Effect of Glimepiride and Glibenclamide on S-Warfarin 7-Hydroxylation by Human Liver Microsomes, Recombinant Human CYP2C9.1 and CYP2C9.3

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The effect of glimepiride on metabolism of S-warfarin to 7-hydroxywarfarin was studied using human liver microsomes and recombinant cytochrome P450 2C9 microsomes (CYP2C9.1 and CYP2C9.3), and was compared with the results from the experiments using glibenclamide as an inhibitor. S-Warfarin 7-hydroxylation by recombinant CYP2C9.1 and CYP2C9.3 was inhibited by glimepiride competitively. The apparent K_i value of glimepiride was lower at CYP2C9.3 than at CYP2C9.1. Glimepiride also inhibited 7-hydroxylation of S-warfarin in a competitive manner by microsomes from human liver which showed the genotypes of CYP2C9, as CYP2C9*1/*1 or CYP2C9*1/*3. The apparent K_i value of glimepiride was lower than that of glibenclamide. These results may provide valuable information for optimizing the anticoagulant activity of warfarin when glimepiride is co-administered to patients.

Key words warfarin; glimepiride; liver microsome; CYP2C9; drug interaction; drug metabolism

Warfarin is an anti-coagulation drug as administered as racemate. S-Enantiomer of warfarin has 3 to 5 times higher anticoagulant activity than the R-enantiomer. Therefore, changes in the disposition of S-warfarin will affect more significantly to its anti-coagulation activity of warfarin than that of R-warfarin. S-Warfarin is mainly metabolized to 7-hydroxylated metabolite by cytochrome P450 2C9 (CYP2C9). More than 10 genotypes of CYP2C9 are demonstrated, and major genotypes of CYP2C9 are CYP2C9*1 and CYP2C9*3 in Asian people. Sulfonlurea anti-diabetic drugs such as glimepiride, glibenclamide, and tolbutamide were also metabolized by CYP2C9. Recently, it has been reported that persons having CYP2C9*3 genotypes show higher AUC and longer elimination half-life of both glimepiride and glibenclamide after oral administration of these drugs. Tolbutamide and glibenclamide inhibit the metabolism of warfarin to 7-hydroxyl metabolite by human liver microsomes in a competitive manner. However, little information of the metabolic interaction between warfarin and glimepiride has been obtained. We have investigated the effect of glimepiride on metabolism of S-warfarin to 7-hydroxywarfarin using recombinant cytochrome P450 2C9 microsomes (CYP2C9.1 and CYP2C9.3) and human liver microsomes, and have compared with the results from the experiments using glibenclamide as an inhibitor of CYP2C9.

MATERIALS AND METHODS

Materials Warfarin racemate, warfarin enantiomers, glibenclamide, naproxen, NADP+, glucose-6-phosphate, MgCl2, and glucose-6-phosphate dehydrogenase were obtained from Wako Pure Chemical Ind. (Osaka, Japan). Tolbutamide and sulfaphenazole were purchased from Sigma-Aldrich (MO, U.S.A.). Glimepiride was obtained from Sanofi-Aventis (Tokyo, Japan). Human liver microsomes (3 samples from CYP2C9*1/*1 subjects (1/#1; HG30, HH86, HH48) and 3 samples from CYP2C9*1/*3 subjects (1/#3; HH1, HH31, HH9)) and human CYP2C9+P450 reductase microsomes derived from baculovirus expression system (CYP2C9.1 and CYP2C9.3) were purchased from BD Gentest (MA, U.S.A.).

Inhibition Study According to the method by Takahashi et al.,3) metabolic inhibition study was performed. The time of incubation and concentration of microsomal protein in the study were determined to be in a linear range for the rate of formation of 7-hydroxywarfarin. Initially, microsomes (final protein concentration: 0.1 mg/ml or 0.1—0.2 mg/ml for CYP2C9 or human liver microsomes, respectively) and warfarin racemate (2—20 μM) in 50 mM Tris–HCl (pH 7.4) with or without inhibitor, such as glimepiride (1.0—4.0 μM), glibenclamide (1.0—4.0 μM), tolbutamide (100—400 μM), or sulfaphenazole (0.25—1.0 μM), were preincubated for 10 min at 37 °C, then NADPH regenerating system (NADP+ 0.5 mM, glucose-6-phosphate 2 mM, MgCl2 4 mM, glucose-6-phosphate dehydrogenase 1 U/ml) was added to the preincubated mixture at 37 °C. The total incubation volume was 0.25 ml. After 30 min the incubation was terminated by the addition of acetonitrile (0.25 ml) containing 2.7 μM naproxen as an internal standard. The mixture was centrifuged for 5 min at 18500 g at 4 °C. An aliquot of the supernatant was used for analysis of warfarin metabolites by HPLC system. The incubations were performed in duplicate and the mean value and S.D. from three experiments were provided.

HPLC Analysis According to a slightly modified method reported by Takahashi et al.,3) the contents of 7-hydroxywarfarin enantiomers were analyzed by HPLC using a chiral column. HPLC analyses were performed using Shimadzu SCL-10A VP system controller, SIL-10A VP auto-injector, LC-10A VP pump, RF-10A XL fluorescence detector, and Daicel Chiralcel OD-RH column (4.6×150 mm, 5 μm). The mobile phase was acetonitrile : phosphate (pH 2.0) = 35 : 65. The flow rate was 0.5 ml/min. The column temperature was 40 °C. The eluent was monitored fluorometrically (excitation: 320 nm, emission: 415 nm). The retention time of 7-hydroxy R-warfarin, naproxen (internal standard),...
7-hydroxy S-warfarin, R-warfarin, and S-warfarin was 16, 26, 28, 43, and 61 min, respectively.

**Kinetic Analysis.** Kinetic parameters were estimated from the fitted curve using a non-linear analysis program (MULTI2).

**RESULTS AND DISCUSSION**

Initially, we compared the kinetic parameters of 7-hydroxylation metabolism of S-warfarin by CYP2C9.1 and CYP2C9.3. The $K_m$ value (4.9 ± 1.0 μM; mean ± S.D.) of CYP2C9.3 was 2-times higher value (2.2 ± 0.3 μM) of that of CYP2C9.1. The $V_{\text{max}}$ value (62.1 ± 6.9 pmol/min/nmol P450) of CYP2C9.1 was 50% higher value of CYP2C9.3 (41.8 ± 5.0 pmol/min/nmol P450). Therefore, The $V_{\text{max}}/K_m$ value (apparent intrinsic clearance; 8.2 ± 0.6 μl/min/nmol P450) of CYP2C9.3 was about 30% value of CYP2C9.1 (28.1 ± 2.3 μl/min/nmol P450). The $K_m$ values were comparable to the previous value reported by using recombinant CYP2C9 from yeast. The $V_{\text{max}}/K_m$ of R-warfarin enantiomer by CYP2C9.1 and CYP2C9.3 was about 3% of that of S-warfarin enantiomer, indicating stereoselective metabolism of warfarin by CYP2C9.

Next, the inhibitory effects of glimepiride, glibenclamide, sulfaphenazole and tolbutamide on 7-hydroxylation metabolism of S-warfarin (2 μM) by CYP2C9.1 and CYP2C9.3 were compared (Table 1). The IC$_{50}$ value of these drugs was the following order: tolbutamide > glibenclamide > glimepiride > sulfaphenazole. Three human liver microsomal samples from CYP2C9*1/*1 subjects (CYP2C9*1/*1) and 3 samples from CYP2C9*1/*3 subjects (CYP2C9*1/*3) were used to compare the inhibitory effect of glimepiride, glibenclamide, sulfaphenazole and tolbutamide on 7-hydroxylation metabolism of S-warfarin. Table 1 also indicates the IC$_{50}$ value at 2 μM S-warfarin was the following order: tolbutamide > glibenclamide > glimepiride > sulfaphenazole. This order from human liver microsomes was similar to the previous result from the recombinant CYP2C9. The IC$_{50}$ value of sulfaphenazole was comparable to the previously reported value. Figure 1 shows the Dixon plot of the inhibitory effect of glibenclamide and glimepiride on S-warfarin 7-hydroxylation by CYP2C9.1 and human liver microsomes. Glibenclamide and glimepiride inhibited the metabolism by CYP2C9 in a competitive manner. In a previous study by Wang *et al.*, tolbutamide showed essentially no binding with the 0.1 mg/ml microsomal protein, so we supposed the negligible interaction.

![Fig. 1](image-url) Effect of Glibenclamide and Glimepiride on S-Warfarin 7-Hydroxylation by CYP2C9.1 and Human Liver Microsomes from a Subject (CYP2C9*1/*1) (Dixon Plot) (Left: Glibenclamide (GLB), Right: Glimepiride (GLP))

Warfarin concentration (○: 2 μM, ■: 4 μM, △: 10 μM, ●: 20 μM). Each point represents mean value (n=3).
binding of glimepiride or glibenclamide to microsomal proteins at low protein concentration (0.1—0.2 mg/ml) and calculated the apparent inhibitory constant ($K_i$) of glimepiride and glibenclamide. The apparent $K_i$ value of glimepiride on $S$-warfarin 7-hydroxylation by both CYP2C9.1 and CYP2C9.3 was lower than that of glibenclamide (Table 1). The apparent $K_i$ value of glimepiride was lower at CYP2C9.3 than at CYP2C9.1. Figure 1 also shows the Dixon plot of the inhibitory effect of glibenclamide and glimepiride on $S$-warfarin 7-hydroxylation by human liver microsome sample whose CYP2C9 genotype was *1/*1. Glibenclamide and glimepiride inhibited the metabolism by human liver microsomes in a competitive manner. The apparent $K_i$ value of glimepiride on $S$-warfarin 7-hydroxylation by human liver microsomes was lower than that of glibenclamide (Table 2). The apparent $K_i$ value of glimepiride at recombinant CYP2C9 showed a tendency to have a lower value than that at human liver microsomes, however, the reasons of the apparent difference in $K_i$ value between CYP2C9 and liver microsomes are remained to be studied.

In summary, $S$-warfarin 7-hydroxylation by recombinant CYP2C9.1 and CYP2C9.3 was inhibited by glimepiride competitively. The apparent $K_i$ value of glimepiride was lower at CYP2C9.3 than at CYP2C9.1. Glimepiride also inhibited 7-hydroxylation of $S$-warfarin in a competitive manner by microsomes from human liver which showed the genotypes of CYP2C9, as CYP2C9*1/*1 or CYP2C9*1/*3.

Table 2. Apparent $K_i$ of Glibenclamide and Glimepiride on $S$-Warfarin 7-Hydroxylation by Recombinant CYP2C9 and Human Liver Microsomes

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<th>Recombinant CYP2C9</th>
<th>Human liver microsomes</th>
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<tr>
<td></td>
<td>CYP2C9.1</td>
<td>CYP2C9.3</td>
</tr>
<tr>
<td>Glibenclamide</td>
<td>2.01±0.34</td>
<td>1.92±0.41</td>
</tr>
<tr>
<td>Glimepiride</td>
<td>0.70±0.03*</td>
<td>0.46±0.11**</td>
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Each value represents mean±S.D. ($n$=3). *$p$<0.05 and **$p$<0.01 compared with corresponding value of glibenclamide. $r p$<0.05 compared with corresponding value of CYP2C9.1.

The apparent $K_i$ value of glimepiride was lower than that of glibenclamide. These results may provide valuable information for optimizing the anticoagulant activity of warfarin when glimepiride is co-administered to patients.

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**REFERENCES**