP2C19) examined exerted TB oxidation activities, the kinetic profile of CYP2C8 was most similar to that of marmoset P450 M-2C (Table 2). The results of the inhibition study demonstrated that quercetin, a CYP2C8 inhibitor, was the most effective inhibitor for TB oxidation by P450 M-2C as well as by marmoset liver microsomes, followed by omeprazole, a CYP2C19 inhibitor. TB *p*-methylhydroxylation was kinetically

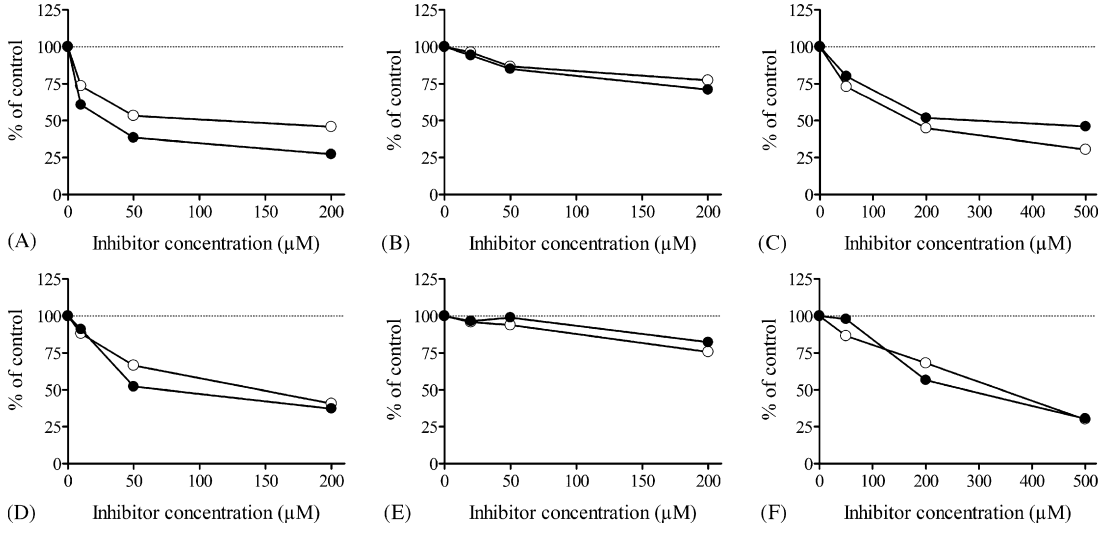

Fig. 5 – The effects of human CYP2C enzyme inhibitors on TB *p*-methylhydroxylation by marmoset liver microsomes (upper panels) and by P450 M-2C (lower panels). The final inhibitor concentrations used were 10, 50 and 200 μM for quercetin (A and D) 20, 50 and 200 μM for sulfaphenazole (B and E) and 50, 200 and 500 μM for omeprazole (C and F). The substrate concentrations used were 100 (open circles) and 1000 μM (closed circles). Each point represents the mean of two independent determinations.

analyzed to be monophasic, and the apparent K_m values were similar between the marmoset liver microsomes and the recombinant P450 M-2C, indicating that P450 M-2C is the major TB *p*-methylhydroxylase in the marmoset liver.

It is well known that CYP2C9 is the major TB *p*-methylhydroxylase in the human liver [26]. However, TB *p*-methylhydroxylation gave biphasic kinetics in the pooled human liver microsomes used in the present study. The apparent K_m value for TB *p*-methylhydroxylation by recombinant CYP2C9 was 340 μM in this study, which was close to the K_m values of purified CYP2C9 reported by Lasker et al. [29] (180–400 μM) and of recombinant CYP2C9 (410 μM) reported by Flanagan et al. [30] for TB *p*-methylhydroxylation. Therefore, it is reasonable to think that some CYP enzyme(s) with a lower K_m value of around 70 μM together with CYP2C9 with a higher K_m value of 340 μM are responsible for TB *p*-methylhydroxylation in the human liver microsomal fractions used.

As described above, for TB *p*-methylhydroxylation, P450 M-2C and CYP2C8 showed similar kinetic profiles in the present study. In contrast, P450 M-2C did not show any detectable activity for PT 6α-hydroxylation, which was catalyzed by CYP2C8. Fig. 6 shows the active sites of P450 M-2C and CYP2C8. In a modeling study on PT 6α-hydroxylation by CYP2C8, Tanaka et al. [31] proposed that there are two distal sites (1 and 2) in addition to the proximal site occupying the space just above the heme iron in the active site of CYP2C8. They thought

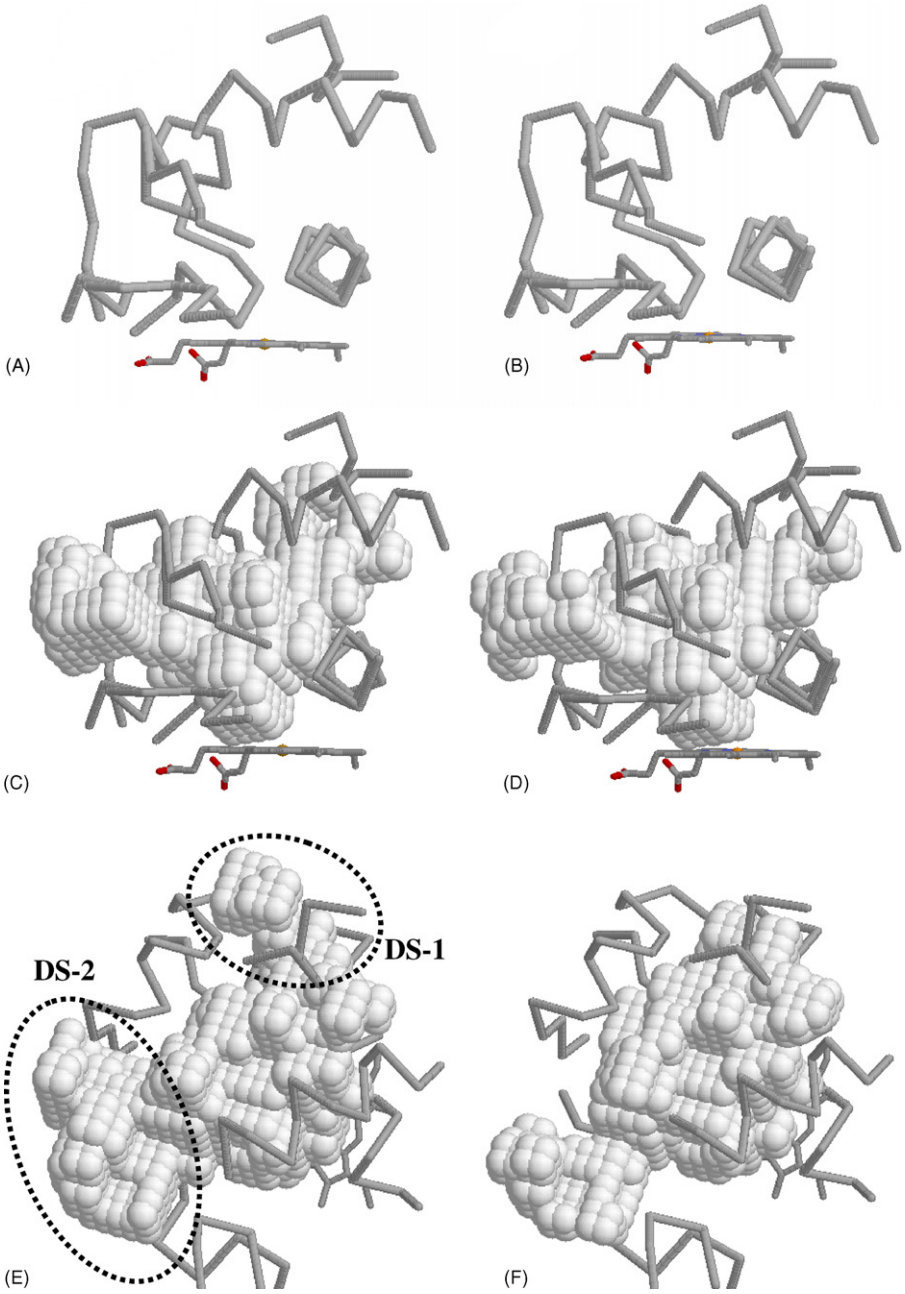

Fig. 6 – Comparison of the active site structures (A and B) and the active site cavities (C–F) between CYP2C8 (left panels) and P450 M-2C (right panels). The active site conformation was depicted using RasMol Version 2.6-ucb 1.0. DS, distal site.

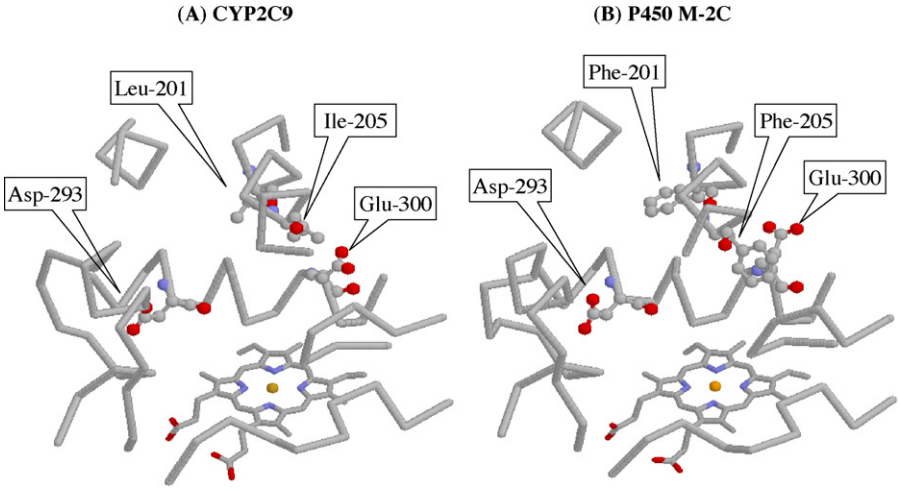

Fig. 7 – Comparison of the active site structures between CYP2C9 (A) and P450 M-2C (B). Proteins are depicted as backbone form, and Phe-201 and Asp-293 for CYP2C8 and Phe-205 and Glu-300 for P450 M-2C as ball and stick form using RasMol Version 2.6-ucb 1.0.

it possible that the *N*-benzoyl-3-phenylisoserine side-chain of PT binds to distal site 2, resulting in oxidation at the 6-position of the taxol ring.

The active site conformations of CYP2C8 and P450 M-2C which are shown with backbone depiction on RasMol are very similar (Fig. 6A and B). However, the shapes of the active site cavities of the two enzymes are considerably different from each other (Fig. 6C–F), especially, the shapes viewed from above (Fig. 6E and F) show clear differences in both distal sites 1 and 2. That is, the sizes of both distal sites 1 and 2 of CYP2C8 are larger than those of P450 M-2C. It is feasible that the smaller size of the active site cavity, particularly of the distal site 2 of P450 M-2C, makes it impossible for PT to appropriately dock in the active site, resulting in undetectable PT oxidation activity.

Fig. 7 shows the active sites of CYP2C9 (left panel) and P450 M-2C (right panel). The active site of CYP2C8 is almost the same as that of P450 M-2C. In the active site cavity of CYP2C9, there are two acidic amino acids, *i.e.*, Asp-293 and Glu-300, whose carboxylate groups may interact ionically with basic nitrogen atoms of TB. As a result, the *p*-methyl group of TB to be oxidized comes close to the heme iron, yielding *p*-hydroxymethyl-TB efficiently. In the active site cavity of P450 M-2C as well as of CYP2C8 having Asp-293 and Glu-300, however, there are two aromatic amino acids, Phe-201 and Phe-205. The phenyl group of Phe-205, in particular, is located just in front of the carboxylate group of Glu-300, which seems to block the ionic interaction between Glu-300 and the basic nitrogen atom of TB. Furthermore, these phenylalanine residues may cause hydrophobic interaction with the aromatic ring of TB, making the tolyl group of TB far from the heme ion, which may result in low capacities of P450 M-2C and CYP2C8 for TB *p*-methylhydroxylation.

In summary, we cloned a cDNA encoding a novel CYP2C enzyme, called P450 M-2C, from the marmoset liver. The deduced amino acid sequence showed high identities to human CYP2C8 (87%), CYP2C9 (78%) and CYP2C19 (77%). Yeast cell microsomal P450 M-2C catalyzed *p*-methylhydroxylation of only TB among four substrates, PT, DF, TB and S-MP, for human CYP2C enzymes. Marmoset liver microsomes exerted monophasic kinetics for TB, and its apparent K_m value was similar to that of the recombinant P450 M-2C. Although three human recombinant CYP2C enzymes, CYP2C8, CYP2C9 and CYP2C19, also showed TB *p*-methylhydroxylation, the kinetic profile of CYP2C8 was most similar to that of P450 M-2C. TB oxidation by the marmoset microsomes and the recombinant P450 M-2C was similarly inhibited by quercetin, a CYP2C8 inhibitor. These results indicate that P450 M-2C (marmoset CYP2C8) is the major TB *p*-methylhydroxylase in the marmoset liver.

Acknowledgments

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