PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2002;8(2):318-322
World Journal of Gastroenterology
 ISSN 1007-9327

• BASIC RESEARCH •

Cloning of cytochrome P-450 2C9 cDNA from human liver and its expression in CHL cells

Ge-Jian Zhu, Ying-Nian Yu, Xin Li, Yu-Li Qian

Ge-Jian Zhu, Ying-Nian Yu, Department of Pathophysiology and Laboratory of Medical Molecular Biology, Zhejiang University School of Medicine, Hangzhou 310031, Zhejiang Province, China
Xin Li, Department of pharmaceutical analysis & drug metabolism, College of Pharmacology Science, Zhejiang University, Hangzhou 310031, Zhejiang Province, China
Yu-Li Qian, Present address: Center of laboratory, Women's hospital, School of Medicine, Zhejiang University, Hangzhou 310031, Zhejiang Province, China
Supported by National Natural Science Foundation of China, No.39770868 and Natural Science Foundation of Zhejiang Province, No.397490
Correspondence to: Prof. Ying-Nian Yu, Department of Pathophysiology and Laboratory of Medical Molecular Biology, Zhejiang University School of Medicine,Hangzhou 310031,Zhejiang Province,China. ynyu@mail.hz.zj.cn
Telephone: +86-571-87217149 Fax: +86-571-87217149
Received 2001-11-15 **Accepted** 2001-12-12

Abstract

AIM: **Using bacterial, yeast, or mammalian cell expressing a human drug metabolism enzyme would seem good way to study drug metabolism-related problems. Human cytochrome P-450 2C9(*CYP2C9*) is a polymorphic enzyme responsible for the metabolism of a large number of clinically important drugs. It ranks among the most important drug metabolizing enzymes in humans. In order to provide a sufficient amount of the enzyme for drug metabolic research, the *CYP2C9* cDNA was cloned and expressed stably in CHL cells.**

METHODS: **After extraction of total RNA from human liver tissue, the human *CYP2C9* cDNA was amplified with reverse transcription-polymerase chain reaction (RT-PCR), and cloned into cloning vector pGEM-T. The cDNA fragment was identified by DNA sequencing and subcloned into a mammalian expression vector pREP9. A transgenic cell line was established by transfecting the recombinant vector of pREP9-*CYP2C9* into CHL cells. The enzyme activity of *CYP2C9* catalyzing oxidation of tolbutamide to hydroxy tolbutamide in S9 fraction of the cell was determined by high performance liquid chromatography(HPLC).**

RESULTS: **The amino acid sequence predicted from the cDNA segment was identical to that of *CYP2C9**1, the wild type *CYP2C9*. However, there were two base differences, i.e. 21T>C, 1146C>T, but the encoding amino acid sequence was the same, L7, P382. The S9 fraction of the established cell line metabolizes tolbutamide to hydroxy tolbutamide; tolbutamide hydroxylase activity was found to be 0.465±0.109 μmol·min^{-1}·g^{-1} S9 protein or 8.62±2.02mol·min^{-1}·mol^{-1} CYP, but was undetectable in parental CHL cell.**

CONCLUSION: **The cDNA of human *CYP2C9* was successfully cloned and a cell line of CHL-*CYP2C9*, efficiently expressing the protein of *CYP2C9*, was established.**

Zhu GJ, Yu YN, Li X, Qian YL. Cloning of cytochrome P-450 2C9 cDNA from human liver and its expression in CHL cells. *World J Gastroenterol* 2002; 8(2) :318-322

INTRODUCTION

Cytochrome P-450 (CYP) is a heme-containing enzyme widely distributed from bacteria to mammals, which catalyzes oxidative or reductive metabolism of a wide variety of substances including endogenous as well as exogenous compounds. Mammalian CYP present in liver microsomes is one of the key enzymatic mechanisms for the matabolism of drugs, pesticides, environmental pollutants, and carcinogens[1]. Mammals possess at least 17 distinct CYP gene families that together code for an estimated 50-60 individual CYP genes in any given species[2]. The human CYP2C subfamily comprises four members, *CYP2C8*, *CYP2C9*, *CYP2C18* and *CYP2C19*[3], accounting for 20% of the total CYP in human liver. *CYP2C9* is a polymorphic enzyme responsible for the metabolism of a large number of clinically important drugs such as S-warfarin, phenytoin, tolbutamide, torsemide, losartan, fluoxetine, dapsone[4], cyclooxygenase-2 inhibitor: celecoxib[5,6], nonpeptide angiotensin II receptor antagonist: irbesartan[7] and numerous nonsteroidal anti-inflammatory drugs. It ranks among the most important drug metabolizing enzymes in humans[8].

The combination of gene technology and cell culture technology has provided new opportunities for studying proteins because any gene from any species encoding an protein may be cloned and expressed in bacterial, yeast, or mammalian cells in a defined way[9-18]. This approach to drug metabolism is of particular importance because some of the enzymes are difficult to purify and prepare in sufficient quantities, or expression levels are low, expression is organ-specificity, or the enzyme-product organs are very scarce. These restrictions apply especially for human enzymes. The heterologous expression of the cDNA allows to bypass these restrictions[19]. Human *CYP2C9* previously has been expressed in E. *coli*[20], Salmonella typhimurium[21], yeast[22] COS-1[3], human liver epithelial cell THLE[23], and human hepatic cell line HepG2[24]. Several cell lines stably expressing human CYP1A1[25], CYP2B6[25], CYP2A6[26], CYP3A4[27], *CYP2C18*(in press) and a phase II metabolism enzyme UDP-glucuronosyltransferase, UGT1A9[28] have been established in our laboratory. In this study human *CYP2C9* cDNA was amplified with reverse transcription-polymerase chain reaction (RT-PCR), and a transgenic cell line stably expressing *CYP2C9* was established.

MATERIALS AND METHODS

Materials

Restriction endonucleases, Moloney murine leukemia virus (M-MuLV) reverse transcriptase were purchased from MBI Fermentas AB, Lithuania. Taq plus I DNA polymerase, dNTPs, PCR primers, DNA sequence primers and random hexamer primer were supplied or synthesized by Shanghai Sangon Biotechnology Corp. DNA sequencing kit was supplied by Perkin-Elmer Corp. The TRIzol reagent, G418, minimum essential media(MEM) and newborn bovine calf sera from Gibco. NADPH from Roche Molecular Biochemicals. Diethyl pyrocarbonate (DEPC), tolbutamide and hydroxytolbutamide were obtaineded from Sigma Chemical Company. T4 DNA ligase and pGEM-T vector system were supplied by

Promega. Other chemical reagents used were all of analytical purity from commercial sources.

Methods

Cloning of human CYP2C9 cDNA from a Chinese human liver

The total RNA was extracted from a surgical specimen of human liver with TRIzol reagent according to the manufacture's instructions, and then the first strand of cDNA was reverse transcripted from mRNA. Procedure: 5μg of the total RNA and 2μg of random hexamer primer in deionized DEPC-treated water were denatured at 65℃ for 15min, then 4μL 5×reverse transcription buffer, 3μL 10mmol·L^{-1} dNTP, 1μL M-MuLV reverse transcriptase (200u) and essential deionized DEPC treated water was added to a total volume of 20μL. The reaction was performed at 25℃ for 10min, then at 42℃ for 1h, and finally at 70℃ for 10min to inactivate reverse transcriptase. The reactant then was stored at 4℃. To amplify the human *CYP2C9* cDNA by PCR, 2μL of the reactant were mixed with 2μL of 10mmol·L^{-1} each of dNTPs, 20pmol of PCR primers and 4u of Taq plus I DNA polymerase in 1×PCR buffer containing 1.5mmol·L^{-1} $MgCl_2$. A total volume of 100μL was reached by adding deionized water. Two specific 32 mer and 28 mer oligonucleotide PCR primers were designed according to the cDNA sequence of *CYP2C9* clone 25 reported by Romkes *et al*[3] (GenBank accession no. M61855, J05326). The sense primer corresponding to base position 1 to 32 was 5'-GAGAAGGTACCAATGGATTCTCTTGTGGTCCT- 3', with a restriction site of *Kpn* I, and the anti-sense one, corresponding to the base position from 1513 to 1540, was 5'-AGAGGAAAGAGAGCTCGAGGGACTGCAC-3' with a restriction site of *Xho* I. The PCR was performed at 94℃ 2min, then 35 cycles of 94℃ 60s, 60℃ 60s, 72℃ 2min, and lastly 72℃ 10min. The product was stored at 4℃. An aliquot of 10μL from the PCR was subjected to electrophoresis in a 10g·L^{-1} agarose gel stained with ethidium bromide.

Construction of recombinant pGEM-CYP2C9 and sequencing of CYP2C9 cDNA[29]

The PCR product of about 1.5 kb in length, recovered and purified by electroelution into dialysis bag was ligated with a clone vector, pGEM-T (Promega), by T4 DNA ligase. E.coli DH5α was transformed with the resulted recombinant pGEM-*CYP2C9* and the replicated plasmid was harvested from the bacteria screened by ampicillin resistant and blue-white selection with X-gal and IPTG. The cDNA of *YP2C9* cloned in pGEM-T was sequenced on both strands by dideoxy chain-termination method marked with BigDye with primers of T7 and SP6 promoters and two specific primers of 5'-TGCCTTGTGGAGTTG-AGA-3'(463-483),and 5'-ACAGAGACGACAAGCACAAC -3' (907-926). The termination products were resolved and detected using an automated DNA sequencer (Perkin-Elmer-ABI Prism 310).

Construction of the pREP9 based expression plasmid for CYP2C9

The *Kpn* I/ *Xho* I fragment of the human *CYP2C9* cDNA cleaved from the recombinant pGEM-*CYP2C9* recovered and purificated by electroelution into dialysis bag was cloned directly into a unique site *Kpn* I/ *Xho* I within the multiple cloning sites of the mammalian expression vector pREP9 (Invitrogen) with T4 DNA ligase. The recombinant was transformed to *E. coli* Top 10, and screened by ampicillin resistant. The recombinant was identified by restriction mapping.

Transfection and selection[29]

Chinese hamster lung(CHL) cells were transfected with the resultant recombinant, pREP9-*CYP2C9*, using a modified calcium phosphate method. After 24h incubation in MEM containing 10% newborn bovine calf sera at 37℃, the culture was rinsed and re-fed with fresh growth medium. After 72h post-transfection, the culture were split and then selected in the culture medium containing the neomycin analogue G418 (400mg·L^{-1}). The selective medium was changed every 3-4d to remove dead cells and to allow the growth of resistant colonies. After 1mo, surviving colonies (termed CHL-*CYP2C9*) were harvested as a pool and propagated in medium containing G418.

Preparation of S9 of CHL-CYP2C9

CHL-*CYP2C9* cells grown in the culture medium containing G418 (400mg·L^{-1}) were rinsed with phosphate balanced solution (PBS), scraped and collected from the bottle with 11.5g·L^{-1} KCl aqua solution and then sonicated in 200W, 5 s for 10 times with 10s of interval break. The resulted homogenate was centrifuged at 9000g at 4℃ for 20min and the postmitochondrial supernatant (S9) was transferred carefully to a clean tube for assay or storage under -70℃. The protein in S9 was determined by the method described by Lowry *et al*, with bovine serum albumin as standard. CYP was measured spectrally using the method of Johannesen *et al*[30].

Tolbutamide hydroxylase assay[22,31]

The *CYP2C9* tolbutamide hydroxylase activity of S9 fraction was determined by high performance liquid chromatography (HPLC). The assay was performed in a total volume of 500μL containing final concentrations of 5mmol·L^{-1} HEPES (pH 7.4), 1.5mmol·L^{-1} $MgCl_2$, 0.1mmol·L^{-1} EDTA, 0.25mg S9 and 1mmol·L^{-1} sodium tolbutamide. The reaction was initiated with 0.5mmol·L^{-1} NADPH and terminated after 60min at 37℃ by the addition of 50μL of 4mmol·L^{-1} HCl. Reaction product was extracted by vortex-mixing of 3mL of water-saturated ethyl acetate with the mixture for 2min. The organic layer was collected after centrifugation in a table top centrifuge at 1000g for 5min. After most of the ethyl acetate extract had air-dried, the rest was removed in a heating block at 75℃. The residue was resolubilized in 200μL of methanol, and reaction product, hydroxytolbutamide was then assayed using HPLC by injecting 20μL of the solubilized extract on to a reversed phase column (Shim-pack CLC-ODS 15cm×0.6cm id, 10μm particle size), using 0.5g·L^{-1} phosphoric acid, pH 2.6, acetonitrile (6:4/V:V) as the mobile phase with a flow rate of 1mL·min^{-1}. The column elution was monitored at 230nm, and rates of product formation were determined from standard curves prepared by adding varying amounts of hydroxytolbutamide to incubations conducted without NADPH.

RESULTS

Construction of recombinants

The recombinant of pGEM-*CYP2C9* (Figure 1) was constructed with the human *CYP2C9* cDNA inserted into the cloning site of vector pGEM-T between the promoters of T7 and SP6. Selection and identification of the recombinant was carried out by *Kpn* I/*Xho* I endonuclease digestion and agarose gel electorphoresis (Figure1). The cloned cDNA segment was sequenced. In comparison with the CYP2C9 clone 65 cDNA sequence reported by Romkes *et al*[3] (GenBank accession no. M61857, J05326), our preparation showed two base differences, i.e. 21T>C, 1146C>T, but the encoding amino acid sequence was the same, *i.e.* L7, P382 respectively.

The *Kpn* I/*Xho* I fragment (1.5 kb) containing the complete *CYP2C9* cDNA was subcloned into the *Kpn* I/*Xho* I site of mammalian expression vector pREP9 (Figure 2). Selection and identification of the recombinants were carried out by *Kpn* I/*Xho* I endonuclease digestion and agarose gel electrophoresis (Figure 2). The resulting plasmid was designated as pREP9-*CYP2C9* and

contained the entire coding region, along with 2 bp of the 5'end and 41 bp of the 3'end untranslated region of the *CYP2C9* cDNA, respectively.

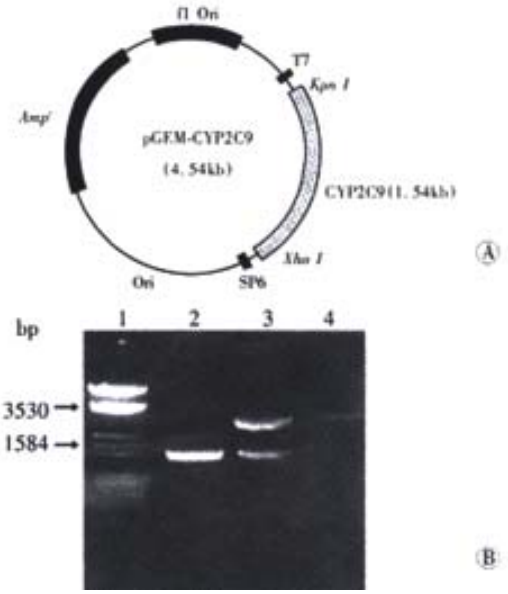

Figure 1 Scheme and electrophoresis identification of recombinant pGEM-*CYP2C9*. A: Scheme of recombinat of pGEM-CYP2C9; B: Electrophoresis identification of recombinant pGEM-*CYP2C9*; 1: Marker (λ/*Eco*RI and *Hin*d III); 2: PCR product of *CYP2C9*(1.54 kb); 3: Recombinant of pGEM-*CYP2C9* digested by *Kpn* I and *Xho* I; 4: pGEM-T vector

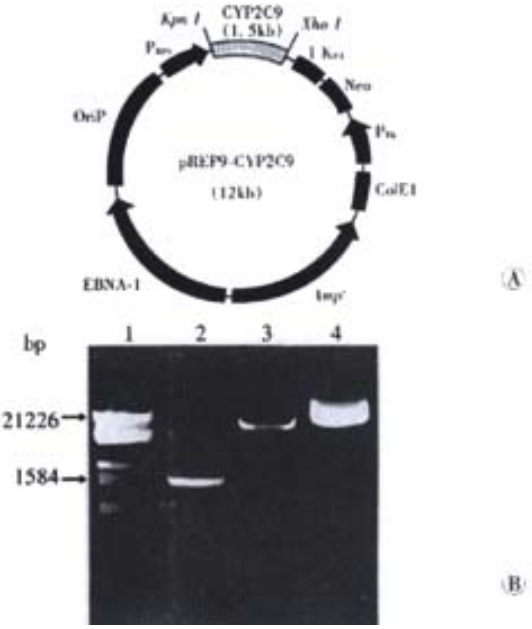

Figure 2 Scheme and electrophoresis identification of recombinant pREP9-*CYP2C9*. A: Scheme of pREP9-*CYP2C9*; B: Electrophoresis identification of recombinant pREP9-*CYP2C9*; 1: Marker (λ/*Eco*R I and *Hin*d III); 2: PCR product of *CYP2C9*(1.54 kb); 3: Recombinant of pREP9-*CYP2C9* digested by *Kpn* I and *Xho* I; 4: pREP9 vector

Establishment of transgenic cell lines with CYP2C9 enzyme activity

CHL cells were transfected with pREP9-*CYP2C9*, and selected with G418 (400mg·L^{-1}). The surviving colonies were propagated and a cell line termed CHL-*CYP2C9* was established. The tolbutamide hydroxylase activity of *CYP2C9* in S9 fraction of CHL-*CYP2C9* cells was assayed by HPLC.A typical elution profile of hydroxytolbutamide in extracts is shown (Figure 3). *CYP2C9* enzyme activity with tolbutamide was found to be 0.465±0.109μmol·min^{-1}·g^{1} S9 protein or 8.62±2.02mol·min^{-1}·mol^{-1} CYP (n=3), but none was detectable in parental CHL cells. The CYP content was 57.7nmol·g^{1} S9 protein from CHL-*CYP2C9* but no detectable CYP was present in CHL cell.

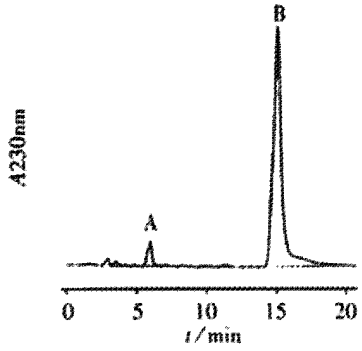

Figure 3 Representative chromatogram of extractsA Shim-pack CLC-ODS column (15cm×0.6cm i.d.) was used. The mobile phase was constituted with phosphoric acid (pH 2.6), acetonitrile (6:4/V:V) with the flow rate at 1mL·min^{-1}. Hydroxytolbutamide was monitored at 230nm. A: hydroxytolbutamide; B: tolbutamide

DISCUSSION

Direct cloning of human *CYP* cDNAs from cDNA libraries generally has been successful using anti-rodent or anti-human *CYP* antibodies and rodent *CYP*cDNA probes. But these cloning procedures are applicable only for the most abundantly expressed *CYP* mRNAs. Using the RT-PCR to clone low abundance *CYP*cDNA is a simple and direct method. *CYP2C9* mRNA was present in human liver[32], HepG2 cells[33], kidney, testes, adrenal gland, prostate, ovary, duodenum[34], and brain tumors[35]. The pGEM-T vector system possesing single 3'-T overhangs at the insertion site greatly improves the efficiency of ligation of a PCR product into the plasmid by preventing recircularization of the vector and providing a compatible overhang for PCR products generateed by Taq Plus I DNA polymerases.

The human *CYP2C9* gene is located on chromosome 10q24. Up to date, 12 *CYP2C9* alleles have been identified (see: Table 1 and *CYP2C9* alleles nomenclature http://www.imm.ki.se/CYPalleles/cyp2c9.htm). *CYP2C9**1 is the wild type of human *CYP2C9*. *CYP2C9**2 exhibit a base substitute 430C>T, resulting in a R144C substitution which has been suggested to affect the interaction between the CYP enzyme molecule and the cytochrome P450 reductase[36]; this may explain the slower metabolism of some *CYP2C9* substrates such as S-warfarin and tolbutamide[8, 37]. *CYP2C9**3 has a base substitute 1075A>C, which leads to an I359L substitute. Takanashi *et al*[38] expressed the *CYP2C9**1 and *CYP2C9**3 cDNA in yeast and examined the kinetics of seven individual metabolic reactions by wild-type *CYP2C9**1 and its *CYP2C9**3 variant. Their results indicated that the I359L exchange significantly reduces the catalytic activity with all *CYP2C9*-mediated substrates studied, although the extent of the reduction in activity and kinetic parameters varied between different substrates. Interestingly Kidd *et al*[39] reported a male Caucasian, homozygous for *CYP2C9**3, who poorly metabolized phenytoin and glipizide/tolbutamide. This study establishes that the I359L mutation is responsible for the poor metabolizer phenotype. The *CYP2C9**2 and *CYP2C9**3 are responsible for the poor metabolizing celecoxib[5], losartan[40], torsemide[41]. *CYP2C9**4[42] has a base substitute of 1076T>C,

leading to a I359T substitution. Ieiri *et al*[43] evaluated the catalytic activity of three variants (I, L, and T) at codon 359 of *CYP2C9* enzymes expressed in a yeast cDNA expression system. The specific catalytic activities were assessed by diclofenac-4'-hydroxylation. The *in vitro* study revealed that recombinant I359, L359, and T359 (2 batches) exhibited a mean Km of 2.0, 16.5 and (3.8 and 2.9) μmol and Vmax of 12.4, 17.9 and (4.4 and 5.1) nmol·min^{-1}·nmol^{-1}*CYP*, respectively. The *CYP2C9**5 variant is derived from a 1080C>G transversion in exon 7 of *CYP2C9* that leads to a D360E substitution in the encoded protein[44]. The *CYP2C9**5 variant was found to be expressed in African-Americans with a frequency of approximately 3% in this population group. This variant was expressed in, and purified from, insect cells infected with a recombinant baculovirus. The *in vitro* data suggest that carriers of the *CYP2C9**5 allele would eliminate *CYP2C9* substrates at slower rates compared to individuals expressing the wild-type protein[44]. Kidd *et al*[45] reported a new *CYP2C9* allele (*CYP2C9**6) with the deletion of an adenine at base pair 818 of the cDNA. The clearance of phenytoin in this individual is estimated to be approximately 17% of that observed in normal patients. The frequency of this allele was 0.6% in 79 African-Americans and 0% in 172 Caucasians. Shintani *et al*[46] reported that mutations in the 5'-flanking region of the human *CYP2C9* gene appear to contribute to the large interindividual variability in drug metabolism activity. Compared with *CYP2C9**1 cDNA, there are two base differences in the *CYP2C9* cDNA cloned by us, but the encoded amino acid sequence remains unchanged. This obviously is the molecular basis for full enzymatic activity.

Table 1 Nomenclature and characteristics of CYP2C9 alleles

Allele	Protein	Nucleotide changes	Effect	Enzyme activity		References
				In Vivo	*In vitro*	
*CYP2C9*1*	*CYP2C9*.1	None		Normal	Normal	3
*CYP2C9*2*	*CYP2C9*.2	430C>T	R144C		Decr	36
*CYP2C9*3*	*CYP2C9*.3	1075A>C	I359L	Decr	Decr	37,38,39,43
*CYP2C9*4*	*CYP2C9*.4	1076T>C	I359T			42,43
*CYP2C9*5*	*CYP2C9*.5	1080C>G	D360E	Decr		44
*CYP2C9*6*		818delA	frame shift	Decr		45
*CYP2C9*7-*12*						*In press*

To express the functional activity of a CYP, a cell evidently must have adequate heme supply, either by intracellular biosynthesis or extracellular provision[47]. CYPs also require other enzymatic components for full activity, including the flavoprotein NADPH-P450 oxidoreductase (OR) and, in some cases, cytochrome b_5. The OR must interact directly with the CYP to transfer the required two electrons from NADPH. Cytochrome b5 is necessary for increasing electron transfer for certain CYP forms and specific substrates. The CHL is the cell line originally derived from the lung of a newborn female Chinese hamster and has no or very limited activities of CYP enzymes, but has adequate OR and cytochrome b5 levels to support CYP activities.

To achieve high expression levels of *CYP2C9*, the *CYP2C9* cDNA was cloned into the eukaryotic expression vector pREP9, which had previously been used in this laboratory for the expression of human CYP1A1, CYP2B6, CYP2A6, CYP3A4 and UGT1A9 in CHL cells[25-28]. The salient feature of this vector has an Epstein Barr Virus origin of replication and nuclear antigen (EBNA-1) to allow high-copy episomal replication in mammal cell lines. The Rous sarcoma virus long terminal repeat (RSV LTR) early promoter controls the expression of the *CYP2C9* cDNA.

Tolbutamide (1-butyl-3-p-tolylsulfonylurea) is an oral hypoglycemic agent which is being used in the treatment of diabetes. In humans it undergoes CYP-catalyzed hydroxylation of the tolyl methyl group which is the initial and rate-limiting reaction followed by further oxidation by cytosolic dehydrogenases yielding carboxytolbutamide. Overall this pathway accounts for up to 85% of tolbutamide clearance in humans. Evidence that *CYP2C9* is solely responsible for tolbutamide hydroxylation is convincing and tolbutamide is widely accepted as a prototypic substrate for the assessment of hepatic *CYP2C9* activity, both *in vitro* and *in vivo*[8].

We used tolbutamide as a substrate for evaluating the expressing of human CYP2C9 activity in CHL-*CYP2C9* cell. The tolbutamide hydroxylase activity was 0.465±0.109μmol·min^{-1}·g^{-1} S9 protein or 8.62±2.02mol·min^{-1}·mol^{-1} *CYP*. These value, were somewhat higher than these obtained with recombinant *CYP2C9* purified from *E. coli*: 4.67-4.96nmol·min^{-1}·nmol^{-1} *CYP*[48] or human liver microsomes: 0.189±0.0083nmol·min^{-1}·mg^{-1} microsome[49] and 2.29-4.33nmol·min^{-1} ·nmol^{-1} CYP[48]. This clearly stated that CHL-*CYP2C9* expressed the *CYP2C9* efficiently and this may be a useful tool for further studies of its enzymatic function and mechanism.

REFERENCES

1 Anzenbacher P, Anzenbacherova E. Cytochromes P450 and metabolism of xenobiotics. *Cell Mol Life Sci* 2001;58:737-747
2 Nelson DR. Cytochrome P450 and the individuality of species. *Arch Biochem Biophys* 1999; 369: 1-10
3 Romkes M, Faletto MB, Blaisdell JA, Raucy JL, Goldstein JA. Cloning and expression of complementary DNAs for multiple members of the human cytochrome P450IIC subfamily. *Biochemistry* 1991; 30: 3247-3255
4 Winter HR, Wang Y, Unadkat JD. *CYP2C8*/9 mediate dapsone N-hydroxylation at clinical concentrations of dapsone. *Drug Metab Dispos* 2000; 28: 865-868
5 Tang C,Shou M,Rushmore TH,Mei Q,Sandhu P,Woolf EJ,Rose MJ, Gelmann A,Greenberg HE,De-Lepeleire I,Van-Hecken A, De-Schepper PJ,Ebel DL,Schwartz JI,Rodrigues AD. In-vitro metabolism of celecoxib, a cyclooxygenase-2 inhibitor, by allelic variant forms of human liver microsomal cytochrome P450 2C9:correlation with *CYP2C9* genotype and in-vivo pharmacokinetics. *Pharmacogenetics* 2001;11:223-235
6 Tang C, Shou M, Mei Q, Rushmore TH, Rodrigues AD. Major role of human liver microsomal cytochrome P450 2C9 (*CYP2C9*) in the oxidative metabolism of celecoxib, a novel cyclooxygenase-II inhibitor. *J Pharmacol Exp Ther* 2000; 293: 453-459
7 Bourrie M, Meunier V, Berger Y, Fabre G. Role of cytochrome P-4502C9 in irbesartan oxidation by human liver microsomes. *Drug Metab Dispos* 1999; 27: 288-296
8 Miners JO, Birkett DJ. Cytochrome P450 2C9: an enzyme of major importance in human drug metabolism. *Br J Clin Pharmacol* 1998; 45: 525-538
9 Liu XF, Zou SQ, Qiu FZ. Construction of HCV-core gene vector and its expression in cholangiocarcinoma. *World J Gastroenterol* 2002; 8:135-138
10 Wu C, Zou QM, Guo H, Yuan XP, Zhang WJ, Lu DS, Mao XH. Expression, purification and immuno-characteristics of recombination UreB protein of *H. pylori*. *World J Gastroenterol* 2001; 7:389-393
11 Li XJ, Wu JG, Si JL, Guo DW,Xu JP. High-level expression of human calmodulin in E.coli and its effects on cell proliferation. *World J Gastroenterol* 2000; 6:588-592
12 Cheng J, Zhong YW, Liu Y, Dong J, Yang JZ, Chen JM. Cloning and sequence analysis of human genomic DNA of augmenter of liver regeneration. *World J Gastroenterol* 2000; 6:275-277
13 Guo SP, Ma ZS, Wang WL. Construction of eukaryotic expression vector of HBV x gene. *World J Gastroenterol* 1999; 5:351-352
14 Qu S, Li QF, Deng YZ, Zhang JM, Zhang J. Cloning and expression of HLA-B7 gene. *World J Gastroenterol* 1999; 5:345-348
15 Liu ZG, Yang JH, An HZ, Wang HY, He FT, Han ZY, Han Y, Wu HP, Xiao B, Fan DM. Cloning and identification of an angiostatic molecule IP10/crg-2. *World J Gastroenterol* 1999; 5:241-244
16 He Y, Zhou J, Dou KF. Construction of hepatocyte growth factor expression vector and detection of expression in human hepatocytes. *Shijie Huaren Xiaohua Zazhi* 2001; 9:1143-1146
17 Pan X, Pan W, Ke CW, Zhang B, Cao GW, Qi ZT. Tetracycline controlled DT/VEGF system gene therapy mediated by adenovirus vector. *Shijie Huaren Xiaohua Zazhi* 2000; 8:1121-1126
18 Lu JG, Lin C, Huang ZQ, Wu JS, Fu M, Zhang XY, Liang X, Yao X, Wu M. Inhibitory effects of human cholangiocarcinoma cell line by recombinant adenoviruses P16 with CDDP. *Shijie Huaren Xiaohua Zazhi* 2000; 8: 641-645
19 Crespi CL, Miller VP. The use of heterologously expressed drug metabolizing enzymes-state of the art and prospects for the future. *Pharmacol Therapeutics* 1999;84:121-131
20 McGinnity DF, Griffin SJ, Moody GC, Voice M, Hanlon S, Friedberg T, Riley RJ. Rapid characterization of the major drug-metabolizing human

hepatic cytochrome P-450 enzymes expressed in Escherichia *coli*. *Drug Metab Dispos* 1999; 27: 1017-1023
21 Fujita K, Kamataki T. Role of human cytochrome P450(CYP) in the metabolic activation of N-alkylnitrosamines: application of genetically engineered Salmonella typhimurium YG7108 expression each form of CYP together with human NADPH-cytochrome P450 reductase. *Mutat Res* 2001; 483: 35-41
22 Goldstein JA, Faletto MB, Romkes-Sparks M, Sullivan T, Kitareewan S, Raucy JL, Lasker JM, Ghanayem BI. Evidence that *CYP2C19* is the major (S)-mephenytoin 4'-hydroxylase in humans. *Biochemistry* 1994; 33: 1743-1752
23 Bort R, Castell JV, Pfeifer A, Gomezlechon MJ, Mace K. High expression of human CYP2C in immortalized human liver epithelial cells. *Toxicol in Vitro* 1999; 13: 633-638
24 Yoshitomi S, Ikemoto K, Takahashi J, Miki H, Namba M, Asahi S. Establishment of the transformants expressing human cytochrome P450 subtypes in HepG2, and their applications on drug metabolism and toxicology. *Toxicol in Vitro* 2001; 15: 245-256
25 Wu JM, Dong HT, Cai ZN, Yu YN. Stable expression of human cytochrome CYP2B6 and CYP1A1 in chinese hamster CHL cells: their use in micronucleus assays. *Chin Med Sci J* 1997; 12:148-155
26 Yan LQ, Yu YN, Zhuge J, Xie HY. Cloning of human cytochrome P450 2A6 cDNA and its expression in mammalian cells. *Zhongguo Yaolixue Yu Dulixue Zazhi* 2000; 14:31-35
27 Chen Q, Wu J, Yu Y, Establishment of transgenic cell line CHL-3A4 and its metabolic activation. *Zhonghua Yufang Yixue Zazhi* 1998; 32: 281-284
28 Li X, Yu YN, Zhuge J, Qian YL. Cloning of UGT1A9 cDNA from liver tissues and its expression in CHL cells. *World J Gastroenterol* 2001; 7:841-845
29 Sambrook J, Fritsch EF, Maniatis T. Molecular cloning, a laboratory manual. 2nd ed. New York: Cold Spring Harbor Laboratory Press, 1989;6:28-29, 1:63-86, 16:39-40
30 Johannesen KA, DePierre JW. Measurement of cytochrome P-450 in the presence of large amounts of contaminating hemoglobin and methemoglobin. *Anal Biochem* 1978; 86: 725-732
31 Ho JW, Moody DE. Determination of tolbutamide hydroxylation in rat liver microsomes by high-performance liquid chromatography: effect of psychoactive drugs on *in vitro* activity. *Life Sci* 1993; 52: 21-28
32 Rodriguez-Antona C, Donato MT, Pareja E, Gomez-Lechon MJ, Castell JV. Cytochrome P-450 mRNA expression in human liver and its relationship with enzyme activity. *Arch Biochem Biophys* 2001; 393: 308-315
33 Sumida A, Fukuen S, Yamamoto I, Matsuda H, Naohara M, Azuma J. Quantitative analysis of constitutive and inducible CYPs mRNA expression in the HepG2 cell line using reverse transcription-competitive PCR. *Biochem Biophys Res Commun* 2000; 267: 756-760
34 Klose TS, Blaisdell JA, Goldstein JA. Gene structure of *CYP2C8* and extrahepatic distribution of the human CYP2Cs. *J Biochem Mol Toxicol* 1999; 13: 289-295
35 Knupfer H, Knupfer MM, Hotfilder M, Preiss R. P450-expression in brain tumors. *Oncol Res* 1999; 11: 523-528
36 Crespi CL, Miller VP. The R144C change in the CYP2C9*2 allele alters interaction of the cytochrome P450 with NADPH:cytochrome P450 oxidoreductase. *Pharmacogenetics* 1997; 7:203-210
37 Aithal GP, Day CP, Kesteven PJ, Daly AK.Association of polymorphisms in the cytochrome P450 *CYP2C9* with warfarin dose requirement and risk of breeding complications. *Lancet* 1999; 353:717-719
38 Takanashi K, Tainaka H, Kobayashi K, Yasumori T, Hosakawa M, Chiba K. *CYP2C9* Ile359 and Leu359 variants: enzyme kinetic study with seven substrates. *Pharmacogenetics* 2000; 10: 95-104
39 Kidd RS, Straughn AB, Meyer MC, Blaisdell J, Goldstein JA, Dalton JT. Pharmacokinetics of chlorpheniramine, phenytoin, glipizide and nifedipine in an individual homozygous for the *CYP2C9*3 allele. *Pharmacogenetics* 1999; 9: 71-80
40 Yasar U, Tybring G, Hidestrand M, Oscarson M, Ingelman-Sundberg M, Dahl ML, Eliasson E. Role of *CYP2C9* polymorphism in losartan oxidation. *Drug Metab Dispos* 2001; 29: 1051-1056
41 Miners JO, Coulter S, Birkett DJ, Goldstein JA. Torsemide metabolism by *CYP2C9* variants and other human CYP2C subfamily enzymes. *Pharmacogenetics* 2000; 10: 267-270
42 Imai J, Ieiri I, Mamiya K, Miyahara S, Furuumi H, Nanba E, Yamane M, Fukumaki Y, Ninomiya H, Tashiro N, Otsubo K, Higuchi S. Polymorphism of the cytochrome P450 (CYP) 2C9 gene in Japanese epileptic patients: genetic analysis of the *CYP2C9* locus. *Pharmacogenetics* 2000;10:85-89
43 Ieiri I, Tainaka H, Morita T, Hadama A, Mamiya K, Hayashibara M, Ninomiya H, Ohmori S, Kitada M, Tashiro N, Higuchi S, Otsubo K. Catalytic activity of three variants (Ile, Leu, and Thr) at amino acid residue 359 in human CYP2C9 gene and simultaneous detection using single-strand conformation polymorphism analysis. *Ther Drug Monit* 2000; 22: 237-244
44 Dickmann LJ, Rettie AE, Kneller MB, Kim RB, Wood AJ, Stein CM, Wilkinson GR, Schwarz UI. Identification and functional characterization of a new CYP2C9 variant (CYP2C9*5) expressed among African Americans. *Mol Pharmacol* 2001; 60:382-387
45 Kidd RS, Curry TB, Gallagher S, Edeki T, Blaisdell J, Goldstein JA. Identification of a null allele of *CYP2C9* in an African-American exhibiting toxicity to phenytoin. *Pharmacogenetics* 2001; 11: 803-808
46 Shintani M, Ieiri I, Inoue K, Mamiya K, Ninomiya H, Tashiro N, Higuchi S, Otsubo K. Genetic polymorphisms and functional characterization of the 5'-flanking region of the human *CYP2C9* gene: *in vitro* and *in vivo* studies. *Clin Pharmacol Ther* 2001;70:175-182
47 Correia MA, Farrell GC, Schmid R, Ortiz-de-Montellano PR, Yost GS, Mico BA. Incorporation of exogenous heme into hepatic cytochrome P-450 *in vivo*. *J Biol Chem* 1979; 254: 15-17
48 Lasker JM, Wester MR, Aramsombatdee E, Raucy J. Characterization of CYP2C19 and *CYP2C9* from human liver: respective roles in microsomal tolbutamide, S-mephenytoin, and omeprazole hydroxylations. *Arch Biochem Biophys* 1998;353:16-28
49 Easterbrook J, Fackett D Li AP. A comparison of aroclor 1254-induced and uninduced rat liver microsomes in phenytoin O-deethylation, coumarin 7-hydroxylation,tolbutamide 4-hydroxylation, S-mephenytoin 4'-hydroxylation, chloroxazone 6-hydroxylation and testosterone 6β-hydroxylation. *Chem Biol Interact* 2001; 134: 243-249

Edited by Schmid R