Regular Article

Functional Role of Ile264 in CYP2C8: Mutations Affect Haem Incorporation and Catalytic Activity

Rajinder SINGH1, Jonathan G. TING1, Yan PAN1, Loy Kek TEH2, Rusli ISMAIL3 and Chin Eng ONG1,*

1School of Pharmacy and Health Sciences, International Medical University, Kuala Lumpur, Malaysia
2Faculty of Pharmacy, Universiti Teknologi MARA, Selangor, Malaysia
3Pharmacogenetics Research Group, Institute for Research in Molecular Medicine, Universiti Sains Malaysia, Kelantan, Malaysia

Summary: The work described in this study aimed to express CYP2C8 wild-type and mutant proteins in bacterial expression system and to use the expressed proteins to investigate the structural and functional consequences of a reported allele CYP2C8*4 (carrying Ile264Met substitution) on protein activity. Ile264 was replaced by three different amino acids resulting in three mutant constructs, 2C8I264M, 2C8I264R and 2C8I264D. The presence of isoleucine at position 264 in CYP2C8 was found to be important for proper haem insertion and protein folding; whereas bulkier or charged residues were highly disruptive resulting in inactive proteins with minimum spectral and catalytic activities. This was evidenced from the low levels of Soret peak at 450 nm and negligible levels of tolbutamide methylhydroxylase activity. Kinetic study using paclitaxel indicated that all three mutants exhibited only 9.7 to 35.4% of the activity level observed in the wild-type. In addition, the mutants were more sensitive to proteinase K digestion, indicating a possible alteration of conformation. The combined effects of protein instability and compromised catalytic activity resulted in defective CYP2C8 protein which may have clinical implications in carriers of CYP2C8*4, particularly in terms of their capacity to clear potent drugs and their susceptibility to adverse drug reactions.

Keywords: genetic polymorphism; CYP2C8*4; site-directed mutagenesis; paclitaxel; pharmacogenomics

Introduction

Interindividual variability in drug metabolism is a major cause of altered drug response. In many cases, such variability is linked to polymorphisms in genes encoding drug-metabolizing enzymes. Among the enzyme subfamilies that comprise the cytochrome P450 (CYP) system, CYP2C assumes particular importance in drug metabolism. CYP2C enzymes account for about 20% of the total CYP in liver microsomes1) and are involved in the metabolism of about 20% of clinically used drugs. All members of the CYP2C gene subfamily, namely CYP2C8, CYP2C9, CYP2C18 and CYP2C19, are polymorphic.2)

CYP2C8 genetic polymorphism has only recently been described (an updated list of allelic variants is available at http://www.imm.ki.se/CYPalleles/). Nine variant alleles, designated as CYP2C8*2 to *10, along with the wild-type CYP2C8*1, have been reported.3-6) Except for CYP2C8*5 and CYP2C8*7, which carry nucleotide changes resulting in early introduction of a translational stop codon leading to truncated proteins, the rest of the variants carry missense mutations causing amino acid substitutions in their primary sequences. The CYP2C8*2 allele has been reported primarily in Afro-American subjects, with an estimated allele frequency of about 18% in this population. Heterologously expressed, CYP2C8*2, was shown to cause impaired paclitaxel metabolism. In the same study, the CYP2C8*3 variant, with an allele frequency of 13% in Caucasians and 2% in
Afro-Americans, was found to exhibit markedly defective metabolism of paclitaxel and arachidonic acid, corresponding to only 15% and 35% of the wild-type CYP2C8*1 activity, respectively.\(^3\) Both CYP2C8*2 and CYP2C8*3 have also been reported recently in Asian populations.\(^4\) The CYP2C8*5 variant has a base deletion at position 475 resulting in a frameshift at codon 159 and the early introduction of a stop codon at residue 177. The predicted enzyme lacks 64% of the protein structure, including the haem binding site and 5 of 6 substrate recognition sites and, therefore, is most probably inactive.\(^5\) CYP2C8*5 was detected in a population of Japanese subjects with an allele frequency of just 0.25%\(^6\). CYP2C8*6 to *10 were described by Hichiya and coworkers\(^6\) in a study involving 201 Japanese subjects. CYP2C8*7 has a base substitution resulting in an early stop codon leading to expression of a truncated protein lacking 60% of the C-terminal region, including the haem-binding site. The other four variants all encode full-length proteins carrying a single amino acid substitution that compromise paclitaxel metabolism to a variable extent.

The CYP2C8*4 allele has a frequency of 8% in Caucasians, but has not been detected in Asian populations.\(^3\)\(^,\)\(^7\)\(^,\)\(^8\) The protein product CYP2C8*4 has Met for Ile at position 264 and no assessment of its activity toward CYP2C8 probe substrates has been made either in vitro or in vivo. Hence the functional and structural consequences of this mutation remain to be elucidated. In this study, three site-specific mutants (I264M, I264R and I264D) were generated in order to replace Ile264 with bulkier, positively and negatively charged residues, using the full-length CYP2C8 construct in a bacterial expression system. The wild-type and mutant CYP2C8s were functionally characterised for enzyme activities (tolbutamide methylhydroxylation and paclitaxel 6α-hydroxylation), susceptibility to protease degradation, and reduced CD-difference spectra. The results suggest that Ile264 is important for proper haem insertion and protein folding in CYP2C8; and mutation in this amino acid has caused structural perturbation resulting in inactive proteins with minimum spectral and catalytic activities.

### Materials and Methods

**Materials:** GeneEditor\(^\text{TM}\) in vitro site-directed mutagenesis system and isopropyl-β-D-thiogalactopyranoside (IPTG) were purchased from Promega (Madison, WI, US). Paclitaxel and 6α-hydroxypaclitaxel were acquired from BD Biosciences (Woburn, MA, US). E. coli DH5α cells, oligonucleotide primers, Luria-Bertani and Terrific broth media were purchased from Invitrogen Corporation (Carlsbad, CA, US). HPLC-grade solvents and reagents were from Fischer Scientific (Loughborough, Leicestershire, UK). All other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, US).

**In vitro site-directed mutagenesis of CYP2C8 cDNA:** Site-directed mutagenesis of the CYP2C8 cDNA was performed using the GeneEditor\(^\text{TM}\) in vitro site-directed mutagenesis system according to the manufacturer’s protocol (Promega, Madison, WI, US). Briefly, the mutagenesis reaction involved annealing of the selection oligonucleotide (provided by the manufacturer) and the mutagenic oligonucleotide to the DNA template, followed by synthesis of the mutant strand with T\(_4\) DNA polymerase and T\(_4\) DNA ligase. Selection oligonucleotide(s) provided with the system encode mutations that alter the ampicillin resistance gene, creating an additional resistance to the GeneEditor\(^\text{TM}\) Antibiotic Selection Mix, which was also provided by the supplier. During the mutagenesis reaction, the selection oligonucleotide was annealed to the DNA template at the same time as a mutagenising oligonucleotide. Subsequent synthesis and ligation of the mutant strand links the two oligonucleotides. The resistance of the GeneEditor\(^\text{TM}\) Antibiotic Selection Mix encoded by this mutant DNA strand thus facilitated selection of the desired mutation. The heteroduplex DNA was then transformed into the repair minus E. coli strain BMH 71–18 mutS, and these bacteria were grown in selective media to select for clones containing the mutant plasmid. Plasmids resistant to the novel GeneEditor\(^\text{TM}\) Antibiotic Selection Mix were then isolated and transformed into the final host strain, JM109, using the same selection conditions. The DNA template used was pCW-CYP2C8(17a) vector that has been described previously.\(^9\) Table 1 lists the sequences of the oligonucleotides used for mutagenesis. Primer 2C8I264M contained ATG to ATG substitution that encoded Ile264Met substitution in the expressed CYP2C8 protein. Similarly, primers 2C8I264R and 2C8I264D contained the respective ATG to CGC and ATG to GAC substitutions allowing expression of mutant CYP2C8 proteins carrying the Ile264Arg and Ile264Asp substitutions. One of the clones obtained in each mutagenesis was subjected to DNA sequencing of the full-length cDNA to confirm that the desired mutation, but not additional mutations, was present in the clones.

**Table 1. Oligonucleotide primers used for site-directed mutagenesis in this study**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2C8 native sequence:</td>
<td>5′-GAT GTT ACC AAT CCT CGG GAC TTT ATC GAT TGC TTC CTG ATC-3′</td>
</tr>
<tr>
<td>Mutagenic oligonucleotide primers:</td>
<td></td>
</tr>
<tr>
<td>2C8I264M</td>
<td>5′-GAT GTT AAC AAT CCT CGG GAC TTT ATG GAT TGC TTC CTG ATC-3′</td>
</tr>
<tr>
<td>2C8I264R</td>
<td>5′-GAT GTT AAC AAT CCT CGG GAC TTT CGC GAT TGC TTC CTG ATC-3′</td>
</tr>
<tr>
<td>2C8I264D</td>
<td>5′-GAT GTT AAC AAT CCT CGG GAC TTT GAC GAT TGC TTC CTG ATC-3′</td>
</tr>
</tbody>
</table>

\(^a\) Nucleotides that were changed to introduce desired mutations are emboldened. The underlined nucleotides represent the native or altered Clal restriction sites in the sequences.
Co-expression of CYP2C8 and OxR in Escherichia coli: In order to achieve self-sufficient expression system, a two-plasmid co-expression system was adopted where CYP2C8 co-expressed with the NADPH-cytochrome P450 reductase (OxR) in the cell cultures. The cDNA coding for the rat NADPH-cytochrome P450 reductase (rOxR), used in all constructs, consists of the OmpA signal sequence fused upstream of the full length native rOxR sequence10) inserted in the plasmid pACYC184 as described by Boye et al.30) The pACYC184 plasmid has a different origin of replication and selective marker conferring compatibility with co-transformed pCW-based expression plasmids. The individual CYP2C8 plasmid constructs carrying the desired mutation, pCW-2C8I264M, pCW-2C8I264R and pCW-2C8I264D were subsequently co-transformed with pACYC-rOxR into DH5α cells. Overnight cultures (5 ml) grown in Luria-Bertani broth with ampicillin (50 mg l⁻¹) and chloramphenicol (50 mg l⁻¹) at 37°C were used to inoculate a 50 ml culture of Terrific broth containing 0.4 mg DH5α with pACYC-rOxR into DH5α cells. The expression of replication and selective marker conferring compatibility with co-transformed pCW-based expression plasmids was under the control of the lac promoter. The individual CYP2C8 plasmid constructs carrying the desired mutation, pCW-2C8I264M, pCW-2C8I264R and pCW-2C8I264D were subsequently co-transformed with pACYC-rOxR into DH5α cells. Overnight cultures (5 ml) grown in Luria-Bertani broth with ampicillin (50 mg l⁻¹) and chloramphenicol (50 mg l⁻¹) at 37°C were used to inoculate a 50 ml culture of Terrific broth containing 50 mg l⁻¹ ampicillin and 50 mg l⁻¹ chloramphenicol. Cultures were grown at 37°C with shaking (200 rpm) for 4 h, or until an optical density of approximately 0.7 at 600 nm was attained, at which time isopropyl-β-D-thiogalactopyranoside (1 mM) and δ-aminolevulinic acid (0.5 mM) were added. Cultures were subsequently grown at 30°C with shaking at 200 rpm for an additional 24 h. Cells were then harvested and membranes prepared according to an established protocol.11) Membranes were stored at −20°C in a 1:1 mixture of TES (Tris/EDTA/sucrose buffer) and distilled water. Membranes in which both CYP and OxR were expressed were diluted in water at 0°C to give an appropriate CYP concentration before addition to the reaction mixtures.

Enzyme assays: Tolbutamide methylhydroxylase activity was measured using a modification of a published protocol.12) A standard 0.5 ml incubation mixture contained 0.4 mg DH5α membrane protein, NADPH generating system (1 mM NADP, 10 mM glucose 6-phosphate, 2 IU glucose 6-phosphate dehydrogenase and 5 mM MgCl₂) and was incubated at 37°C for 120 min. Tolbutamide was dissolved in methanol and the final concentration of methanol in all assay mixtures was 1.5%. This concentration was shown in preliminary incubation experiments to have little effect on reaction rate of the assay. All reactions were initiated by the addition of NADPH-generating system. Reactions were terminated by placing the samples on ice followed by addition of 0.1 M phosphoric acid (1.0 ml). Samples were then extracted with 8 ml of hexane: chloroform: iso-amyl alcohol (1000:250:1 v/v) to remove unchanged tolbutamide. After centrifuging the mixture for 5 min at 1000 x g, the tube containing the mixture was placed in a −80°C freezer for 10 min and the organic layer was discarded. One hundred µl of chloropropamide (10 mg ml⁻¹), the internal standard, and 8 ml of diethyl ether were added to the thawed sample and extracted by vortex mixing. The sample was then centrifuged for 5 min at 1000 x g and then placed in a −80°C freezer for 15 min. The resulting organic layer was decanted into a conical tube and evaporated to dryness under a stream of N₂. The residue was reconstituted in 60 µl of the mobile phase, and 20 µl was injected onto the HPLC column. Chromatography was performed using an Inertsil™ C18 column (15 cm × 4.6 mm inner diameter, 4 micron particle size, GL Sciences, USA) and eluted with acetone:buffer (10 mM, pH 4.3; acetoniitrile (78:22) at flow rate of 2.0 ml min⁻¹. The column was connected to a Perkin Elmer HPLC Series 200 system comprising a pump and a UV detector. Absorbance was monitored at 230 nm. Under these conditions the retention times for hydroxytolbutamide and chloropropamide were 16.2 and 30.6 min, respectively. Standard curves for hydroxytolbutamide were constructed in the range of 4.5–25.0 µM. Unknown concentrations of hydroxytolbutamide were determined by comparison of hydroxytolbutamide: chloropropamide peak area ratios with those of the standard curve. Under this procedure the mean recovery of hydroxytolbutamide, calculated by comparing the peak area for extracted compound with that of an equal amount injected directly into the chromatograph, was 90.3 ± 6.3% for eight samples in the concentration range 5.0 to 20.0 µM. Within-day coefficients of variation for spiked incubation mixtures containing 8 and 20 µM hydroxytolbutamide (n = 5 at each concentration) were 9.1 and 4.7% respectively. The corresponding values for inter-day variation were 11.4 and 6.5% respectively. The effects of incubation time and protein concentration were also investigated. Hydroxytolbutamide formation was linear for incubation times to 150 min and for protein concentrations to 1.2 mg ml⁻¹.

Paclitaxel 6α-hydroxylase activity was determined by a modification of the published procedure of Crespi et al.13) Briefly, a standard 0.50 ml incubation mixture contained 10–50 pmol of the expressed CYP and NADPH generating system (1 mM NADP, 10 mM glucose 6-phosphate, 2 IU glucose 6-phosphate dehydrogenase and 5 mM MgCl₂) in 0.05 M phosphate buffer, pH 7.4. Paclitaxel was dissolved in ethanol and typically added to incubation mixtures from a 5 mM stock. The final concentration of paclitaxel was normally 50–100 µM, but for kinetic studies paclitaxel concentrations ranged from 15 to 100 µM. The final concentrations of ethanol in incubation mixtures were ≤ 2%. These concentrations were shown not to affect rates of paclitaxel metabolite formation in control experiments. All reactions were initiated by the addition of membrane protein and were carried out in air at 37°C in a metabolic shaker for 20 min. The reaction was terminated by the addition of 150 µl of acetonitrile and cooling on ice. The reaction mixture was then centrifuged at 10 000 x g in a microcentrifuge for 4 min to precipitate protein. Aliquots of the supernatant fraction (100 µl) were injected onto the HPLC for quantification of metabolite formation. The chromatography was performed using a Hypersil™ C18 column (25 cm × 4.6
described kinetic data. Experiments a single enzyme Michaelis-Menten model best
gram EZ-Fit (Perrella Scientific, Amherst, USA), and in all
further data for both assays were model-fitted using the pro-
Cytochrome P450 CYP2C8/9/19 polyclonal antibody
the membranes were reacted with the rabbit anti-human
TTBS (0.05% Tween 20 in Tris-buffered saline). Proteins on
lulose membranes which were treated in 5% nonfat milk in
fate-polyacrylamide gel electrophoresis and immunoblot-
ations were subsequently subjected to sodium dodecyl sul-
phenylmethylsulfonyl fluoride to each tube and incubated at
room temperature for 10 min. The digested protein prepa-
rations of 6α-hydroxypaclitaxel and paclitaxel were 22.0 and 25.7 min respectively. Standard curves for
hydroxyaipaclitaxel were constructed in the range 1.0–
00 μM using authentic metabolite. Unknown concentra-
tions of 6α-hydroxyaipaclitaxel were determined by compari-
son of the metabolite peak areas with those of the standard
curve. Using the procedure described, the intra-day
coefficients of variation were 6.3 and 3.0% for metabolite
centrations of 5.0 and 50.0 μM respectively. The corre-
sponding inter-day values were 9.1 and 5.0% respectively.
The mean recoveries of 6α-hydroxyaipaclitaxel ranged from
97.3 to 115.4% at two concentrations investigated (5.0 and
50.0 μM). Linearity studies indicated that the reaction rate
was linear with incubation times and protein concentrations
to 305 min and 1.05 mg ml⁻¹, respectively.
Rate of metabolite formation versus substrate concentra-
tion data for both assays were model-fitted using the pro-
gram EZ-Fit (Perrella Scientific, Amherst, USA), and in all
experiments a single enzyme Michaelis-Menten model best
described kinetic data.
Proteinase K digestion and immunoblotting of
CYP2C8 proteins: Bacterial membrane fractions (50 μg)
containing wild-type or mutant CYP2C8 proteins were in-
cubated with varying concentrations of proteinase K (0.05
mg ml⁻¹, 0.1 mg ml⁻¹, 0.2 mg ml⁻¹, and 0.4 mg ml⁻¹) at
37°C for 30 min in separate PCR tubes. The digestion was
carried out in 50 mM Tris-HCl (pH 7.5) containing 10 mM
CaCl₂. The reaction was stopped by addition of 4 mM
phenylmethylsulfonyl fluoride to each tube and incubated at
room temperature for 10 min. The digested protein prepa-
rations were subsequently subjected to sodium dodecyl sul-
ate-polyacrylamide gel electrophoresis and immunoblot-
ing. Briefly, proteins in the gel were transferred to nitrocel-
lulose membranes which were treated in 5% nonfat milk in
TTBS (0.05% Tween 20 in Tris-buffered saline). Proteins on
the membranes were reacted with the rabbit anti-human
cytochrome P450 CYP2C8/9/19 polyclonal antibody
(Chemicon™, USA) at 1 to 6000 dilution at room tempera-
ture for 1 h followed by three 5-min washes in TTBS. The
membranes were then soaked in peroxidase-labelled goat
anti-rabbit antibody (1 to 3000 dilution) at room tempera-
ture for 1 h before washing with TTBS. After draining ex-
cess solution, the membranes were overlaid with detection
reagents (4-chloro-1-naphthol) for band visualization. The
immunoblots were scanned and the intensity of each band
was measured by densitometry (GS-670 imaging densitome-
ter, Bio-Rad, USA), and the results were expressed as ar-
bitrary units of optical density.
Other methods: Protein concentrations were deter-
mined according to Bradford. The CYP content of mem-
brane fractions was determined spectrophotometrically.
The rate of reduction of cytochrome c was used as a mea-
sure of OxR activity in membrane fractions. All statistical
analyses were carried out with the SPSS statistical program
(SPPS Inc., USA).

Results
Expression of CYP2C8 proteins in Escherichia coli:
DH5α frozen stocks harbouring each of the CYP2C8
mutant constructs were grown and membrane fractions
prepared. The yield and expression levels of recombinant
proteins from routine culturing using 300 ml culture media
are shown in Figure 1. Cytochrome c reductase activity, a
measure of OxR expression level, did not differ significantly
among the four preparations. The OxR levels achieved in
preparations containing mutant proteins ranged from 98.9
to 129.4% of the level achieved in the wild-type protein.
Control protein (bacterial membrane isolated from stocks
transformed with pCWori + plasmid) on the other hand
showed an OxR activity of 16.9 ± 6.9 nmol/min/mg, a mere

![Figure 1](image)

**Fig. 1. Expression of CYP2C8 and OxR proteins in E. coli cells**
The cytochrome c reductase activity and the spectral content were
determined as described under ‘Materials and Methods’. The
reductase activity and spectral content for 2C8WT were 1016.2 ±
217.0 nmol min⁻¹ mg⁻¹ and 1873.8 ± 653.3 pmol mg⁻¹
respectively. Bars represent mean values ± S.D. based on three inde-
dependent determinations.
1.7% of the wild-type figure, indicating a low constitutive OxR level in the bacterial cells. The yields of spectrally-active CYP obtained from the three mutant constructs, 2C8I264M, 2C8I264R and 2C8I264D, were very low compared to the wild-type protein ranging from 1.9 to 3.5% of the wild-type. Representative reduced-CO spectra determined in the four preparations including that of the wild-type (2C8WT) are shown in Figure 2. 2C8WT protein exhibited a typical CYP optical spectrum with a large Soret band at 450 nm (Fig. 2(A)). A relatively minor absorbance peak was observed at 420 nm indicating low level of inactive CYP protein in the preparations. Control cells which were transformed with blank pCW vector (without any gene of interest) did not show any absorbance peak. In contrast to the wild-type preparation, all the mutant proteins showed only minor peaks at 450 nm with relatively large peaks at 420 nm (Fig. 2(B)). This indicated that a large proportion of the proteins in mutant preparations were that of denatured or inactive forms.

Proteinase K digestion of the CYP2C8 wild-type and mutant proteins: Digestion of CYP2C8 proteins expressed in DH5α membranes with increasing concentrations of proteinase K was carried out to probe the protein stability of expressed proteins. Proteinase K concentrations of 0.05, 0.1, 0.2 and 0.4 mg ml⁻¹ were chosen to evaluate the rate of degradation in CYP2C8 proteins. As shown in Figure 3, 2C8WT did not show any appreciable degradation as evidenced from approximately the same band density when the proteins were digested with increasing proteinase K concentrations. In contrast, the three mutants, 2C8I264M, 2C8I264R and 2C8I264D were apparently sensitive to digestion K digestion as indicated by the decreasing band densities in the presence of increasing proteinase K concentrations (especially at 0.2 and 0.4 mg ml⁻¹). The pattern of density changes however was not uniform in the

![Fig. 2. Reduced-CO difference spectra showing expression of the (A) CYP2C8 wild-type and (B) mutant proteins](image)

Spectrum for the control cells (bacterial membrane isolated from stocks transformed with pCWori + plasmid) was included in (A) for comparison.

![Fig. 3. Proteinase K digestion of wild-type CYP2C8 and its mutants expressed in DH5α cells](image)

Bacterial membranes (50 μg of proteins) containing either wild-type (A), or the mutant proteins, 2C8I264M (B), 2C8I264R (C) and 2C8I264D (D) were digested with various concentrations of proteinase K (from 0.05 to 0.4 mg ml⁻¹) as indicated on top of the lanes at 37°C for 30 minutes. The digestion products were separated by gel electrophoresis and subjected to immunoblotting as described under ‘Materials and Methods’.
Mean the indicated proteinase K concentrations. Each bar is shown as a comparison experiments (n = 3), analysed and between-group statistical comparisons were made. Figure 4 shows the densitometric readings of CYP2C8 wild-type and mutant enzymes following treatment with proteinase K. No statistical difference was observed in all lanes for 2C8WT whereas in the case of 2C8I264M, 2C8I264R and 2C8I264D, there were statistically significant differences when comparisons of between-group variation were made for each of the proteins (P < 0.01, one-way ANOVA). Further post-hoc analysis using a 2-sided Dunnett’s t-test was carried out to compare the densities of treated bands with that of the control (the first band with no proteinase K added) for each mutant. As indicated in Figure 4, statistically significant differences were noted for bands at higher proteinase K concentrations in all the three mutants (i.e. 0.2 and 0.4 mg ml⁻¹ for 2C8I264M, 0.2 and 0.4 mg ml⁻¹ for 2C8I264R, and 0.1 and 0.2 mg ml⁻¹ for 2C8I264D). Overall, the proteinase K assay showed that while the wild-type CYP2C8 was relatively stable towards proteinase K digestion, the three mutants were sensitive to the protease treatment, thus indicating their inherent instability of the protein structures possibly due to the mutations introduced in their sequences.

**Enzymatic activities of the CYP2C8 wild-type and mutant proteins:** Although CYP2C8 was shown to play a minor role in tolbutamide hydroxylation, it has been used as an activity marker in a number of studies.17,18) Catalytic activity of the 2C8WT was examined at tolbutamide concentration ranging from 400 to 2000 μM. Hydroxytolbutamide formation by 2C8WT was found to best fit the single Michaelis-Menten kinetic, with apparent Km and Vmax values of 1157.6 ± 47.9 μM and 6.5 ± 0.6 pmol min⁻¹ nmol CYP⁻¹ respectively (n = 3). These data were in the similar range as reported for CYP2C8 in the literature.17,18) Subsequently, tolbutamide hydroxylase activity was determined for the mutant CYP2C8s in comparison to 2C8WT at a fixed substrate concentration (1 mM). All of the mutants lacked activity (see Table 2). The limit of detection, defined by a signal to noise ratio of 3, was 0.3 μM in our study. This corresponds to 0.03 nmol of tolbutamide metabolite on the column in 100 μl of injection volume, which is within the range reported by other investigators.19,20)

In order to examine further the mutant catalytic activity, paclitaxel 6α-hydroxylation was subsequently used as the

### Table 2. Enzymatic activities of CYP2C8 wild-type and mutants

<table>
<thead>
<tr>
<th>Enzyme assays</th>
<th>Expressed CYP2C8 proteinsᵃᵈ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tolbutamide methylhydroxylation</td>
<td>2C8WT</td>
</tr>
<tr>
<td>1.0 mM</td>
<td>1.46 ± 0.18</td>
</tr>
</tbody>
</table>

| Paclitaxel 6α-hydroxylation | | | |
| 70.0 μM | 25.09 ± 3.06 | 3.82 ± 0.34 | 8.89 ± 0.41 | 4.14 ± 0.33 |
| (100.0) | (15.2) | (35.4) | (16.5) |
| 15.0 μM | 17.78 ± 4.64 | 1.83 ± 1.03 | 4.25 ± 0.68 | 1.73 ± 1.32 |
| (100.0) | (10.3) | (23.9) | (9.7) |

ᵃ Values are shown as the mean ± SD from three independent experiments in pmol min⁻¹ nmol CYP⁻¹ for tolbutamide methylhydroxylation and pmol min⁻¹ pmol CYP⁻¹ for paclitaxel 6α-hydroxylation. ᵇ ND—not detectable ᶜ Second figures in parentheses represent the percentage. ᵈ No detectable activity was observed in control protein (bacterial membrane isolated from stocks transformed with pCWori + plasmid) for both assays.

**Fig. 4.** Densitometric readings of immunoblot bands for CYP2C8 wild-type and mutant proteins

Results are mean band intensity expressed as arbitrary units (AU) obtained from immunoblots of proteins (50 μg each) treated with the indicated proteinase K concentrations. Each bar is shown as mean ± SD from 3 independent experiments. **P < 0.05; ***P < 0.01 versus the respective controls (the first bars with no treatment with proteinase K).
second marker. As expected, 2C8WT exhibited high catalytic activity towards the substrate. 6α-Hydroxypaclitaxel formation followed Michaelis-Menten kinetics, showing apparent $K_m$ and $V_{max}$ values of $11.4 \pm 1.2$ $\mu M$ and $34.8 \pm 4.9$ pmol min$^{-1}$ pmol CYP$^{-1}$ respectively ($n=3$). The $K_m$ value was in the similar range as those previously reported for heterologously expressed CYP2C8 (7–10 $\mu M$) and human liver microsomes (12–15 $\mu M$). Catalytic activities of the three mutant proteins, 2C8I264M, 2C8I264R and 2C8I264D were subsequently examined and compared with the wild-type protein. Two concentrations, 70 and 15 $\mu M$, were chosen for investigation of catalytic activities to reflect the high and low substrate concentrations. Concentrations higher or lower than these two were not attempted due to the problems of substrate solubility and limit of detection of the HPLC system. Hence full kinetic characterization to derive kinetic parameters (i.e. $K_m$ and $V_{max}$) were not carried out for the mutants. Table 2 shows the paclitaxel 6α-hydroxylase activities of the mutants compared to the wild-type. All the substitutions at Ile264 resulted in greatly reduced activity in CYP2C8. At 70 $\mu M$, the catalytic activities for 2C8I264M, 2C8I264R and 2C8I264D were 15.2, 35.4 and 16.5% of the wild-type respectively. The enzymatic activities of the mutants at 15 $\mu M$ paralleled that of 70 $\mu M$ with activity levels of 10.3, 23.9 and 9.7% of the wild-type. Kinetics characterisation was not carried out at paclitaxel concentrations higher than 100 $\mu M$ as these concentrations are close to the limit of solubility in aqueous media. Similarly, incubation at concentrations lower than 15 $\mu M$ was not performed due to the limit of detection of the HPLC system. The limit of detection, defined by a signal to noise ratio of 3, was 0.5 $\mu M$ in our study. This corresponds to 0.05 nmol of 6α-hydroxypaclitaxel on the column in 100 $\mu l$ of injection volume, which corresponds closely the limit of detection reported by others.

### Discussion

The overall aims of this study were to express CYP2C8 mutant alleles carrying Ile264Met, Ile264Arg and Ile264Asp in E.coli cells, and to characterise protein catalytic activity using paclitaxel and tolbutamide as the substrate probes and determine the protein integrity and stability using reduced-CO spectrophotometric and proteinase K assays. These aims were achieved with the successful co-expression of OxR with 2C8WT and the three mutants (2C8I264M, 2C8I264R and 2C8I264D) in separate membrane preparations in DH5α cells.

CYP2C8*4 carrying Ile264Met substitution is of interest because no detailed investigation has been conducted to investigate the structural and functional effects of the mutation on protein activity. Analyses on the CYP2C8 crystal structure and several homology models have indicated that Ile264, located in helix H, is unlikely to bind haem or form part of the substrate binding or contact domains. Helix H is located on protein surface away from regions involved in haem and substrate binding, thus residues in this helix are unlikely to play critical role in direct substrate catalysis. However, numerous studies have shown that amino acids residing in non-SRS (substrate recognition site) regions were in fact important for proper functions and folding of many CYP isoforms. We therefore sought to investigate whether Ile264 in CYP2C8 could play a role in other aspects apart from catalysis.

The first evidence of structural influence of Ile264 on CYP2C8 was revealed by the CO-reduced spectra of the mutant proteins. All amino acid substitutions resulted in diminished yields of CYP2C8 holoenzyme when expressed in bacterial system. The level achieved in 2C8WT (1873.8 pmol/mg membrane) was high and comparable to the levels reported in the literature for CYP2C8 and other isoforms. The CO-reduced difference spectra of the three mutants indicated minor peaks at 450 nm and relatively much larger peaks at 420 nm (Fig. 2). This indicated that a large proportion of the proteins in mutant constructs were in denatured forms that were not folded properly and these failed to bind haem. Without proper folding and haem incorporation into the apoenzymes, these proteins are expected to be nonfunctional and catalytically inactive even though they may be expressed at full length as detected on immunoblots (Fig. 3). On the other hand, 2C8WT showed the typical optical spectrum with a large absorption peak at 450 nm. This indicated that a large proportion of the protein was holoenzyme which exhibited proper protein folding and haem incorporation. The inability of expression observed in the mutants suggested that the Ile264 residue is possibly involved in proper folding of the protein, at least when expressed in prokaryotic cells.

Proteinase K assay is commonly used in protein stability studies and has been applied to study the effect of amino acid mutations on the stability of some CYP isoforms. Hsu and coworkers investigated the stability of wild-type and mutant P450c21 proteins using a protocol similar to that described in this study. They found increased sensitivity of the mutants to proteinase K digestion when compared to the wild-type enzyme. The result from our study was consistent with these previous results, that is, 2C8I264M, 2C8I264R and 2C8I264D exhibited significant degree of degradation in comparison to wild-type CYP2C8. Our observation was based on visual inspections of band intensity of the proteins on the immunoblots which was followed by a more objective measurement of band density using densitometer. Densitometric readings in the mutants, in general showed statistically significant lower densities when compared to the wild-type. The proteolytic sensitivity towards proteinase K however differed among the three mutants. Although 2C8I264M and 2C8I264R showed increased rate of degradation in the presence of increasing protease concentration, 2C8I264D unexpectedly resisted digestion at higher concentration. Proteinase K is known to work optimally at certain concentration range, typically 0.05–0.2
mg ml\(^{-1}\),\(^{31,32}\) At higher concentration, the proteinase K:protein ratio may not be optimum for protease action. It is possible that this ratio changes with different proteins showing different folding patterns, and thus the effect seen in this study may be protein-specific. Considered collectively, the spectra and data from the proteinase K assay demonstrated that the introduced mutations had affected to a great extent the ability of the mutant proteins to bind haem and to properly fold into stable tertiary structures. This implied that mutations introduced at amino acid 264 had caused certain degree of conformational change making them susceptible to protease digestion. As residue 264 is located in helix H on the surface, it is likely that mutations at this location may have caused partial unfolding and exposed certain sections of the polypeptide chain for proteolytic activity by protease K. Initial digestion on the surface may have resulted in generation of proteolytic fragments which were then further digested to completion after disruption of the tertiary structure of the protein. This is evidenced from the lack of visible bands in mutant proteins which were treated with higher protease K concentrations (see lane 4 for 2C8I264M in Fig. 3). 2C8WT, on the other hand, was fold-ed properly into an intact structure with limited protease entry sites on its surface. Hence the negligible rate of degradation seen in our study.

Functional consequence of Ile264 mutations was further studied using paclitaxel 6α-hydroxylase and tolbutamide methyhydroxylase assays. Since the identification of CYP2C8 as the major isoform involved in 6α-hydroxylation, the major metabolic pathway for paclitaxel, was published,\(^{29}\) this drug has established its role as a unique activity marker for the isoform and has been used in a number of studies to investigate CYP2C8 activity.\(^{3,33,34}\) Tolbutamide has also been used in some studies although CYP2C8 is known to play a minor role in its methyhydroxylation.\(^{18}\) Our results indicated that mutations at Ile264 led to the loss of tolbutamide methyhydroxylation and substantial reduction of paclitaxel 6α-hydroxylase activities in CYP2C8, confirming the important of Ile residue in this isoform.

It is apparent from this study that mutations in helix H at position 264 may have affected the folding of these neighbouring secondary structures, hence the effect on protein stability and haem binding. The importance of Ile264 is also evidenced from the fact that this residue is highly conserved among members from the CYP2 family in the location (see Fig. 5). Isoleucine is found at this position in 16 of the 20 members in CYP2 family with all human CYP2C isoforms (i.e. CYP2C8, CYP2C9 and CYP2C19) having Ile264. Moreover, the three mutations studied here involved non-conservative changes to Ile264, from relatively small residue to a hydrophobic one in the case of 2C8I264M, a change to positively charged residue in 2C8I264R, and a change to negatively charged residue in 2C8I264D. Substitution with amino acids of different physical characteristics (in terms of mass, volume and charge nature) is expected to have greater influence on structural integrity of the protein than the conservative substitutions. Our spectral data seem to suggest that these introduced substitutions have caused alteration in association of the various neighbouring structures surrounding helix H leading to improper protein folding, failure in haem binding and formation of the denatured apoproteins as evidenced from the large absorbance peak at 420 nm. Data from both proteinase K and enzyme assays, when considered collectively, has further substantiated the structural role played by Ile264 in maintaining intact structure of the enzyme. It is likely that Ile264 together with other residues in helix H in their native configuration are able to provide a folding motif that lock other neighbouring secondary structures for proper haem and substrate binding. The three-dimensional structure of CYP2C8 as revealed in the X-ray crystallography illustrates that helix H is located adjacent to many other structures that form part of the substrate access and binding sites, including the helices F, F', G and I.\(^{27}\) Consequently, introduction of a larger and charged side chain at the 264 position may have disrupted this part of the protein, causing altered association and spatial repositioning of these associated structures. Hence the steric arrangement of the haem group relative to enzyme would be disturbed, and the morphology of substrate access channel and binding pocket would be changed. This subtle rearrangement in the active site pocket, coupled with compromised haem binding, may be the reason for the impaired activities of the mutants observed in this study. In other words, Ile264 may be involved in 'long-range' interactions that are transmitted to residues in the surrounding structures which contact the haem and substrates. This type of interaction is not unique and has been reported for other CYP isoforms. For example,
and coworkers were able to demonstrate that substituting a residue located far from the haem moiety in the active site, Glu351, a residue located in helix K of CYP21A2, has been found to be important for haem binding even though it is located far from the haem moiety in the active site. Glu351Lys mutation introduced to the isoform was shown to result in the loss of enzyme activity towards the substrate 17α-hydroxyprogesterone. In another study involving CYP2A13, He and coworkers were able to demonstrate that substitutions made at amino acids Ser208, His372, and Pro465 had resulted in reduced catalytic activities towards the substrate nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in the mutants. These residues were known to be located far from the active site and did not contact the bound substrate in the three-dimensional model of CYP2A13. Results from these studies are consistent with our findings and indicate that residues not involved in substrate and haem binding may play important functional or structural role in CYP catalysis.

In short, our study with this single CYP2C8 variant (CYP2C8*4) underscores the importance of investigating the functional consequences of genetic polymorphisms at the level of the proteins for prediction of potential consequences in clinical settings. Polymorphic alleles of CYP2C8 (including CYP2C8*4) may potentially lead to variation in clinical response and the toxicity of the anticancer drug paclitaxel and other drugs metabolized by CYP2C8, such as the antidiabetic drug rosiglitazone. Of relevant importance is that many of the CYP2C8 substrates are potent and commonly used drugs. Any factor such as genetic polymorphism in the drug metabolic pathways could lead to variation in plasma level, and hence occurrence of undesirable adverse effects. The results of these types could be used to increase efficacy of some treatment regimens and decrease the extent and severity of adverse drug reactions.

Acknowledgments: The authors thank Professor John Miners (Flinders University, Adelaide, Australia) and Professor Donald Birkett (Johnson & Johnson Research Pty Ltd., Sydney, Australia) for providing the vectors pCW-CYP2C8(17α) and pACYC184-rOxR, and for their helpful discussions in manuscript preparation.

References


18) Veronese, M. E., Doecke, C. J., Mackenzie, P. I., McManus, M.
23) Taniguchi, R., Kumai, T., Matsumoto, N., Watanabe, M., Ka-
22) Melet, A., Marques-Soares, C., Schoch, G. A., Macherey, A. C.,
20) Hansen, L. L. and Brouns, K.: Quantitative determination of tol-