Relevance of non-synonymous CYP2C8 polymorphisms to 13-cis retinoic acid and paclitaxel hydroxylation

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Running title page

Running title: Effect of CYP2C8 SNPs on 13cisRA and paclitaxel metabolism

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Abreviations: CYP, Cytochrome P450; 13cisRA, 13-cis retinoic acid; LCMS, Liquid chromatography-mass spectrometry.
Abstract
Cytochrome P450 (CYP) 2C8 has a major role in the metabolism of the anticancer agents 13-cis retinoic acid (13cisRA) and paclitaxel. There is evidence that polymorphisms in the CYP2C8 gene contribute to observed inter-individual differences in paclitaxel metabolism. However, no studies have been performed to determine the relevance of CYP2C8 polymorphisms to 13cisRA metabolism. In the current study, the effect of two common nonsynonymous CYP2C8 polymorphisms, CYP2C8*3 (R139K and K399R) and *4 (I264M), on the metabolism of 13cisRA and paclitaxel was examined using an *E. coli* expression system with co-expression of human cytochrome P450 reductase. No statistically significant differences in the level of 13cisRA 4-hydroxylase activity were associated with either CYP2C8 allelic variant compared to the wild-type CYP2C8.1 enzyme. Furthermore, no differences were observed for the CYP2C8.3 or CYP2C8.4 enzymes with respect to paclitaxel 6α-hydroxylase kinetics compared to wild-type CYP2C8.1. However, when the effects of the individual polymorphisms making up the *CYP2C8*3 allele were considered, a significantly lower level of paclitaxel 6α-hydroxylase activity was associated with the K399R enzyme. A lower level of activity was also seen for the R139K enzyme, although this difference was not significant. No differences were observed with respect to 13cisRA 4-hydroxylase activity. We conclude that common CYP2C8 polymorphisms are unlikely to explain reported interindividual variation in 13cisRA or paclitaxel pharmacokinetics.
Introduction

Chemotherapy with 13-cis retinoic acid (13cisRA, isotretinoin) is integral to the treatment of high-risk neuroblastoma (Matthay et al., 1999; Matthay et al., 2009). Previous studies have demonstrated a high degree of inter-patient variability in the pharmacokinetics and metabolism of 13cisRA (Veal et al., 2007). Thus, identification of factors predictive of 13cisRA pharmacokinetics and metabolism may allow the optimization of 13cisRA therapy.

Several cytochromes P450 (CYPs) have been reported to catalyse the oxidation of 13cisRA, including CYPs 3A7, 2C8, 4A11, 1B1, 2B6, 2C9, 2C19 and 3A4 (Chen et al., 2000; Marill et al., 2002). Based on both hepatic expression and overall activity, CYP2C8 is considered to be a major isoform involved in 13cisRA metabolism. Indeed, CYP2C8 plays a major role in the metabolism of a number of therapeutically important drugs and is the principal enzyme responsible for the oxidative metabolism of the anticancer agent paclitaxel (Rahman et al., 1994). To date, sixteen CYP2C8 alleles have been described (www.cypalleles.ki.se), two of which, CYP2C8*3 and *4, include non-synonymous polymorphisms commonly seen in Caucasians (Dai et al., 2001; Bahadur et al., 2002; Hennigsson et al., 2005). Both the *3 and *4 alleles have been associated with a lower rate of paclitaxel metabolism, based on in vitro approaches using expressed enzymes or liver microsomes, although not all studies agree on the extent of this effect (Dai et al., 2001; Soyama et al., 2001; Bahadur et al., 2002; Taniguchi et al., 2005; Parikh et al., 2007; Rodriguez-Antona et al., 2008; Singh et al., 2008). More recent pharmacokinetic studies with repaglinide and rosiglitazone report that individuals heterozygous for CYP2C8*3 show higher drug clearance, suggesting that the effects of this allele on enzyme activity might be substrate dependant (Niemi et al., 2003; Kirchheiner et al., 2006). In contrast, studies on paclitaxel metabolism in cancer patients found no difference in clearance between CYP2C8*3 heterozygotes and wild-type individuals (Hennigsson et al., 2005). While less information is
available on effects of the rarer CYP2C8*4, no significant change in paclitaxel metabolism in liver microsomes (Bahadur et al., 2002) or differences in repaglinide or paclitaxel pharmacokinetics compared with wild-type were seen in heterozygotes (Niemi et al., 2003).

In view of the lack of clarity on the functional effects of polymorphisms in CYP2C8*3 and *4 and the absence of data on their relevance to 13cisRA metabolism, we undertook a further assessment using an E. coli expression system with 13cisRA and paclitaxel as substrates. Our aim was to provide a basis for future clinical pharmacogenetic studies on 13cisRA pharmacokinetics.
Materials and Methods

Materials

All solvents and chemicals were obtained from commercial sources. A CYP2C8*4 cDNA clone was kindly provided by Dr Frank J. Gonzalez of the National Institute of Health, Bethesda, US. The expression vector pB13 and a human P450 reductase cloned in pACYC184 were kindly provided by Dr Thomas Friedberg of the University of Dundee. Anti-human CYP2C8 and human cytochrome P450 reductase bactosomes were supplied by CYPEX (Dundee, UK). Anti-human cytochrome P450 reductase was purchased from Stressgen (Victoria, Canada).

Construction of expression plasmids

The CYP2C8 cDNA used was the CYP2C8*4 variant cloned into the bacterial expression plasmid p91023 (B) (Kimura et al., 1987). The cDNA was modified to include a sequence encoding the bacterial ompA leader sequence by two-step PCR, as previously described (Pritchard et al., 1997). PCR involved the use of reverse, linker and forward primers flanking the natural EcoRI site in this cDNA. PCR products were individually subcloned into pGEM®-T (Promega), prior to insertion into plasmid pB13 for expression in E. coli. Details of all PCR primers used for the subcloning are provided in Table 1. Base changes were introduced by site-directed mutagenesis using a QuikChange Multi Kit (Stratagene). The starting DNA template was the CYP2C8*4 variant which has a C to G base substitution at position 792. To change the G at position 792 to C, the mutagenic primer 5`-CAATCCTCGGGACTTTATcGATTGCTTCCTGATC was used. This “wild-type” cDNA was subsequently used as the template for all further reactions. To generate a CYP2C8*3 cDNA it was necessary to introduce two base changes, at positions 416 (G to A) and 1196 (C to T). The mutagenic primers 5`-

GAATTTTGGGATGGGAAGAaGAGCATTGAGGACCGTG and 5`-
CTGACTTCCGTGCTACATGAGAATTTCC were used for the changes at position 416 and 1196, respectively. In addition, two further cDNAs termed CYP2C8*1 (R139K) and CYP2C8*1 (K399R), which included either the 416 mutation only or the 1196 mutation only, were prepared. The presence of desired mutations as well as the complete sequence was confirmed by sequencing (MWG Biotech, Milton Keynes, UK).

Co-expression of recombinant CYP2C8 and P450 reductase in E. coli

CYP2C8 cDNA clones were co-expressed in E. coli with cytochrome P450 reductase. The reductase was modified with the bacterial leader sequence pelB and was cloned into plasmid pACYC184. E. coli strain JM109 was transformed with each expression plasmid, as described previously (Gillam et al., 1993).

CYP2C8 content was measured using Fe²⁺-CO vs. Fe²⁺ difference spectra (Omura and Sato, 1964). The yield of active P450 reductase was determined using a spectrophotometric assay to measure cytochrome c reductase (Strobel and Dignam, 1978). Protein concentrations were determined using a modified Bradford assay. Levels of recombinant CYP2C8 and P450 reductase were also assessed by Western Blot analysis using either rabbit anti-CYP2C8 or rabbit anti-P450 reductase antibody and HRP-linked donkey anti-rabbit IgG secondary antibody. Samples were run alongside a BenchMark Pre-Stained Protein Ladder (10 – 200 kDa). Detection was by enhanced chemiluminescence.

Determination of kinetic parameters for 4-hydroxy 13cisRA and 6α-hydroxy paclitaxel formation

Kinetic parameters for 4-hydroxy 13cisRA and 6α-hydroxy paclitaxel formation were determined using wild-type CYP2C8.1 and CYP2C8.1 (R139K), CYP2C8.1 (K399R), CYP2C8.3 and CYP2C8.4 constructs. Conditions for linearity with respect to time and protein concentration were optimized in preliminary studies. The incubation mixture consisted of recombinant CYP2C8 (10 pmol), 13cisRA (2.5 – 50 µM) or paclitaxel (2.5 – 15
µM), NADPH (1 mM), MgCl₂ (0.25 mM) made up to a final volume (200 µl) with phosphate buffer (0.1 M, pH 7.4). Incubations were carried out in a shaking water bath at 37°C for 30 min. Reactions, performed in duplicate, were initiated by the addition of NADPH and terminated with ice-cold acetonitrile (400 µl). Samples were centrifuged at 17,900 g for 5 min and supernatant (200 µl) was removed and evaporated to dryness. For incubations performed with 13cisRA, residue was reconstituted in 50:0.1 acetonitrile:glacial acetic acid (200 µl). For incubations performed with paclitaxel, residue was reconstituted in 75:0.1 acetonitrile:formic acid (200 µl). Substrate concentration and velocity data for 13cisRA oxidation were fitted by the hyperbolic Michaelis-Menten model to obtain $K_m$ and $V_{max}$ values. As an authentic standard was unavailable for 4-hydroxy 13cisRA, $V_{max}$ units were given as the peak area generated, as determined by LCMS analysis, in counts*sec per sec (c*s/s). For paclitaxel oxidation, substrate concentration and velocity data could not be fitted by the hyperbolic Michaelis-Menten model due to product inhibition at paclitaxel concentrations > 15 µM. For concentrations ≤ 15 µM, paclitaxel 6α-hydroxylase activity was linear. A comparison of catalytic activities of recombinant CYP2C8 allelic variants was performed by measuring the mean rate of formation of 6α-hydroxy paclitaxel at 10 µM. GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA) was used for all calculations.

**LCMS analysis**

Analysis of 13cisRA and 4-hydroxy 13cisRA was carried out using a Perkin Elmer LC System (Beaconsfield, UK) coupled to an API-2000 LC/MS/MS Triple Quadrupole Mass Spectrometer (Applied Biosystems, Warrington, UK), with Analyst software for data acquisition and analysis, as described previously (Armstrong et al., 2007). Since there was no authentic standard available for the metabolite, 4-hydroxy 13cisRA was synthesised as previously described (Samokyszyn et al., 2000). The identity of the metabolite was confirmed.
by chromatographic retention time and LCMS analysis, and its formation was quantified by chromatographic peak area. Quantification of paclitaxel and 6α-hydroxy paclitaxel was carried out as described for retinoids with the LC system coupled to an API-4000 LC/MS/MS Triple Quadrupole Mass Spectrometer. Separation was performed on an Inertsil ODS-2 column (50 x 4.6 mm, 5µm) (Waters Corp., Milford, MA, USA) by reversed-phase chromatography. The mobile phase was isocratic and consisted of 75% acetonitrile, 0.1% formic acid. Analysis was performed at a constant flow rate of 0.2 ml/min. All measurements were carried out in positive mode. The scan mode used multiple reaction monitoring with ion transitions of \( m/z \ 854.38 \rightarrow 569.20 \) for paclitaxel and \( m/z \ 870.47 \rightarrow 585.30 \) for 6α-hydroxy paclitaxel.

**Statistical analysis**

Data were analysed by one-way ANOVA or with the Pearson correlation coefficient, with \( P < 0.05 \) regarded as being statistically significant. Where applicable, Bonferroni corrections were applied.
Results and Discussion

Membranes contained high levels of CYP2C8 and P450 reductase, with content ranging from 117 to 278 pmol P450/mg protein and 170 to 191 nmol cytochrome c reduced per min/mg protein. A typical western blot showing E. coli membranes co-expressing each of the recombinant CYP2C8 allelic variants and P450 reductase is shown in Figure 1. Expression levels of each of the CYP2C8 proteins exceeded the typical CYP2C content measured in human liver microsomes, estimated at 60 pmol P450/mg protein (Inoue et al., 1994), and were consistent with the levels of P450 expressed in E. coli described elsewhere (Pritchard et al., 1998; Smith et al., 1998). The levels of P450 reductase expressed were slightly lower than the typical P450 reductase content of human liver microsomes, estimated at 230 nmol cytochrome c reduced/mg/min (Forrester et al., 1992). The use of membranes prepared by similar protocols from this E. coli expression system has been validated previously for 5 other CYPs and it was concluded that the kinetic parameters of these recombinant enzymes were similar to those for their human liver microsome counterparts (McGinnity et al., 1999). This direct comparison was not carried out for CYP2C8 but, as mentioned above, the levels of expression were also in general agreement with those reported for human liver microsomes, suggesting that the system is appropriate for the studies performed. Although cytochrome b5 has been shown to affect the activity of CYP enzymes with some substrates, the impact of this cofactor was not investigated here.

All CYP2C8 variants metabolised 13cisRA to 4-hydroxy 13cisRA. Typical Michaelis-Menten plots for 13cisRA oxidation by recombinant CYP2C8 allelic variants are shown in Figure 2. Apparent $K_m$, $V_{max}$ and $K_m/V_{max}$ values for CYP2C8.1, CYP2C8.1 (R139K), CYP2C8.1 (K399R), CYP2C8.3 and CYP2C8.4 are given in Table 2. No statistically significant differences in mean $K_m$, $V_{max}$, or $K_m/V_{max}$ values ($p > 0.05$) were observed for the individual variants. The $K_m$ values obtained were broadly in line with those
reported by others for the all-\textit{trans} and 9-\textit{cis} retinoic acid isomers using lymphoblastoid cell and baculovirus expression systems respectively (McSorley and Daly, 2000; Marill et al., 2002), but slightly lower than the 50 µM value for all-\textit{trans} retinoic acid reported by Marill \textit{et al} (Marill et al., 2000).

Although all CYP2C8 variants metabolized paclitaxel to 6\textalpha{}-hydroxy paclitaxel, formation of the metabolite declined with increasing concentration above 15 µM. Although limited solubility of paclitaxel may have contributed to this departure from Michaelis-Menten kinetics, the data were more consistent with the phenomenon of substrate inhibition which has been shown to occur previously for CYP2C8 with the substrate pioglitazone (Tornio et al., 2008). Comparison of catalytic activities of recombinant CYP2C8 allelic variants was performed by measuring the mean formation of 6\textalpha{}-hydroxy paclitaxel over a period of 30 min at 10 µM paclitaxel, which was within the linear range. Typical enzyme kinetic data for paclitaxel oxidation by recombinant CYP2C8 allelic variants are shown in Figure 3. The mean formation values of 6\textalpha{}-hydroxy paclitaxel for CYP2C8.1, CYP2C8.1 (R139K), CYP2C8.1 (K399R), CYP2C8.3 and CYP2C8.4 are given in Table 3. To account for between-experiment variation in kinetic assays, the mean rate of formation of 6\textalpha{}-hydroxy paclitaxel for each of the variants CYP2C8.1 (R139K), CYP2C8.1 (K399R), CYP2C8.3, CYP2C8.4 was subtracted from the mean rate of formation of 6\textalpha{}-hydroxy paclitaxel for the wild-type CYP2C8.1 for each experiment. Differences between variants in 4 independent experiments were tested by one-way ANOVA and were shown to be highly significant, ($p < 0.01$). Therefore each variant was tested against a mean of 0 (the expectation for no difference from wild-type CYP2C8.1) using a one-sample t-test and applying a Bonferroni correction by setting statistical significance to $P \leq 0.0125$ ($0.05/4$). No statistical significance was observed for the CYP2C8.1 (R139K) ($p > 0.0125$), CYP2C8.3 ($p > 0.05$) or CYP2C8.4 ($p > 0.05$) values, although CYP2C8.1 (R139K) gave a value close to significance. Statistical
significance was observed for formation of 6α-hydroxy paclitaxel by the CYP2C8.1 (K399R) allelic variant ($p < 0.01$). Previous studies using expression systems have shown a lower rate of activity of the recombinant CYP2C8.3 enzyme in the metabolism of paclitaxel, arachidonic acid and amodiaquine (Dai et al., 2001; Soyama et al., 2001; Parikh et al., 2007). Absence of activity of recombinant CYP2C8.4 enzyme towards paclitaxel has also been reported (Singh et al., 2008). The data presented in the current study demonstrate no difference for the CYP2C8.3 or CYP2C8.4 enzymes with respect to paclitaxel 6α-hydroxylase kinetics compared with wild-type CYP2C8.1 enzyme. However, a significantly lower level of paclitaxel 6α-hydroxylase activity was associated with the CYP2C8.1 (K399R) enzyme and a lower level of activity was also seen for the CYP2C8.1 (R139K) enzyme. Inconsistencies between data presented here and previously published studies on paclitaxel metabolism using expression systems may be due to differences in the experimental systems employed. To date, no studies have been performed to determine the functional significance of polymorphisms in CYP2C8 on the conversion of 13cisRA to 4-hydroxy 13cisRA in vitro. The present analysis demonstrated that no statistically significant differences in the level of 13cisRA 4-hydroxylase activity were associated with any of the CYP2C8 variants when compared to the wild-type CYP2C8.1 enzyme.

The CYP2C8*3 allele has been the focus of several studies investigating the effect of this variant on the disposition of numerous different drugs in vivo. Clinical studies involving patients treated with paclitaxel failed to identify any evidence to link CYP2C8 genotype with altered pharmacokinetics, toxicity or efficacy (Henningsson et al., 2005). However, additional studies have suggested that the functional effects of CYP2C8*3 are substrate dependent, with a higher clearance in individuals with this genotype reported for repaglinide (Niemi et al., 2003), rosiglitazone (Kirchheiner et al., 2006) and pioglitazone (Tornio et al., 2008).
In summary, findings from the present study suggest that the high degree of observed inter-individual variability in the pharmacokinetics and metabolism of 13\textit{cis}RA (Veal et al., 2007) is unlikely to be attributable to either the CYP2C8.3 or CYP2C8.4 variants. It remains possible that other CYP2C8 polymorphisms or polymorphisms in other P450 genes could have a role to play in determining the extent of variation in 13\textit{cis}RA metabolism in patients.
Acknowledgements

We are grateful to Dr Frank Gonzalez for providing the human CYP2C8*4 cDNA clone, to the late Dr Thomas Friedberg for supplying the expression vector pB13 and a human P450 reductase cloned in pACYC184 and to Hoffman-La Roche for providing 4-oxo 13cisRA.
References


Footnotes

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Legends for figures

**Figure 1. Typical western blot analysis of *E. coli* membrane fractions showing expression of CYP2C8 variants and P450 reductase.**

Blots were probed with rabbit anti-CYP2C8 (A) or rabbit anti-P450 reductase (B) primary antibody, followed by HRP-linked donkey anti-rabbit IgG secondary antibody. Lane 2: membranes isolated from *E. coli* carrying the empty expression vector, Lane 3: CYP2C8.1 + reductase, Lane 4: CYP2C8.1 (R139K) + reductase, Lane 5: CYP2C8.1 (K399R) + reductase, Lane 6: CYP2C8.3 + reductase, Lane 7: CYP2C8.4 + reductase. (A) Lane 1: membranes containing CYP2C8 expressed in lymphoblastoid cell lines. (B) Lane 1: membranes containing P450 reductase expressed in *E. coli*. Samples were run alongside a BenchMark Pre-Stained Protein Ladder (10 – 200 kDa). The protein loading in all lanes was 10 µg.

**Figure 2. Typical Michaelis-Menten plots of the CYP2C8-mediated metabolism of 13cisRA.**

Incubation conditions were 30 min at 37°C with 10 pmol P450 and assays were performed in duplicate for each concentration of 13cisRA. Panel (a) shows data for CYP2C8.1, (b) for CYP2C8.1(R139K), (c) for CYP2C8.1(K399R), (d) for CYP2C8.3 and (e) for CYP2C8.4

**Figure 3. Typical enzyme kinetic data of the CYP2C8-mediated metabolism of paclitaxel.**

Incubation conditions were 30 min at 37°C with 10 pmol P450 and assays were performed in duplicate for each concentration of paclitaxel. Lines were fitted to the data using non-linear least squares regression analysis. Panel (a) shows data for CYP2C8.1, (b) for CYP2C8.1(R139K), (c) for CYP2C8.1(K399R), (d) for CYP2C8.3 and (e) for CYP2C8.4
# Tables

## Table 1. Primers used for the subcloning of *ompA*-CYP2C8

Restriction sites are indicated in bold underlined type. F; forward primer, L; linker primer, R; reverse primer.

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<th>Reaction</th>
<th>Primers</th>
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<td><em>ompA</em>-5′CYP2C8 fusion</td>
<td><strong>F</strong> GGAATTCCATATGAAAAAGACAGCTATCGCG</td>
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<td><strong>L</strong> CAGGACCACAAAAGGTTCCATCGG</td>
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<tr>
<td></td>
<td>GACGGCCTGCCTACGGTAGCGA</td>
</tr>
<tr>
<td></td>
<td><strong>R</strong> GTTTTCATATTTGAAAACTGAC</td>
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<tr>
<td>3′CYP2C8 amplification</td>
<td><strong>F</strong> GGACAACCAAAAGTCA<strong>GAATTCA</strong></td>
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<tr>
<td></td>
<td><strong>R</strong> GTTAATACGAAGCTTAGACAGGGATGAAGCGACGATC</td>
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Table 2. Effect of allelic differences on CYP2C8-mediated 13cisRA metabolism

Metabolism of 13cisRA by *E. coli* membrane fractions co-expressing CYP2C8 variants and P450 reductase. $K_m$ and $V_{max}$ were derived from Michaelis-Menten plots, and are expressed as means ± SD for 6 independent experiments performed with the same batch of enzyme. $V_{max}$ was calculated by dividing the peak area generated in a 30 min period by 1800 to give peak area generated per sec.

<table>
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<tr>
<th>CYP2C8 variant</th>
<th>$K_m$ (µM)</th>
<th>$V_{max}$ (c*s/s)</th>
<th>$V_{max}/K_m$</th>
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<td>CYP2C8.1 (R139K)</td>
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<td>CYP2C8.1 (K399R)</td>
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<td>108.6 ± 53.8</td>
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<tr>
<td>CYP2C8.3</td>
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<td>154.0 ± 115.8</td>
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<td>CYP2C8.4</td>
<td>16.6 ± 4.2</td>
<td>110.2 ± 72.8</td>
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Table 3. Effect of allelic differences on CYP2C8-mediated paclitaxel metabolism.

Metabolism of paclitaxel by *E. coli* membrane fractions co-expressing CYP2C8 variants and P450 reductase.

Data were not consistent with Michaelis-Menten kinetics due to substrate inhibition at concentrations >15 µM paclitaxel. Formation of 6α-hydroxy paclitaxel over a period of 30 min at 10 µM paclitaxel was determined. Values are expressed as means ± SD for 4 independent experiments performed with the same batch of enzyme.

<table>
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<th>CYP2C8 variant</th>
<th>6α-OH paclitaxel (nmol/min/nmol P450)</th>
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<tr>
<td>CYP2C8.1</td>
<td>6.4 ± 2.3</td>
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<tr>
<td>CYP2C8.1 (R139K)</td>
<td>4.4 ± 2.1</td>
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<td>CYP2C8.1 (K399R)</td>
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<td>CYP2C8.3</td>
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<tr>
<td>CYP2C8.4</td>
<td>7.0 ± 2.6</td>
</tr>
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</table>
Figure 1

A

B

55Kd

78Kd