INHIBITION OF S-WARFARIN METABOLISM BY NONSTEROIDAL ANTIINFLAMMATORY DRUGS IN HUMAN LIVER MICROSOMES IN VITRO

Tomoaki TAKIGAWA,a Hitoshi TAINAKA,b Kiyoshi MIHARA,a and Hiroyasu OGATA,a,b
Department of Biopharmaceutics,a Meiji College of Pharmacy, 1-22-1 Yato-cho, Tanashi-shi, Tokyo 188, Japan, and Central Laboratory,b Amersham K.K., 2802-1, Hiratsuka, Shiroi-machi, Inba-gun, Chiba 270-14, Japan.

We studied the inhibition of S-warfarin metabolism by nonsteroidal antiinflammatory drugs (NSAIDs) in human liver microsomes in vitro. After screening for potential inhibitors among ten NSAIDs using human recombinant cytochrome P450, inhibition kinetic parameters were estimated using human liver microsomes. Phenylbutazone and bucolome were suggested to increase the unbound steady-state level of S-warfarin about four- and five-fold, respectively, as estimated from these metabolic parameters.

**KEY WORDS** warfarin metabolism; inhibition; nonsteroidal antiinflammatory drug; human recombinant cytochrome P450; human liver microsomes

More than one hundred drug-drug interactions related to warfarin have been reported in the medical literature.¹ These interactions are mediated by either pharmacokinetic mechanisms altering the hepatic metabolism of warfarin and/or pharmacodynamic mechanisms such as changes in clotting factor synthesis, rather than by displacement of the protein binding of warfarin,¹ under steady-state conditions. Nonsteroidal antiinflammatory drugs (NSAIDs) are considered to prolong bleeding times in patients and healthy volunteers.² However, no study has directly demonstrated inhibition of drug metabolism or clotting factor synthesis by NSAIDs except for phenylbutazone.³ Therefore, in this study we measured inhibition kinetic parameters, which are major determinants of clinical effect, of NSAIDs against the metabolism of S-warfarin using human recombinant cytochrome P450 and human liver microsomes in order to estimate the extent of change in the steady-state unbound concentration of S-warfarin.

**MATERIALS AND METHODS**

**Materials** Warfarin (3-α-acetonylbenzyl-4-hydroxycoumarin) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Seven-hydroxy (7-OH) warfarin was kindly supplied by Eisai Co. (Tokyo, Japan). Human liver microsomes were purchased from XenoTech, LLC (Kansas City, KS, USA). Human recombinant cytochrome P450 2C9(359-Ile) expressed in yeast (Schizosaccharomyces pombe) cells was kindly supplied by Amersham K.K. (Chiba, Japan). Bucolome was obtained from Grelan Pharmaceutical Co. (Tokyo, Japan). All other chemicals were reagent-grade products obtained commercially.

**S-warfarin metabolism and its inhibition** Fifty microliters of 500 mM phosphate buffer, 150 µl of S-warfarin solution, and 25 µl of human microsomes (0.5 mg protein/ml) or human recombinant cytochrome P450 2C9 (359-Ile) (0.5 mg protein/ml) were mixed in an ice-cold tube. The metabolic reaction was started by adding 25 µl of a NADPH-generating system. After incubation for 45 min at 37°C, the reaction was stopped by adding 5 µl of 70% perchloric acid. Immediately after, the tube was centrifuged at 3000 x g for 5 min at 4°C and the supernatant was collected. The effects of various inhibitors on the rate of 7-OH warfarin formation were studied by adding the inhibitors to the reaction mixture. We selected ten NSAIDs: acetaminophen, bucolome, diflunisal, flurbiprofen, ibuprofen, indomethacin, methyl salicylate, oxyphenbutazone, phenylbutazone, and salicylic acid. One hundred microliters of S-warfarin solution and 50 µl of the inhibitor solution were

* To whom correspondence should be addressed. © 1998 Pharmaceutical Society of Japan
added to the reaction solution described above. The solvent of the inhibitor was used for the control study.

**Assay for 7-OH warfarin** Since 80% to 85% of S-warfarin is eliminated via 6-OH and 7-OH warfarin and the extent of the formation of 7-OH warfarin is 3.5 times greater than that of 6-OH warfarin, we measured only 7-OH warfarin to estimate the kinetic parameters. Seven-OH warfarin was assayed using an HPLC system with a fluorometric detector. The excitation and emission wavelengths were 312 and 416 nm, respectively. A reverse-phase C18 column and a mobile phase of acetonitrile: 0.5% phosphoric acid = 60:40 (v/v) were used. After making a calibration curve for 7-OH warfarin, 20 μl of the supernatant obtained from the metabolic reaction was injected into the HPLC system and the concentration of the metabolite formed was determined.

**Data analysis** An Eadie-Hofstee plot was adopted to determine the type of inhibition (competitive or mixed), and then the kinetic parameters were determined by nonlinear regression of unweighted data using the computer program WinNonlin (Scientific Consulting, Inc., Apex, NC, USA). The velocity of 7-OH warfarin formation (V; pmol/min/nmol P450), standardized by the amount of cytochrome P450, was expressed as:

\[
V = \frac{V_{max}S}{K_m(1 + \frac{I}{K_i}) + S} \quad \text{(competitive)} \\
V = \frac{V_{max}S}{K_m(1 + \frac{I}{K_i}) + S(1 + \frac{I}{\alpha K_i})} \quad \text{(mixed)}
\]

where \(V_{max}\) and \(K_m\) are the maximum velocity and Michaelis constant, respectively, \(K_i\) is the inhibition constant, and \(S\) and \(I\) denote the concentrations of S-warfarin and inhibitor, respectively. The term \(\alpha\) was introduced to describe the affinity of the enzyme-warfarin and enzyme-inhibitor complexes for inhibitor and warfarin, respectively.

**RESULTS AND DISCUSSION**

Formation of 7-OH warfarin was found to be linear for at least 60 min, showing that the velocity calculated at 60 min would correspond to the initial velocity.

We carried out the metabolic reaction of S-warfarin (2.5 μM) with or without NSAIDs (25 μM) using human recombinant CYP2C9 in order to screen substrates showing significant inhibition of S-warfarin metabolism. Figure 1 shows the inhibition of S-warfarin metabolism by the ten NSAIDs tested. Six NSAIDs, bucolome, diflunisal, flurbiprofen, ibuprofen, indomethacin, and phenylbutazone, showed more than 50% inhibition. We then performed a metabolic study using pooled human liver microsomes to calculate precisely the inhibition kinetic parameters of S-warfarin (2.5 - 25 μM).

Table I demonstrates the \(K_i\) estimated, unbound fractions (fUB), total level of inhibitors in clinical situations cited from previous reports, free concentration of inhibitor (Ii), and \(1+I/I/K_i\) calculated using \(K_i\) and Ii described above. Because of the low free level of S-warfarin in the human body, the extent of increase in the steady-state average free level of warfarin could be expressed as \(1+I/I/K_i\), regardless of inhibition type. As shown in Table I, only phenylbutazone and bucolome were expected to have a clinically significant effect
on the pharmacokinetics of $S$-warfarin since displacement of the protein binding of warfarin would not increase the free concentration in the steady-state condition.  

Table 1. Estimated Increase in S-Warfarin Free Blood Concentration in the Steady State in Humans

<table>
<thead>
<tr>
<th>Type of inhibition</th>
<th>Ki (µM)</th>
<th>$f_{UB}$</th>
<th>I (µM)</th>
<th>Ir (µM)</th>
<th>$1+Ir/Ki$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bucolome</td>
<td>Com</td>
<td>8.5</td>
<td>0.1</td>
<td>380</td>
<td>38.0</td>
</tr>
<tr>
<td>Diflunisal</td>
<td>Mix</td>
<td>4.9</td>
<td>0.001</td>
<td>280</td>
<td>0.28</td>
</tr>
<tr>
<td>Flurbiprofen</td>
<td>Com</td>
<td>5.1</td>
<td>0.005</td>
<td>25</td>
<td>0.125</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>Mix</td>
<td>17.6</td>
<td>0.007</td>
<td>40</td>
<td>0.28</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>Com</td>
<td>4.6</td>
<td>0.1</td>
<td>1</td>
<td>0.1</td>
</tr>
<tr>
<td>Phenylbutazone</td>
<td>Mix</td>
<td>5.0</td>
<td>0.04</td>
<td>380</td>
<td>15.2</td>
</tr>
</tbody>
</table>

Com: Competitive inhibition. Mix: Mixed inhibition. Ki: Inhibition constant obtained in this study. $f_{UB}$: Unbound fraction of inhibitors. I: Average total blood concentration of inhibitors in steady state in clinical treatments, calculated from the values of clearance and usual dose reported in the literature. Ir: Free blood concentration of inhibitors in steady state, calculated from the values of $f_{UB}$ and I. $1+Ir/Ki$: Estimated increase in the ratio of the free concentration of $S$-warfarin in the steady state.

In conclusion, phenylbutazone and bucolome are suggested to show significant pharmacokinetic interactions with $S$-warfarin, which would increase the free level of $S$-warfarin due to inhibition of $S$-warfarin metabolism in the liver. The other NSAIDs studied induced no significant effect on the pharmacokinetics of $S$-warfarin, showing either pharmacodynamic interaction or no clinically significant effect on coagulant therapy.

REFERENCES

(Received January 29, 1998; accepted March 17, 1998)