Short Communication

CATALYTIC ROLES OF CYP2C9 AND ITS VARIANTS (CYP2C9*2 AND CYP2C9*3) IN LORNOXICAM 5'-HYDROXYLATION

(Received March 28, 2003; accepted September 4, 2003)

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

ABSTRACT:
The effects of allelic variants of CYP2C9 (CYP2C9*2 and CYP2C9*3) on lornoxicam 5'-hydroxylation were studied using the corresponding variant protein expressed in baculovirus-infected insect cells and human liver microsomes of known genotypes of CYP2C9. The results of the baculovirus expression system showed that CYP2C9*3 gives higher $K_m$ and lower $V_{max}$ values for lornoxicam 5'-hydroxylation than does CYP2C9*1. In contrast, $K_m$ and $V_{max}$ values of CYP2C9*1 and CYP2C9*2 for the reaction were comparable. Lornoxicam 5'-hydroxylation was also determined in liver microsomes of 12 humans genotyped for the CYP2C9 gene ($^{*1}/^{*1}, n = 7; ^{*1}/^{*2}, n = 2; ^{*1}/^{*3}, n = 2; ^{*3}/^{*3}, n = 1$). A sample genotyped as $^{*3}/^{*3}$ exhibited 8- to 50-fold lower intrinsic clearance for lornoxicam 5'-hydroxylation than did samples genotyped as $^{*1}/^{*1}$. However, the values for intrinsic clearance for $^{*1}/^{*3}$ were within the range of values exhibited by samples of $^{*1}/^{*1}$. In addition, no appreciable differences were observed in kinetic parameters for lornoxicam 5'-hydroxylation between $^{*1}/^{*1}$ and $^{*1}/^{*2}$.

In conclusion, this study showed that lornoxicam 5'-hydroxylation via CYP2C9 was markedly decreased by the substitution of Ile359Leu (CYP2C9*3), whereas the effect of the substitution of Arg144Cys (CYP2C9*2) was nonexistent or negligible. Additional in vivo studies are required to confirm that individuals with homologous CYP2C9*3 allele exhibit impaired lornoxicam clearance.

Lornoxicam (also known as chlorotentoxicam) [6-chloro-4-hydroxy-2-methyl-n-2-pyridyl-5H-thieno[2,3-c]-[1,2]thiazine-2-carboxamide-1,1-dioxide] is a nonsteroidal anti-inflammatory drug that decreases prostaglandin synthesis by inhibiting cyclooxygenase (Radhofer-Welte and Rabasseda, 2000). Exhibiting analgesic, antipyretic, and anti-inflammatory effects, lornoxicam has been clinically available in certain European countries since 1995. Since no unchanged form is detectable in excreted material, lornoxicam appears to be eliminated predominantly by hepatic biotransformation. The enzyme responsible for the main metabolic pathway, 5'-hydroxylation of lornoxicam, is cytochrome P450 2C9 (CYP2C9) (Bonnabry et al., 1996).

CYP2C9 is the principal enzyme responsible for the metabolism of numerous clinically important drugs, such as amitriptyline, fluoxetine, losartan, phenytoin, S-warfarin, tolbutamide, and many nonsteroidal antirheumatics, including oxicams (Miners and Birkett, 1998). To date, more than 10 allelic variants have been described for the CYP2C9 gene (Goldstein, 2002). Among them, CYP2C9*2 (Arg144Cys), CYP2C9*3 (Ile359Leu), CYP2C9*5 (Asp360Glu), and CYP2C9*6 (frame shift by the deletion of an adenine at the 818 cDNA base pair) have been reported to affect the metabolism and clinical toxicity of drugs in vitro and in vivo (Dickmann et al., 2001; Kidd et al., 2001). However, the degree of reduction of activity and changes in kinetic parameters appear to be highly substrate-dependent (Takanashi et al., 2000). In addition, a few studies have been performed regarding whether heterozygotes of the CYP2C9 alleles exhibit lower metabolic activity than homozygotes of the CYP2C9*1 allele (Bhasker et al., 1997; Yamazaki et al., 1998; Hermida et al., 2002; Lee et al., 2002).

In the present study, we examined the effects of allelic variants of CYP2C9 on lornoxicam 5'-hydroxylation by comparing the kinetic parameters of lornoxicam 5'-hydroxylation with CYP2C9*1, CYP2C9*2, or CYP2C9*3, which are the CYP2C9 proteins corresponding to CYP2C9*1, CYP2C9*2, or CYP2C9*3 alleles, expressed in baculovirus-infected insect cells and liver microsomes of 12 white people genotyped for the CYP2C9 gene.

Materials and Methods

Chemicals. Lornoxicam was synthesized at Taisho Pharmaceutical Co., Ltd. (Saitama, Japan). 5'-Hydroxylornoxicam was supplied by Nycomed (Roskilde, Denmark). Piroxicam was purchased from ICN Biomedicals Inc. (Costa Mesa, CA). Other chemicals and reagents used in this study were of the highest quality commercially available.

Enzyme Preparations. Microsomes from baculovirus-infected insect cells expressing CYP2C9*1, CYP2C9*2, and CYP2C9*3 (catalog numbers, P218, P209, and P242) were obtained from BD Gentest (Woburn, MA). These were coexpressed with NADPH-P4501 oxidoreductase. The P450 contents in recombining CYP2C9*1, CYP2C9*2, and CYP2C9*3 were 667, 426, and 741 pmol P450/mg protein, whereas the values for the cytochrome c reductase activity were 980, 590, and 800 nmol/min/mg protein, respectively. Individual human liver microsomes (HG3, HG23, HG30, HG42, HG43, HG56, HG66, HG70, HG89, HG93, HG912, and HK27) were also purchased from BD Gentest. The genotyping of the liver samples used in this study was carried out for the detection of CYP2C9*2 and CYP2C9*3 by BD Gentest. Table 1 lists the donor genotypes for the CYP2C9 gene.

Lornoxicam 5'-Hydroxylase Activity. Lornoxicam 5'-hydroxylase activities in human liver microsomes or microsomes from the expression system were determined by HPLC. The standard incubation conditions were chosen based on the results of preliminary experiments varying both incubation times and concentrations of microsomal proteins. A typical incubation mixture (0.2 ml of the total volume) contained 100 mM Tris buffer (pH 7.4), an NADPH-
Kinetic parameters for 5'-hydroxylation of lornoxicam in cDNA-expressed CYP2C9 recombinant systems and human liver microsomes

<table>
<thead>
<tr>
<th></th>
<th>$K_m$ (μM)</th>
<th>$V_{max}$ (pmol/min/pmol total P450)</th>
<th>$V_{max}/K_m$ (pmol/min/pmol total P450)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>cDNA-expressed CYP2C9 recombinant systems</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2C9.1</td>
<td>0.83 ± 0.15</td>
<td>0.406 ± 0.016</td>
<td>0.489 ± 0.065</td>
</tr>
<tr>
<td>CYP2C9.2</td>
<td>0.91 ± 0.08</td>
<td>0.495 ± 0.010</td>
<td>0.544 ± 0.041</td>
</tr>
<tr>
<td>CYP2C9.3</td>
<td>1.95 ± 0.09</td>
<td>0.097 ± 0.001</td>
<td>0.050 ± 0.002</td>
</tr>
<tr>
<td><strong>Human liver microsomes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HG23 *1/*1</td>
<td>0.79</td>
<td>0.256</td>
<td>0.324</td>
</tr>
<tr>
<td>HG27 *1/*2</td>
<td>0.81</td>
<td>0.278</td>
<td>0.343</td>
</tr>
<tr>
<td>HG89 *1/*3</td>
<td>0.77</td>
<td>0.189</td>
<td>0.245</td>
</tr>
<tr>
<td>HG93 *1/*2</td>
<td>0.72</td>
<td>0.143</td>
<td>0.199</td>
</tr>
</tbody>
</table>
| **Statistical Analysis.** All experiments were performed in triplicate, and the mean values for each data point were used for analysis. Enzyme kinetic parameters ($K_m$, $V_{max}$) were estimated by curve-fitting metabolite formation rate data by the single-enzyme Michaelis-Menten equation and Eadie-Hofstee plot. All graphical analyses were performed by nonlinear regression using SAS Version 6.1 (SAS Institute, Cary, NC). Differences in kinetic parameters among different CYP2C9 genotypes were evaluated for statistical significance by Dunnett's multiple comparison.**

**Results and Discussion**

Figure 1A shows the formation of 5'-hydroxylation by recombinant systems prepared from the cell line expressing the variants of CYP2C9. Simple Michaelis-Menten kinetics were noted for lornoxicam metabolism in all samples studied. The kinetic parameters of lornoxicam 5'-hydroxylation for CYP2C9.1, CYP2C9.2, and CYP2C9.3 are shown in Table 1. CYP2C9.3 (Leu359) had higher $K_m$ and $V_{max}$ values than CYP2C9.1 (wild-type, Ile359) for lornoxicam 5'-hydroxylation. The differences in $K_m$ and $V_{max}$ values between CYP2C9.1 and CYP2C9.3 were about 2- and 4-fold, respectively.
In terms of intrinsic clearance ($V_{\text{max}}/K_m$, the intrinsic capacity of an enzyme to metabolize a substrate), the values for CYP2C9.3 showed 10-fold smaller $V_{\text{max}}/K_m$ values for the reaction than did CYP2C9.1. This finding is in good agreement with a previous report of Takahashi et al. (2000) in which CYP2C9.3 expressed in yeast cells exhibited lower intrinsic clearance than did CYP2C9.1 for oxidation of other oximac, such as tenoxicam 5'-hydroxylation and piroxicam 5'-hydroxylation. These results suggest that the amino acid substitution of Ile359 for Leu359 also affects the metabolic capacity of CYP2C9 in addition to the affinity of CYP2C9 for lornoxicam. The effect of this change on both the $K_m$ and $V_{\text{max}}$ values may be explained by the proposal that CYP2C9 amino acid 359 lies within putative substrate recognition sequence 5 in the CYP2 family (Lewis, 2002).

In contrast, $K_m$, $V_{\text{max}}$, and intrinsic clearance ($V_{\text{max}}/K_m$) of lornoxicam 5'-hydroxylation for CYP2C9.1 (wild-type, Arg144) and CYP2C9.2 (Cys144) expressed in baculovirus-infected insect cells were comparable (Table 1). There was no significant difference between CYP2C9.1 and CYP2C9.2 in $V_{\text{max}}/K_m$ values for the reaction. This finding is consistent with previous reports that CYP2C9.2 has catalytic functions similar to those of CYP2C9.1 for the oxidation of tolbutamide, S-warfarin, and toremifene (Sullivan-Klose et al., 1996; Miners et al., 2000). However, Rettie et al. (1994) reported that the $V_{\text{max}}$ values of the CYP2C9.2 S-warfarin 7-hydroxylation were lower than that of CYP2C9.1. Crespi and Miller (1997) showed that the magnitude of the difference in $V_{\text{max}}$ for S-warfarin between CYP2C9.1 and CYP2C9.2 depends on the expression system used, and is influenced by the ratio of NADPH-P450 oxidoreductase to P450. Thus, it is necessary to use human liver samples genotyped as *2/*2 to clarify the effect of the substitution of Arg144Cys for CYP2C9 on the activity in human liver microsomes.

We determined the kinetic parameters of lornoxicam 5'-hydroxylation from liver microsomes derived from 12 human samples genotyped for the CYP2C9 gene. The formation of 5'-hydroxylornoxicam by human liver microsomes showed simple Michaelis-Menten kinetic behavior (Fig. 1B). Using Eadie-Hofstee plots, we confirmed that a single kinetic parameter could be determined in the reaction. As shown in Table 1, apparent $K_m$ values for lornoxicam 5'-hydroxylation ranged from 0.76 to 1.28 μM in microsomes of livers genotyped as *1/*1. The $K_m$ values for liver microsomes of samples genotyped as *1/*2 or *1/*3 ranged from 0.72 to 1.72 μM. Only a microsome sample genotyped as *3/*3 exhibited a high $K_m$ value (8.26 μM). The sample genotyped as *3/*3 exhibited a lower $V_{\text{max}}$ value (0.019 pmol/min/pmol total P450) than the other samples genotyped as *1/*1, *1/*2, or *1/*3 (0.069–0.278 pmol/min/pmol total P450). Thus, samples genotyped as *3/*3 showed 8- to 50-fold lower intrinsic clearance ($V_{\text{max}}/K_m$, 0.007 μl/min/pmol total P450) than samples genotyped as *1/*1 and other heterozygous alleles of CYP2C9 (0.054–0.343 μl/min/pmol total P450), although only one sample was genotyped as *3/*3. The substitution of Ile359Leu for CYP2C9 also appears to decrease the intrinsic clearance of lornoxicam 5'-hydroxylation in human liver microsomes. In addition, the present study using human liver microsomes containing samples genotyped as *1/*2 or *1/*3 showed that the heterozygous alleles of CYP2C9*2 or CYP2C9*3 did not always have lower intrinsic clearance for lornoxicam 5'-hydroxylation than *1/*1.

Rare cases of subjects with higher than expected plasma concentrations after the administration of lornoxicam have been reported (Turner and Johnston, 1990; Ravic et al., 1991). Since 5'-hydroxylation is the main biological pathway of lornoxicam in humans, it has been speculated that the increase in plasma concentrations is attributable to defective metabolism of lornoxicam. Our results indicate that it may be possible that certain subjects, exhibiting high area under the curve and $C_{\text{max}}$ values associated with delayed elimination, are the poor-metabolizer phenotypes of CYP2C9, although their actual genotypes were unknown.

In conclusion, the present study showed that the lornoxicam 5'-hydroxylation via CYP2C9 was markedly reduced by the substitution of Ile359 for Leu359 (CYP2C9.3), whereas the effect of the substitution of Arg144 for Cys144 (CYP2C9.2) was nonexistent or negligible. Additional in vivo studies are required to confirm that individuals with the homologous CYP2C9*2 allele exhibit impaired lornoxicam clearance.

**References**


