[PHAR01] Analysis of the catalytic activity of cytochrome P450 2C8: i*n* v*itro* and i*n vivo* population study

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Introduction

Cytochrome P450 2C8 (*CYP2C8*), plays a major role in the metabolism of several exogenous and endogenous substances including paclitaxel, all-*trans* retinoic acid, varapamil, rosiglitazone, cerivastatin, amiodarone, dapsone and amodiaquine (Rahman et al., 1994; McSorley at al., 2000; Yamazaki et al., 1999; Baldwin et al., 1999; Ohyama et al., 2002; Li et al., 2002; Bahadur et al., 2002) and arachidonic acid (Bahadur *et al*, 2002; Zeldin *et al*., 1995) as well as activation of toxicologically important compounds including benzo[*a*]pyrene and parathion (Yun *et al*., 1992; Dai *et al.*, 2001).

CYP2C8 is located on chromosome 10q24.1 and consists of nine exons (Klose *et al*., 1999). It is polymorphic with 20 SNPs identified thus far. However, only *CYP2C8*2, CYP2C8*3, CYP2C8*4* and *CYP2C8*5* were reported to have significant differences in enzyme activity compared to wild type, *CYP2C8**1. *CYP2C8**2 has an Ile269Phe substitution in exon 5 and *CYP2C8**3 includes both Arg139Lys and Lys399Arg amino acid substitutions in exons 3 and 8. They were found primarily in African-American and Caucasians respectively (Rahman *et al*., 1994). *CYP2C8*4* represent mutation that caused an amino acid change (Ile264Met) in exon 5 and occurred primarily in Caucasians (Bahadur *et al*., 2002). Proteins of both *CYP2C8*2* and *CYP2C8*3* variants exhibited a lower turnover number and intrinsic clearance for paclitaxel compared to *CYP2C8*1*. Dai *et al*. (2001) reported markedly impaired metabolism of paclitaxel and arachidonic acid in *CYP2C8**3 enzymes. Liver microsomes from individual heterozygous for *CYP2C8*4* showed activities similar to those for heterozygotes *CYP2C8*3* (Bahadur *et al*., 2002).

*CYP2C8*5* was reported in Japanese population (Nakajima *et al*, 2003) recently after we designed current study thus not studied here. *CYP2C8*5* denotes a deletion of adenine 471, resulting in amino acid alterations from codon 159, causing frameshift mutation, an early stop codon at residue 177. Therefore, the variant enzyme is most likely to be inactive as it lacks 64% of the protein structure, including the heme binding site and five out of six substrate recognition sites (Soyama *et al*., 2002).

Human liver specimens have been used as tools to predict human drug metabolism but the use is limited by several factors, including ethical reasons. Another disadvantage of the use of human livers is the low levels of P450 in these biological materials, which are, in some cases, insufficient to analyze the metabolism of a new drug (Iwata *et al*., 1998). Advances in molecular biology have led the possibility of cloning and heterologously expressing genes of the human CYPs. Enzymes with catalytic activity comparable to those of human liver microsomes have now been expressed in heterologous expression system such as in *Escherichia coli*, insect cells, lymphoblastoid cells, Hep G_2 cells and yeast (Gonzalez and Korzekwa, 1995; Friedberg and Wolf, 1996). These enzymes can be produced in large amounts to meet the demand of the automated high-throughput screening systems for drug metabolism research (Masimirembwa *et al*, 1999).

The objective of our study was to develop and optimize a multiplex PCR method which is economical for rapid, specific and simultaneous detection of common *CYP2C8* variants and with that to determine the allele frequency of three codon changing variants; *CYP2C8*2*, *CYP2C8*3* and *CYP2C8*4* in the Malaysian population. We also aim to clone and express *CYP2C8* and reductase protein in

bacterial expression system for specific *CYP2C8* enzyme kinetic studies.

Materials and Methods

Subjects

The relevant Research Ethics Committee approved our study. Subjects were interviewed regarding their medical history and origin up to 3 generations. They were enrolled if they fulfilled all the inclusion criteria. A written informed consent was obtained.

DNA Isolation

DNA was extracted from subjects' leukocytes using standard methods and subjected to PCR genotyping.

PCR Genotyping

A two-step multiplex PCR method was designed to detect variants of *CYP2C8*2, *3* and **4* simultaneously. All PCR reactions were performed using GeneAmp® PCR system 9700 Perkin Elmer (Applied Biosystems, Foster City). Simultaneous amplification of exon 3, 5 and 8 was successful using 2 × PCR buffer (Biotool®, B&M Lab, S.A), 1.5 mmol/L $MgCl_2$, 0.2 mmol/L dNTP (Promega, Madison), specific primers with optimized concentrations were used, 200 ng (2 µl) genomic DNA as template and 1.0 U DNA Taq polymerase (Biotool®, B&M Lab, S.A) in a total volume of 25 µl. The amplification conditions consist of 38 cycles of denaturation at 94ºC for 1 minute, annealing at 60ºC for 45 seconds, extension at 72ºC for 45 seconds. The PCR products were electrophoresed on 4% agarose gel stained with ethidium bromide, at 80 V for 60 minutes.

One in ten dilution of the first PCR products were used as templates for the two parallel allele specific PCR reactions. The second PCR reaction mixture comprised 1 × PCR buffer, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L dNTP, specific primers with optimized concentrations , 2 µl of the 1 in 10 diluted first PCR product and 1.0 U Biotools® DNA *Taq* Polymerase(Biotool®, B&M Lab, S.A). The PCR conditions were the same as the first PCR except that the annealing temperature was changed to 61ºC and PCR cycles were reduced to 15 cycles. Ten ul of PCR products were electrophoresed on a 4% agarose gel, at 65 V for 90 minutes.

Direct DNA sequencing

Uniplex reaction of first PCR products of the wild-type and variant type of exon 3, 5 and 8 were performed and the products were sent for sequencing. They were purified using QIAquick® PCR purification kit (Qiagen, Hilden) and sequenced on ABI 3700 using Big Dye® Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). The results were compared with the published sequence for *CYP2C8* (Accession number AL359672).

Statistics Analysis

Data were compiled according to the genotype and allele frequencies with the 95% confidence intervals. Expected genotype frequencies were calculated using the Hardy-Weinberg equation from the allele frequencies ($p^2+2pq+q^2=1$). The Chi-square test was used to compare allele frequencies between the subpopulations. A p value of 0.05 or less was regarded as significant. All statistics were done using SPSS for Windows Version 11 on an IBM-PC® compatible computer.

Bacterial Strains and Plasmids

DH5α-max efficiency cells (Stratagene, California), was used for high protein expression. The plasmids pCWori+, inserted with *CYP2C8* 17α cDNA (pCW-*CYP2C8* 17α) and NADPH-P450 reductase were gifts from Profesor Don Birkette, Flinders University, Australia. All chemicals and solvents were of the highest grade commercially available.

Transformation and growth conditions

The pCW-*CYP2C8* 17α and NADPH-P450 reductase were transformed into individual DH5α-max efficiency cells. The transformations were performed using the heat-shock method. E.coli DH5α cells transformed with the pCW-*CYP2C8* 17α and pCW-Reductase were grown overnight in Terrific Broth (TB). When the culture reached approximately 0.7 O.D. (600 nm), the culture was supplemented with 1mM isopropyl β-D-thiogalaxtopyranoside (IPTG) and 0.5 mM δ-aminolivulinic (δ-ala). The cells were shaken at 200 rpm at 30ºC for 48 hours. Prior to this large scale protein expression, a pilot scale

protein expression was performed to vary the incubation period at 24 hours, 48 hours, 72 hours and 96 hours and analyzed using sodium dodecyl sulphate – polyacrylamide (SDS-PAGE) gel electrophoresis. The cells were then chilled on ice for 10 minutes and harvested by centrifuging at 5000g (approximately 3K rpm) at 4ºC for 10 minutes. The cell pellet was resuspended in TES buffer (100mM Tris, pH 7.6, 500mM Sucrose, 0.5mM EDTA) (15ml/g wet cell).

Preparations of membranes containing P450 2C enzymes

Lysozyme (300µg/g cells) was added to the suspension containing spheroplasts. The suspension was spun at 10,000g, 4ºC for 10 minutes and sediments resuspended in Spheroplast Resuspension Buffer (SRB) (100mM phosphate buffer, pH 7.6, 6mM magnesium acetate, 0.1mM DTT, 20% glycerol). Five ml of SRB was used per 50 ml culture. The mixture was then frozen at -70ºC for two hours. The spheroplasts preparations were thawed at room temperature and supplemented with 1mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail (Sigma, U.S.A) (1 ml for 20 ml of cell lysate from 4g (wet weight) of *E.coli* cells). The suspensions were lysed by sonication on ice bath. The cell debris was removed by centrifugation at 10,000g, 4ºC for 20 minutes and the supernatant was carefully removed with a pipette and ultra-centrifuged at 180,000g, 4ºC, 75 minutes (or 1 hour and 45 minutes at 44,000rpm). Membrane fractions were resuspended in 50/50 TES-water by gentle pipetting.

Immunodetection of P450 and the Reductase

The expression of the P450 *CYP2C8*-17α and the NADPH-P450 reductase proteins was confirmed by Western Blot analysis. SDS-PAGE was performed at 100V for 2 hours according to the published method (Laemmli UK, 1970). The resolved proteins were transferred onto a nitrocellulose membrane (Bio-Rad, CA) by running for 250 mA, at 4ºC for 2 hours. Immunoblot detection was performed by incubating the nitrocellulose with the *CYP2C8* and reductase protein and their respective primary antibody for two hours with gentle agitation. The antibody include rabbit anti-human cytochrome P450 *CYP2C8*/9/19, rat CYP2C12 polyclonal antibody (Chemicon International, CA) and rabbit anti-human/rat P450 reductase polyclonal antibody (Chemicon International, CA). The nitrocellulose membrane were then incubated for another two hours in anti-rabbit IgG secondary antibody conjugated to Horse Radish Peroxidase (HRP) and bands detected using the HRP detection system (Bio-Rad, CA).

High performance liquid chromatography (HPLC) method development for Amodiaquine and Desamodiaquine.

The HPLC system consists of Perkin Elmer 200series pump and UV detector and Rheodyne manual injector. The column used was C8 (150mm x 4.6mm)(Waters), 5µm particle size. Mobile phase was methanol: 0.01M phosphate buffer (pH3.0), 20:80 v/v, 0.1% TEA. The solvent flow rate used was 2ml/minute. The UV detector was operated at wavelength of 210nm.

Results

Genotyping

DNA from all the 548 healthy Malaysian volunteers, 137 Malays, 288 Chinese, and 123 Indians were successfully amplified (Figure 1). The prevalence of *CYP2C8* variants among 123 Malaysian Indians were 98 % for *CYP2C8*1*, 0.8 % for *CYP2C8*2* and 1.2 % for *CYP2C8*3*. For Malay and Chinese volunteers, by default only the wild type *CYP2C8*1* was detected (Table 1). None of the volunteers among the 3 races had *CYP2C8*4.* The result was confirmed by direct sequencing. The predicted frequencies of *CYP2C8* genotypes among Malaysian Indians were calculated according to the Hardy-Weinberg equation (Table 2). The genotypes were found to be in equilibrium with the Hardy-Weinberg equation.

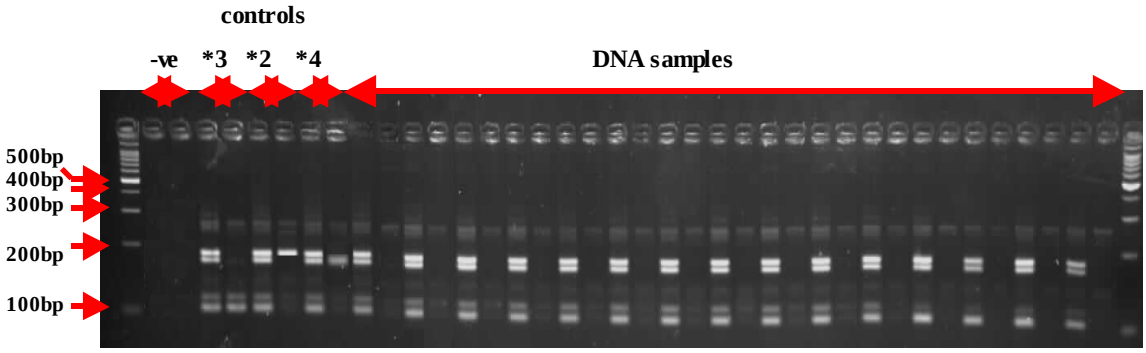

FIGURE 1 Second PCR products for samples screening. Lane 1: 100 bp DNA ladder (Promega Corporation, Madison U.S.A.); lane 2-3: negative controls; lane 4-5: positive control for *CYP2C8*3 (heterozygous){102 and 114 bp}*; lane 6-7: positive control for *CYP2C8*2 (heterozygous){182bp}*; lane 8-9: positive control for *CYP2C8*4 (heterozygous){169 bp}*; lane 10-39: DNA samples; lane 40: 100 bp DNA ladder (Promega Corporation, Madison U.S.A.)

TABLE 1 Study on 548 healthy volunteers

ALLELES	MALAYS(%)	CHINESE(%)	INDIAN(%)
*CYP2C8*1	100	100	98.0
*CYP2C8*2	0	0	0.8
*CYP2C8*3	0	0	1.2
*CYP2C8*4	0	0	0
	100	100	100
N	137	288	123

TABLE 2 Allele frequency together with the observed and predicted genotype frequencies of *CYP2C8* according to Hardy-Weinberg equation in Malaysian Indians (N=123)

Allele	Allele Frequency (%) ± 95% confidence interval (CI)	Genotype	Samples (N)	Observed Genotype Frequency (%) ± 95% confidence interval (CI)	Predicted Genotype Frequency (%) by Hardy-Weinberg Law ± 95% confidence interval (CI)
*CYP2C8*1*	98 ± 1.75	*CYP2C8*1/*1*	118	95.93 ± 3.49	96.04 ± 3.45
*CYP2C8*2*	0.8± 1.11	*CYP2C8*1/*2*	2	1.63 ±2.24	1.568 ± 2.20
*CYP2C8*3*	1.2 ±.1.36	*CYP2C8*1/*3*	3	2.44 ±2.73	2.352 ± 2.68
*CYP2C8*4*	0	*CYP2C8*2/*3*	0	0	0.0192 ± 0.245
		*CYP2C8*2/*2*	0	0	0.0064 ± 0.141
		*CYP2C8*3/*3*	0	0	0.0144 ± 0.212

Protein Expression

The highest protein expression for *CYP2C8* was achieved after an incubation of 48 hours while reductase showed optimum expression after 24 hours. The proteins, *CYP2C8* protein of about 50 kDa, and reductase protein of about 80 kDa was run on a coomasie blue stained 10% SDS-PAGE gel (Figure 2).

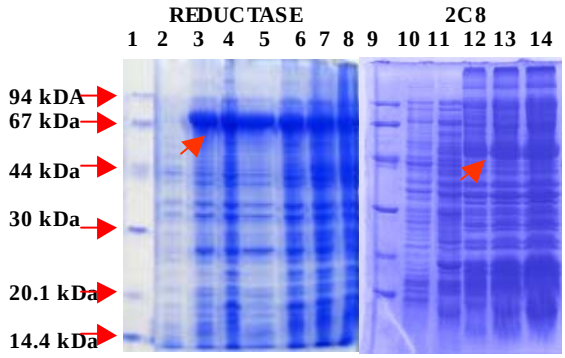

FIGURE 2 Protein expression of reductase and *CYP2C8* Reductase; Lane 1: Protein marker; Lanes 2-5: expression at 24, 48, 72 and 96 hours; Lanes 6-8: Reductase membrane preparation. *CYP2C8*; Lane 9: Protein marker; Lane 10: Expression at zero time; Lane 11: Expression at 48 hours (before membrane preparation); Lanes 12-14: Expression at 48 hours from membrane preparation (concentrated *CYP2C8* protein)

Immunodetection of P450 and the Reductase

Western blot was performed on the *CYP2C8* and reductase protein to confirm the protein produced. A single protein bands were detected for *CYP2C8* and reductase with the expected sizes about 50 kDa and 80 kDa respectively (Figure 3). This confirmed that the *CYP2C8* and Reductase were successfully expressed in *E.coli* cells.

FIGURE 3 Immunobloting for the determination of *CYP2C8* and reductase protein. Protein bands for *CYP2C8* and reductase were positively detected with sizes about 50kDa and 80 kDa respectively.

HPLC method development for amodiaquine and desamodiaquine.

The system detected amodiaquine and its metabolite desamodiaquine with retention times at 6 and 9 minutes respectively. This method will be used for the catalytic studies of *CYP2C8* in later part of this study.

Discussion

The population study of genetic polymorphisms requires rapid, inexpensive, automated methods as it involves the analysis of many samples. We perform multiplex PCR to increase the diagnostic capacity of PCR. In this study, we designed a two step PCR with three pairs of primers to amplify exon 3, exon 5 and exon 8 separately in the first PCR instead of just one pair of primers in 3 different reactions. The two steps nested PCR was designed to give more specific amplification of *CYP2C8* due to close homology of *CYP2C8* and *CYP2C9*.

We failed to detect genetic variation among our Malays and Chinese subjects. However, among the Indians, *CYP2C8*2* and *CYP2C8*3* were found. Thus *CYP2C8* polymorphism is very well conserved among all the 3 races in Malaysia, suggesting that the polymorphic sites were not the hot spots. The occurrence of *CYP2C8* polymorphism in very few other populations also suggest that *CYP2C8* may have evolved much later than other CYP2C members such as *CYP2C9* and *CYP2C19*. Another reason for *CYP2C8* to be well conserved is the fact that it produces an important enzyme involved in the metabolism of physiologically important endogenous compounds such as arachidonic acid. As the major enzyme in the liver and kidney which metabolizes arachidonic acid to biologically active epoxyeicosatrienoic acids (EETs), *CYP2C8* may contribute substantially to circulating levels of EETs. EETs have various physiological roles. Therefore polymorphism in *CYP2C8* could therefore potentially produce dramatic physiological and pathological changes in human (Dai *et al.*, 2001). Thus, to be protected from potential myocardial infarction and hypertension, the *CYP2C8* gene must be very well conserved in the surviving individuals.

Heterologous expression of the P450 enzymes has been important for the characterization of functional properties as

purification of these enzymes from natural sources can be difficult. Although a number of cell systems have been employed for the heterologous expression of P450s, *Escherichia coli* offers a number of advantages if significant expression can be achieved, owing to its ease of manipulation and the low cost of culture (Richardson *et al*., 1995). *CYP2C8* was expressed in E.coli as a chimeric enzyme in which a portion of the N-terminal membrane anchoring 2C8 sequence was replaced with a modified sequence derived from P450 17α (Gillam E.M.J., 1998). The nucleotide sequence encoding the N-terminus of P450 17α was modified to achieve a high level of expression of P450 17α in E.coli. Alteration of the first eight codons of P450 17α had been reported to increases gene expression and minimizes the potential for the formation of stable secondary structure of the corresponding RNA transcript (Richardson *et al*, 1993).

Conclusion

We conclude that we have successfully developed and optimized a multiplex PCR method based on variant specific PCR techniques for a rapid, specific and simultaneous detection of *CYP2C8* allelic variations. This method would be suitable for use in clinical and population studies of *CYP2C8* polymorphism. Thus far, we only detected *CYP2C8*2* and *CYP2C8*3* in Malaysian Indians. *CYP2C8* and reductase were successfully expressed as proteins with sizes about 50kDa and 80kDa respectively with their incubation period for 48 and 24 hours respectively at 30ºC. The *in vitro* enzyme model will be used for elucidation of metabolic pathways of new chemical entities and correlation with in vivo drug metabolism capabilities.

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