THE IMPACT OF CYP2C9 GENETICS AND ORAL CONTRACEPTIVES ON CYTOCHROME P450 2C9 PHENOTYPE

Mia Sandberg, Inger Johansson, Magnus Christensen, Anders Rane, and Erik Eliasson

Karolinska Institutet, Department of Laboratory Medicine, Division of Clinical Pharmacology (M.S., M.C., A.R., E.E.); and Karolinska Institutet, National Institute of Environmental Medicine, Division of Molecular Toxicology (I.J.), Stockholm, Sweden

Received November 17, 2003; accepted January 30, 2004

This article is available online at http://dmd.aspetjournals.org

ABSTRACT:

CYP2C9-dependent drug metabolism is subject to large interindividual variation. To some extent, this is explained by genetic polymorphism with expression of enzyme variants that differ in catalytic activity. The aim of this study was to characterize the variation in CYP2C9 phenotype in relation to genotype, with further analysis of the CYP2C9 gene in metabolic outliers. A study population of 126 healthy white subjects were recruited and genotyped for the CYP2C9*1–3 alleles. In CYP2C9 phenotyping with losartan, three subpopulations were distinguished that differed in the number of CYP2C9*3 alleles (0, 1, or 2). A three-fold higher metabolic ratio (MR; urinary losartan/carboxymetabolite) was found comparing CYP2C9*1/*1 (n = 20) to CYP2C9*1/*3 (n = 81), but there was considerable variation within each genotype. Subjects genotyped as CYP2C9*1/*1, but with an unexpectedly slow oxidation of losartan, were selected for DNA-sequencing analysis of the CYP2C9 gene. Interestingly, single nucleotide polymorphisms (SNPs) could not be identified either in the 5'-flanking region, the nine exons, or exon-intron boundaries. However, sequencing of the CYP2C9 gene was also carried out in patients genotyped as CYP2C9*1/*1 but with an exceptionally low steady-state clearance of losartan. Here, five different SNPs were identified. In further analysis of the healthy volunteers, it became evident that women on oral contraceptives (OCs) had slower oxidation of losartan (MR of losartan: 1.7) than women without OCs (MR of losartan: 0.86). This novel finding was not explained by a different frequency of variant alleles. In summary, CYP2C9 genotype and oral contraceptives both contribute to a large interindividual variation in CYP2C9 activity.
The location of each primer in relation to the translation start is given (LocusLink NT_030059.7).

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Position</th>
<th>Primer Sequence (5′ to 3′)</th>
<th>Primer Sequence (5′ to 3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF 3.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-3603 to -3589</td>
<td>ATGGCTCATCATATAAACGGTCATAC</td>
<td>ATGGCTCATCATATAAACGGTCATAC</td>
</tr>
<tr>
<td>F-3101</td>
<td>-3101 to -3088</td>
<td>ATGATTCACACCGT</td>
<td>ATGATTCACACCGT</td>
</tr>
<tr>
<td>R-3101&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-3088 to -3101</td>
<td>AGCGTTGGAATCAT</td>
<td>AGCGTTGGAATCAT</td>
</tr>
<tr>
<td>SF 3.0</td>
<td>-2967 to -2946</td>
<td>TTATGTAATTACAAATGTCA</td>
<td>TTATGTAATTACAAATGTCA</td>
</tr>
<tr>
<td>R-2600&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-2465 to -2486</td>
<td>GATCTCGCGACCTGTTGAC</td>
<td>GATCTCGCGACCTGTTGAC</td>
</tr>
<tr>
<td>SF 1.9</td>
<td>-1955 to -1931</td>
<td>ATTCAGTTAGATTAGGTTCA</td>
<td>ATTCAGTTAGATTAGGTTCA</td>
</tr>
<tr>
<td>SF 1.0</td>
<td>-1257 to -1274</td>
<td>CAGGAGCTGACATACGTA</td>
<td>CAGGAGCTGACATACGTA</td>
</tr>
<tr>
<td>R 2</td>
<td>-1092 to -1071</td>
<td>AGGACCGCTGCTGAGGATGAC</td>
<td>AGGACCGCTGCTGAGGATGAC</td>
</tr>
<tr>
<td>SF -740</td>
<td>-938 to -955</td>
<td>CAGCCACGAGATGGC</td>
<td>CAGCCACGAGATGGC</td>
</tr>
<tr>
<td>F -342</td>
<td>-734 to -714</td>
<td>TATGACCCTGTTGGCAACCAC</td>
<td>TATGACCCTGTTGGCAACCAC</td>
</tr>
<tr>
<td>R -342&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-342 to -329</td>
<td>TGCATTTCGATCAC</td>
<td>TGCATTTCGATCAC</td>
</tr>
<tr>
<td>R 21&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-329 to -342</td>
<td>CGTATGCAAAAAGC</td>
<td>CGTATGCAAAAAGC</td>
</tr>
<tr>
<td>R 21&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+29 to +7</td>
<td>CAGAGCCACAGAAGGCAAAGGA</td>
<td>CAGAGCCACAGAAGGCAAAGGA</td>
</tr>
</tbody>
</table>

<sup>a</sup> Primers for amplification of 3.5- kilobase-pair fragment.

<sup>b</sup> Reverse primers.

Materials and Methods

**Study Design.** After an overnight fast, losartan (Cozaar; Merck, Darmstadt, Germany), 25 mg, was given as a single oral dose in the morning to 126 healthy volunteers, after they had Voided their bladder. Thereafter, urine was collected during 8 h. All subjects also received four other probe drugs, as part of a so-called cocktail study (Christensen et al., 2003). Except for oral contraceptives, no other medication, including natural remedies, was allowed at least 1 week before study start, and everyone also had to abstain from caffeine-containing food and beverages, alcohol, and grapefruit juice at least 2 days before study start. The participants were recruited among the staff and students at Huddinge University Hospital, Stockholm, Sweden.

In the present study the administration time point of debrisoquine in relation to the other probe drugs was changed, compared with the time point in the previously published cocktail study, to overcome potential interaction risks. Debrisoquine was given in the evening, 9 h before losartan.

All participants were considered to be healthy according to their physical examination, medical history, and clinical laboratory test results. Their mean age was 30 years, and in total there were 49 men and 77 women. The study had been approved by the local ethics committee at Huddinge University Hospital, Karolinska Institutet, Stockholm, and was performed in accordance with the Helsinki Declaration.

**Genotyping.** Blood samples were drawn from all subjects and stored at -20°C. Genomic DNA was extracted from the leukocytes by using a QiAmp DNA blood kit (QIAGEN, Hilden, Germany). A TaqMan assay was used to determine the CYP2C9*1, CYP2C9*2, and CYP2C9*3 genotypes (Yasar et al., 2002d), and the concentrations of reagents were those recommended by the manufacturer (Applied Biosystems, Foster City, CA). The final volume was 11 μL. After an initial DNA denaturation at 95°C for 10 min, the samples were run for 40 cycles at 95°C for 15 s and at 60°C for 1 min.

**Urine Analysis.** Urine samples were stored at -20°C until analyses were performed, according to the method of Yasar et al. (2002c). The CVs for inter- and intraday variation for losartan were less than 6.5 and 13.9%, respectively, and the corresponding figures for the metabolite E-3174 were 10.3 and 6.9%.

**Amplification (PCR I) of the 5′-Flanking Region of CYP2C9.** Fragments of 3.5 kilobase pairs upstream of the initiation codon in the 5′-flanking region of the CYP2C9 gene were amplified by polymerase chain reaction (PCR<sup>1</sup>) on a PerkinElmer GeneAmp PCR System 9700 (PerkinElmer Life and Analytical Sciences, Boston, MA). CYP2C9 gene specific primers (Table 1) were designed in accordance with the published sequence for the 5′-flanking region of the CYP2C9 gene (NT_030059.7). Each PCR contained 100 ng of genomic DNA, 200 μM each dNTP (Invitrogen, Paisley, Scotland, UK), buffer corresponding to 2 mM MgCl<sub>2</sub>, 250 nM each primer, 0.5 μL of Elongase Enzyme Mix (Invitrogen) in a final volume of 25 μL. After an initial DNA denaturation at 95°C for 30 s, the following procedure was applied for 35 cycles: 30 s at 95°C, 30 s at 55°C, and 3.5 min at 68°C with a final extension time of 7 min at 68°C. Electrophoresis on a 1% agarose gel was performed to validate size as well as specificity of the PCR products. After amplification, the DNA was purified (Wizard; Promega, Madison, WI) according to instructions, and the concentrations of the amplified fragments were determined by PicoGreen dsDNA Quantitation Reagent (Molecular Probes, Eugene, OR), using known concentrations of thymus DNA as standard, and measured with a Fluoroscan II microplate reader (Labsystems).

**Amplification (PCR I) of the 9 Exons of CYP2C9.** All nine exons of the CYP2C9 gene, including parts of the introns, were amplified by polymerase chain reaction (PCR) on a PerkinElmer GeneAmp PCR System 9700. CYP2C9 gene specific primers (Table 2) were designed in accordance with the published sequence for the exons of the CYP2C9 gene (NT_030059.8). Each PCR contained approximately 100 ng of genomic DNA, 200 μM concentration of each dNTP (Invitrogen), 2.5 μL of 10X PCR buffer II, MgCl<sub>2</sub> corresponding to 1 to 1.5 mM, 250 nM concentration of each primer, and 1.2 μL of AmpliTaq DNA polymerase (Applied Biosystems) in a final volume of 25 μL. For exon 4 and 6, Elongase Enzyme Mix, 1 μL, and 5× Reaction buffer B (Invitrogen) were used instead. After an initial DNA denaturation at 95°C for 30 s, the following procedure was applied for 35 cycles: 30 s at 95°C, 30 s at 52°C, and 1 min at 68°C with a final extension time of 7 min at 68°C. Electrophoresis on a 1% agarose gel was performed to validate size as well as specificity of the PCR products. After amplification, the DNA was purified with QIAquick PCR purification kit (QIAGEN), and the concentrations were determined on a spectrophotometer (Beckman DU 530 Life Science UV-visible spectrophotometer; Beckman Coulter, Fullerton, CA).

**Sequencing.** The purified fragments were sequenced using ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit and CYP2C9 specific primers (Tables 1 and 2). Purified DNA (75-150 ng) was used in a total volume of 10 μL. Twenty-five cycles of denaturation, annealing, and extension were performed, i.e., 10 s at 96°C, 5 s at 50°C, and 4 min at 60°C. The sequencing reactions were analyzed by an ABI 377 DNA sequencer (Applied Biosystems).

DNA samples were also obtained from Italian patients with exceptionally low clearance of warfarin (Scordo et al., 2002), kindly provided by Dr.

---

1 Abbreviations used are: PCR, polymerase chain reaction; ANOVA, analysis of variance; MR, metabolic ratio; OC, oral contraceptive; SNP, single nucleotide polymorphism.
Gabriella Scordo, Uppsala University, Sweden. The clearances of free S-warfarin in these six patients were less than 400 ml/min and the mean clearance of free S-warfarin in this group of CYP2C9*1/*1 subjects was 660 ± 325 ml/min. These six selected Italian patients of white origin (Scordo et al., 2002) were genotyped as homozygous for the CYP2C9*1 allele, and their 5'-flanking regions and exons of the CYP2C9 gene were sequenced as described above. The study was approved both by the local Ethics Committee at Huddinge University Hospital, Karolinska Institutet, Stockholm and the Ethics Committee at the Azienda Ospedaliera di Padova, and all participants gave their informed consent (Scordo et al., 2002).

**Bioinformatics.** The nucleotide sequence of the 5'-flanking region as well as of the nine exons of CYP2C9 was obtained from the National Center for Biotechnology Information LocusLink database, NT_030059.7 and NT_030059.8, respectively. The former sequence differs in the numbering by one nucleotide, compared with the study by Shintani et al. (2001); i.e., our first nucleotide (−1) upstream of the translation start is numbered as the second nucleotide (−2) upstream of the translation start in the Japanese study (Shintani et al., 2001). NT_030059.7 also refers to −1188T as being the “normal” sequence, contrary to −1188C in the Japanese study. Multiple sequence alignments were performed using ClustalW (http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html). The specificity of the primers was checked in BLASTN at the National Center for Biotechnology Information. A search of potential binding sites for transcription factors was performed using MatInspector V2.2 based on Transfac 4.0 (http://transfac.gbf.de).

**Statistical Analysis.** Metabolic ratios (logarithmic values) of losartan were compared across genotype groups by performing one-way analyses of variance (ANOVA). Since ANOVA resulted in a statistically significant difference (p < 0.05), Scheffe’s post-hoc test was applied to statistically verify the differences between pairs of groups, as appropriate. Factorial ANOVA was used to determine the influence of contraceptive pills and genotype on the log-transformed MR of losartan in women. Linear or multiple linear regression analysis, using logarithmic values, was used for calculating correlation coefficients (r). p < 0.05 was considered as statistically significant. Logarithmic transformation was used for all calculations of mean metabolic ratios, and the results were presented as the antilog. For all other results, descriptive statistics were used. The software utilized for statistical calculations was Statistica 6.1 (StatSoft, Tulsa, OK).

**Results**

In the test panel of 126 healthy volunteers, the distribution of individual CYP2C9 genotypes was the following: *1/*1 = 81, *1/*2 = 19, *2/*2 = 2, *2/*3 = 2, *1/*3 = 20, and *3/*3 = 2. These numbers result in allele frequencies of 16.1% and 16.8% for the CYP2C9*2 and CYP2C9*3 alleles, respectively, i.e., somewhat higher than the previously reported allele frequencies in whites. Considering the CYP2C9 phenotype, as measured by the 8-h urinary ratio of losartan to carboxy-metabolite (MR), a statistically significant difference (p < 0.05) was apparent between individuals genotyped as CYP2C9*1/*1 and CYP2C9*1/*3. The two subjects genotyped as CYP2C9*3/*3 had extremely high MRs when compared with all other genotypes (see Table 3). The lowest MR value (0.30) was recorded in a subject belonging to the CYP2C9*1/*1 group, but a MR as high as 8.9 was observed within the same genotype group. The association between genotype and log MR of losartan was statistically significant, with an adjusted R2 value of 0.30, when all subjects except women using oral contraceptives were included.

Taken together, subjects carrying one or two *3-alleles (including two 2C9*2/*3 subjects) had a mean MR of 3.1, which is significantly higher than the MR of 0.97 in subjects without any *3-alleles, i.e., genotyped as either CYP2C9*1/*1, CYP2C9*1/*2, or CYP2C9*2/*2. However, an overlap in MR between these two population groups was apparent as shown in Fig. 1, A and B. To find an explanation for the slow metabolism of losartan observed in some of the subjects not carrying any *3-alleles, six individuals (five from the CYP2C9*1/*1 group and one genotyped as CYP2C9*2/*2), with a MR of losartan above 3.0, were selected for further genetic analyses. Complete sequencing of 3604-base pair fragments of the 5'-flanking region of the CYP2C9 gene was performed in all six individuals, except a stretch from approximately −2342 to −2000. This region was excluded since it was rich in adenine nucleotides and difficult to sequence. Interestingly, none of the five subjects homozygous for the CYP2C9*1 allele carried any polymorphisms in the 5'-flanking region of the CYP2C9 gene. The single CYP2C9*2/*2 subject, however, carried three different heterogeneous single nucleotide polymorphisms (SNPs), located at −A1096G, −T485A, and −C484A. Importantly, no SNPs were found in any of the nine different exons or in exon-intron boundaries in any of the sequenced DNA samples from slow metabolizers.

The CYP2C9 gene was also analyzed in Italian patients genotyped as CYP2C9*1/*1 but with exceptionally slow metabolism of S-warfarin (Scordo et al., 2002). Here, novel SNPs were identified in the 5'-flanking region. The identified polymorphisms were located at −T3360C, −G3089A, and −T1188C, as well as at −A1096G. Two subjects were found to be homozygous for −3089A and −1188C (confirmed by additional sequencing of the complementary DNA strand), whereas one individual was heterozygous for the polymorphisms at −3360 and −1096. However, similar to the situation with slow metabolizers of losartan (see above), no polymorphisms were
found in any of the nine exons or exon-intron boundaries of CYP2C9 among these patients.

In addition to genotype, other factors were considered that might contribute to the significant variation in CYP2C9 phenotype observed in healthy test subjects. Indeed, a very important relationship between losartan MR and intake of oral contraceptives (OCs) was found in female test subjects. Comparing subjects carrying either \( *1, *2, \) or \( *3 \) alleles, an approximately 2-fold \((p < 0.05)\) higher losartan MR was observed in women on OCs \((1.7 \pm 2.8)\) compared with those without OC treatment \((0.86 \pm 2.0)\). The difference between these test subjects is also illustrated in Fig. 2. Among the total of 34 women on OCs, there were 29 who were genotyped as either \( CYP2C9*1/*1 \) or \( CYP2C9*1/*2 \), and among the 45 women not taking any OCs, the corresponding figure was 37. None of the women were genotyped as \( CYP2C9*2/*2 \). In total there were three women who were genotyped as \( CYP2C9*1/*3 \) in the group taking oral contraceptives, whereas the corresponding figure was eight, in the group of women not taking oral contraceptives. One single homozygous \( *3 \) subject was present in the group of women taking OCs and none in the other group. According to statistical analysis, no interaction effect between genotype or use of oral contraceptives was present. Finally, the MR of women without OCs was compared with that of men, to look for possible gender differences in CYP2C9 phenotype, but no statistically significant difference was found.

### Discussion

The Effect of Oral Contraceptives on CYP2C9 Phenotype.

Losartan oxidation, as a specific marker of CYP2C9 in vivo, was significantly slower in healthy female test subjects taking oral contraceptives as compared with those not taking oral contraceptives. This represents a novel finding, unrelated to CYP2C9 genotype, suggesting either an inhibitory effect of OCs on CYP2C9 gene expression or a direct drug-drug interaction at the cytochrome P450 enzyme level. In support of the latter mechanism, it was in fact recently shown that both ethinylestradiol and medroxyprogesterone inhibit CYP2C9-dependent drug metabolism in human liver microsomes in vitro (Laine et al., 2003). In the present investigation, it was not recorded what specific OCs were taken by the healthy volunteers, since this was not the main scope of the study. However, it seems very likely that the vast majority contained ethinylestradiol, based on the current Swedish sales statistics (www.apoteket.se). According to available data from the year 2002, the DDD/1000 women/day for combination formulations of gestagens and estradiols was 60% higher than for gestagens only. From these results it is, however, impossible to define which of these two components is responsible for the outcome, but estradiol is most likely involved. Follow-up studies, clarifying this issue, are clearly of interest.

### CYP2C9 Genotype/Phenotype Relationship

To date, this is the largest phenotyping study based on losartan as a probe drug for CYP2C9 in vivo. The study size contributes to a better understanding of the interindividual and intragenotype variation in CYP2C9 activity. However, the results on metabolic ratios in different CYP2C9 genotypes agree well with previous findings, i.e., there was a significantly lower MR of losartan in subjects homozygous for the \( CYP2C9*1 \) allele than in subjects carrying either one or two \( CYP2C9*3 \) alleles, as reported for many of the CYP2C9 substrates both in vitro and in vivo (Yasar et al., 2001b; Lee et al., 2002; Sandberg et al., 2002). However, the range in MR was wide for subjects in the \( CYP2C9*1/*1 \) group, reflecting the great phenotypic heterogeneity among these subjects. As is evident from the frequency distribution of losartan MR in Fig. 1, three phenotypic subpopulations can be distinguished; one with subjects genotyped as either \( CYP2C9*1/*1, *1/*2, \) or \( *2/*2; \) one with subjects homozygous for the \( *3 \) allele; and one with subjects homozygous for the \( *3 \) allele. Since there were only two subjects homozygous for the \( *3 \) allele, no clear trimodal distribution could be observed in the probit-plot (Fig. 1), but it would probably be apparent if more subjects genotyped as \( 2C9*3/*3 \) were included. In conclusion, the present data on losartan underscores the importance of the \( CYP2C9*3 \) allele as a predictor of impaired CYP2C9-dependent drug metabolism in vivo.

The frequency of the \( CYP2C9*2 \) and \( CYP2C9*3 \) alleles among the 126 test subjects does not represent the true distribution of these two alleles in the Swedish population, as compared with data from a previous, larger investigation (Yasar et al., 1999). Even though it does not influence the interpretation of our results, it seems likely that a selection bias has occurred. By including more participants, we would probably overcome this skewed distribution of slightly higher-than-expected frequency of \( CYP2C9*2 \) and \( *3 \) alleles.

### Additional SNP Analyses

To find a genetic explanation to the intragenotype variation in losartan MR, we selected individuals with a \( CYP2C9*1/*1 \) genotype but a slow phenotype, and looked for novel SNPs in the \( CYP2C9 \) gene. As described above, the findings were negative among the healthy losartan test subjects, but four SNPs were identified in warfarin-treated patients. Two of the polymorphisms, at \(-3360 \) and \(-3089 \), were here identified for the first time, whereas the others have been described before (Shintani et al., 2001). Their significance or functional relevance is unclear at this stage. However, in additional experiments performed in vitro using luciferase reporter constructs, it was found that the SNP at \(-1188 \) did not affect gene expression (data not shown). It has been reported that the transcription factors AP-1 and C/EBP have potential binding sites in the \(-5000 \) \(-flanking region of the \( CYP2C9 \) gene (\(-362 \) to \(-155 \)) (Goldstein, 2001), but in contrast to the results presented by Shintani et al. (2001), we could not identify any SNPs in this region. This discrepancy might be related to the different ethnic populations under study. Potential glucocorticoid response elements have been described to reside in the \( 5' \)-flanking region of \( CYP2C9 \) (Kimura et al., 1998; Gerbal-Chaloin et al., 2002), but none of these seem to colocalize with any of the identified SNP sites. Results presented by Shintani et al. (2001) showed that reporter constructs containing certain SNPs in the \( 5' \)-flanking region caused lower luciferase activity in HepG2 cells. However, most of these SNPs were linked to the \( CYP2C9*3 \) allele, whereas their overall influence on the CYP2C9 activity probably is minor. The polymorphism located at position \(-1911 \) was suggested to reside at a potential binding site for HNF-1, accounting for a 58% lower gene expression in HepG2 cells, but the SNP at \(-1911 \) was only one of several SNP variants included in the same reporter constructs. Furthermore, the \(-1911 \) SNP was also closely linked to the \( CYP2C9*3 \) SNP, which was confirmed in our samples (unpublished data). In addition, a recently published study demonstrated that the expression of HNF-1 could not be detected in HepG2 cells (Rodriguez-Antonio et al., 2001).
arguing against a functional role of the SNP at -1911 in CYP2C9 expression.

In summary, our data show that the use of oral contraceptives as well as 2C9 genotype are both factors that have a clear impact on CYP2C9 phenotype. The genotypic influence is still not fully explained, however, probably due to the large variation within subjects genotyped as CYP2C9*1/*1. The present data from 126 healthy test subjects also contribute to a better understanding of the intragenotype variation in CYP2C9 activity. The complete genetic explanation for this variability remains unclear but will need clarification, to establish a genetic test that will better predict the CYP2C9 phenotype in vivo.

However, an important reminder from this study is that drug interactions should never be overlooked as an important explanation for interindividual differences in drug metabolism.

Acknowledgments. We thank Professor Magnus Ingelman-Sundberg for scientific input and valuable advice on the manuscript. We also thank Professor Leif Bertilsson for initiative and support to the Karolinska Cocktail Study, as well as Dr. Maria Gabriella Scorso and Professor Roberto Padrini for providing us with DNA samples from warfarin-treated patients that were included in a previously published study (see above).


