Short Communication

Relevance of Nonsynonymous CYP2C8 Polymorphisms to 13-cis Retinoic Acid and Paclitaxel Hydroxylation

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ABSTRACT:
CYP2C8 has a major role in the metabolism of the anticancer agents 13-cis retinoic acid (13-cisRA) and paclitaxel. There is evidence that polymorphisms in the CYP2C8 gene contribute to observed interindividual differences in paclitaxel metabolism. However, no studies have been performed to determine the relevance of CYP2C8 polymorphisms to 13-cisRA 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 13-cisRA and paclitaxel was examined using an Escherichia coli expression system with coexpression of human cytochrome P450 reductase. No statistically significant differences in the level of 13-cisRA 4-hydroxylase activity were associated with either CYP2C8 allelic variant compared with 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 with 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 allele. 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 13-cisRA 4-hydroxylase activity. We conclude that common CYP2C8 polymorphisms are unlikely to explain reported interindividual variation in 13-cisRA or paclitaxel pharmacokinetics.

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

Several cytochromes P450 (P450s) have been reported to catalyze the oxidation of 13-cisRA, including CYP3A7, 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 13-cisRA metabolism. Indeed, CYP2C8 plays a major role in the metabolism of a number of therapeutically important drugs, and it 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 previously (www.cypalleles.ki.se), two of which, CYP2C8*3 and *4, include nonsynonymous polymorphisms commonly seen in whites (Dai et al., 2001; Bahadur et al., 2002; Henningsson 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-Antonio et al., 2008; Singh et al., 2008). More recent pharmacogenetic studies with repaglinide and rosiglitazone report that individuals heterozygous for CYP2C8*3 show higher drug clearance, which suggests that the effects of this allele on enzyme activity might be substrate dependent (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 (Henningsson et al., 2005). Although less information is available on the effects of the rarer CYP2C8*4, there was 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 13-cisRA metabolism, we undertook a further assessment using an Escherichia coli expression system with 13-cisRA and paclitaxel as substrates. Our aim was to provide a basis for future clinical pharmacogenetic studies on 13-cisRA 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 (National Institutes of Health, Bethesda, MD). The expression vector pBl3 and a human P450 reductase cloned in pACYC184 were kindly provided by Dr. Thomas Friedberg (University of Dundee, Dundee, UK). Antihuman CYP2C8 and human cytochrome P450 reductase Bactosomes were supplied by Dr. Thomas Friedberg (University of Dundee, Dundee, UK). Antihuman CYP2C8 and human cytochrome P450 reductase Bactosomes were supplied by

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ABBREVIATIONS: 13-cisRA, 13-cis retinoic acid; P450, cytochrome P450; PCR, polymerase chain reaction; LCMS, liquid chromatography-mass spectrometry.
TABLE 1  
Primers used for the subcloning of ompA-CYP2C8

<table>
<thead>
<tr>
<th>Restriction sites are indicated in bold underlined type.</th>
<th>Reaction</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ompA-5’CYP2C8 fusion</strong></td>
<td>F: GGAATTCCATATGAAAAAGACAGCATGCGG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>L: CAGGACCAGAAAAAGGTTCCATCGGAGCGGCTCGGACGTACGGCAAA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: GTTTCACATATTGAATTCTGAC</td>
<td></td>
</tr>
<tr>
<td><strong>3’CYP2C8 amplification</strong></td>
<td>F: GGAACACAAAAAGTCGAGATTC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: GTTAATACGAGCTTACGACAGGATGAAAGCAGATC</td>
<td></td>
</tr>
</tbody>
</table>

F, forward primer; L, linker primer; R, reverse primer.

CYP2C8 (Dundee, UK). Antihuman cytochrome P450 reductase was purchased from Stressgen (Victoria, Canada).

**Construction of Expression Plasmids.** The CYP2C8 cDNA was used as 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 polymerase chain reaction (PCR), as described previously (Pritchard et al., 1997). PCR involved the use of reverse, linker, and forward primers flanking the natural EcoR I site in this cDNA. PCR products were individually subcloned into pGEM-T (Promega, Madison, WI), before being inserted into plasmid pBl3 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 (Agilent, Stockport, UK). 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′-CAATCTCGGACTTATGATT-GCTTCTGATC was used (the site of the modified base is shown in lowercase bold). This “wild-type” cDNA was subsequently used as the template for all further reactions. To generate a CYP2C8*5 cDNA, it was necessary to introduce two base changes, at positions 416 (G to A) and 1196 (C to T). The mutagenic primers 5′-GATTTTTGGGATGGGAAGCAGCTATCGGGACGGCCTGCGCTACGGTAGCGAA and 3′-CTGACTTCCGCTGTCATAGTGACAGCAGAGATTTCC-3′ 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).

**Coexpression of Recombinant CYP2C8 and P450 Reductase in E. coli.** CYP2C8 cDNA clones were coexpressed 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 (Gilliam et al., 1993).

CYP2C8 content was measured using Fe**3+**-CO versus Fe**3+** difference spectra (Omura and Sato, 1964). The yield of active P450 reductase was determined using a spectrophotometric assay to measure cytochrome c reductase (Strobil and Dignam, 1978). Protein concentrations were determined using a modified Bradford assay (Bradford, 1976). 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 horseradish peroxidase-linked donkey anti-rabbit IgG secondary antibody. Samples were run alongside a BenchMark PreStained 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), and 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 17,900g 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 *Kₘ* and *V_{max}*. Values because an authentic standard was unavailable for 4-hydroxy 13cisRA, *V_{max}*-units were given as the peak area generated, as determined by liquid chromatography-mass spectrometry (LCMS) analysis, in counts · second per second. 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-hydroxylation 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) was used for all calculations.

**LCMS Analysis.** Analysis of 13cisRA and 4-hydroxy 13cisRA was carried out using a PerkinElmer LC System (PerkinElmer Life and Analytical Sciences, Beaconsfield, UK) coupled to an API-2000 LC/MS/MS Triple Quadrupole Mass Spectrometer (Applied Biosystems, Warrington, UK), with Analyst software (Applied Biosystems) for data acquisition and analysis, as described previously (Armstrong et al., 2007). Because there was no authentic standard available for the metabolite, 4-hydroxy 13cisRA was synthesized as described previously (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 × 4.6 mm, 5 μm) (Waters, Milford, MA) by reverse-phase chromatography. The mobile phase was isocratic and consisted of 75% acetonitrile and 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 → 569.20 for paclitaxel and *m/z* 870.47 → 585.30 for 6α-hydroxy paclitaxel.

**Statistical Analysis.** Data were analyzed by one-way analysis of variance or with the Pearson correlation coefficient, and *p < 0.05* was considered 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 coexpressing each of the recombinant CYP2C8 allelic variants and P450 reductase is shown in Fig. 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 per 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 five other P450s, 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, which suggests that the system is appropriate for the studies performed. Although cytochrome b₅ has been shown to affect the activity of P450 enzymes with some substrates, the impact of this cofactor was not investigated here.

All CYP2C8 variants metabolized 13cisRA to 4-hydroxy 13cisRA. Typical Michaelis-Menten plots for 13cisRA oxidation by recombinant CYP2C8 allelic variants are shown in Fig. 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-trans and 9-cis retinoic acid isomers using lymphoblastoid cell and baculovirus expression systems, respectively (McSorley and Daly, 2000; Marill et al., 2002), but they were slightly lower than the 50 \mu M value for all-trans retinoic acid reported by Marill et al. (2000).

Although all CYP2C8 variants metabolized paclitaxel to 6α-hydroxy paclitaxel, formation of the metabolite declined with increasing concentration above 15 \mu M. 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 previously been shown to occur 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 6α-hydroxy paclitaxel over a period of 30 min at 10 \mu M paclitaxel, which was within the linear range. Typical enzyme kinetic data for paclitaxel oxidation by recombinant CYP2C8 allelic variants are shown in Fig. 3. The mean formation values of 6α-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 6α-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 6α-hydroxy paclitaxel for the wild-type CYP2C8.1 for each experiment. Differences between variants in four independent experiments were tested by one-way analysis of variance 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 < 0.0125 (0.05/4).

**TABLE 2**

<table>
<thead>
<tr>
<th>CYP2C8 Variant</th>
<th>(K_m) (\mu M)</th>
<th>(V_{max}) (C · s⁻¹)</th>
<th>(V_{max}/K_m) (C · s⁻¹/\mu M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2C8.1</td>
<td>13.8 ± 5.0</td>
<td>134.6 ± 70.0</td>
<td>10.1 ± 6.0</td>
</tr>
<tr>
<td>CYP2C8.1 (R139K)</td>
<td>10.7 ± 2.9</td>
<td>154.0 ± 95.4</td>
<td>15.8 ± 12.7</td>
</tr>
<tr>
<td>CYP2C8.1 (K399R)</td>
<td>11.3 ± 3.9</td>
<td>108.6 ± 53.8</td>
<td>9.8 ± 4.6</td>
</tr>
<tr>
<td>CYP2C8.3</td>
<td>11.7 ± 6.4</td>
<td>154.0 ± 115.8</td>
<td>15.2 ± 11.3</td>
</tr>
<tr>
<td>CYP2C8.4</td>
<td>16.6 ± 4.2</td>
<td>110.2 ± 72.8</td>
<td>7.2 ± 5.0</td>
</tr>
</tbody>
</table>

\(c · s/\text{s}, \text{counts} \cdot \text{second} \cdot \text{second}^{-1}\).
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 in recombinant CYP2C8.4 enzyme toward 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-hydroxylation kinetics compared with wild-type CYP2C8.1 enzyme. However, a significantly lower level of paclitaxel 6α-hydroxylation 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 a previously published study on paclitaxel metabolism using expression systems may be due to differences in the experimental systems used. 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 with the wild-type CYP2C8.1 enzyme.

The CYP2C8*3 allele has been the focus of several studies that investigated the effect of this variant on the disposition of several 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 clearance in individuals with this genotype reported for repaglinide (Niemi et al., 2003), rosiglitazone (Kirchheiner et al., 2006), and gold (Goldstein JA (2001) Polymorphisms in human CYP2C8 decrease metabolism of the anticancer drug paclitaxel and its metabolites. J Pharmacol Exp Ther 298:55–62.

In summary, findings from the present study suggest that the high degree of observed interindividual variability in the pharmacokinetics and metabolism of 13cisRA (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 13cisRA metabolism in patients.

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

References


