PHARMACOKINETIC INTERACTION BETWEEN WARFARIN AND A URICOSURIC AGENT, BUCOLOME: APPLICATION OF IN VITRO APPROACHES TO PREDICTING IN VIVO REDUCTION OF (S)-WARFARIN CLEARANCE

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ABSTRACT:
A uricosuric agent, bucolome, has been shown to intensify the anticoagulant effect of warfarin. The aims of the present study were to clarify its mechanism(s) and to apply in vitro approaches for predicting this potentially life-threatening interaction in vivo. An in vivo study revealed that Japanese patients given warfarin with bucolome (300 mg/day, n = 21) showed a 1.5-fold greater international normalized ratio than those given warfarin alone (n = 34) despite that the former received a 58% smaller warfarin dose than the latter. Enantioselective assays revealed that bucolome increased plasma unbound fractions of (S)- and (R)-warfarin by 2-fold (P < .01), reduced unbound oral clearances of (S)- and (R)-warfarin by 84 (P < .01) and 26% (P < .05), respectively, and inhibited the unbound formation clearance for (S)-warfarin 7-hydroxylation by 89% (P < .01). In contrast, bucolome elicited no appreciable changes in the plasma unbound (S)-warfarin concentration versus the international normalized ratio relationship. In vitro studies with recombinant human cytochrome P-450 C29 and liver microsomes showed that bucolome was a potent mixed-type inhibitor for (S)-warfarin 7-hydroxylation, with Ki values of 8.2 and 20.2 μM, respectively. An in vitro model incorporating maximum unbound bucolome concentration in the liver estimated as a sum of hepatic artery and portal vein concentrations and in vitro Ki made an acceptable prediction for bucolome-induced reductions in in vivo total (bound + unbound) oral clearance, unbound oral clearance, and unbound formation clearance for (S)-warfarin. In conclusion, the augmented anticoagulant effect of warfarin by bucolome due to the metabolic inhibition for pharmacologically more potent (S)-warfarin may be predictable from in vitro data.

Warfarin is a widely prescribed anticoagulant possessing a narrow therapeutic index. Because potentiation of its anticoagulant effect by coadministered drugs could lead to life-threatening major bleeding (Hirsh et al., 1998), studies have been carried out for more than 250 different therapeutic agents to assess whether coadministration of these agents might intensify the anticoagulant effect of warfarin (Harder and Thürmann, 1996). However, because some of them were either clinical observations of a small number of patients or animal experiments, exact clinical implications of their results often remain obscure (Wells et al., 1994). Bucolome (5-n-butyl-1-cyclohexyl-2,4,6-trioxopyrimidine), Paramidine) is one of the pyrazolidine derivatives (e.g., phenylbutazone) that has been used as a uricosuric and/or anti-inflammatory agent in Japan since 1967 (Yashiki, 1971) and in some other countries. Besides its original clinical indication, the drug has been used anecdotally as an enhancer for the anticoagulant effect of warfarin, particularly in patients whose anticoagulant response to commonly recommended doses of warfarin (e.g., less than 5 mg/day) is still insufficient in Japan (Mashima et al., 1974). Many clinical reports have indicated that the coadministration of bucolome with warfarin substantially augmented its anticoagulant effect as long as coadministered.

Abe et al. (1995) suggested that displacement of warfarin from its plasma protein binding site(s) by bucolome could be responsible for this interaction because both drugs bind extensively to human albumin and possibly share common binding site(s) for acidic drugs (Kakemi et al., 1966; Wilting et al., 1980). However, theory predicts that the potentiation of pharmacological responses via a binding displacement mechanism should be transient despite long-term administration of the displacer drug (Aarons and Rowland, 1981). Thus the warfarin-bucolome interaction cannot be explained simply by the displacement mechanism. An alternative and more persuasive explanation for the interaction would be a bucolome-induced inhibition of warfarin metabolism, particularly for its pharmacologically more active (S)-enantiomer (O’Reilly, 1974). (S)-Warfarin is extensively metabolized by the liver to its major metabolite, (S)-7-hydroxywarfarin, in humans (Toon et al., 1986; Heimark et al., 1992; O’Reilly et al., 1992).

According to the physiological model (Wilkinson and Shand, 1975),
unbound plasma concentration \((C_u)\) of \((S)\)-warfarin at steady state after oral dosing is dependent solely on the hepatic intrinsic clearance \((C_{int,h})\) representing hepatic enzyme activity. To our knowledge, however, no attempts have been made to assess whether bucolome would possess an inhibitory potency for the 7-hydroxylation of \((S)\)-warfarin either in vitro or in vivo. Thus we decided to carry out a clinical study to clarify the mechanism(s) of the warfarin-bucolome interaction in light of changes not only in the unbound fraction in plasma \((f_u)\) of warfarin enantiomers but also in unbound oral clearance \((C_{po,u})\) and unbound formation clearance \((C_{m,u})\) for \((S)\)-warfarin 7-hydroxylation.

Numerous attempts have been made to predict in vivo drug interaction caused by a reversible metabolic inhibitor based on in vitro data (for review, see von Molke et al., 1998). For instance, von Molke et al. (1996) forecasted ketoconazole-induced reductions in in vivo triazolam clearance by using the in vitro inhibitory constant \((K_i)\). Recently, Sugiyama et al. (1996) and Ito et al. (1998) have suggested that a maximum unbound concentration of inhibitor at the entrance of the liver estimated from its total plasma drug concentration \((C_p)\). In contrast, Kunze and Trager (1996) made a prediction based on \(K_i\) value for an inhibitor and its unbound plasma inhibitor concentrations both of which were obtained from in vivo studies. At present, it remains uncertain whether either an estimated total liver concentration of metabolic inhibitor or unbound plasma inhibitor concentration would be a better substitute for actual unbound concentrations of inhibitors at the intracellular hepatic enzyme sites \((I_s)\).

In this context, we decided to study whether the magnitude of bucolome-induced reductions in total (bound + unbound) oral clearance \((C_{po})\), \((C_{po,u})\), and \((C_{m,u})\) for \((S)\)-warfarin may be predicted based on \(f_u\) estimated by using Sugiyama’s equation and in vitro \(K_i\) of bucolome for 7-hydroxylation of \((S)\)-warfarin determined with recombinant human cytochrome P-450 (CYP) 2C9 and human liver microsomes. We also discuss the applicability of the present approach for predicting an interaction between warfarin and another metabolic inhibitor for CYP2C9, fluconazole.

### Materials and Methods

**Chemicals.** \((R/S)\)- and \((S)\)-warfarin and \((R/S)\)-7-hydroxywarfarin were provided by Eisai Co., Ltd. (Tokyo, Japan). \(^{14}\text{C}\)(\((R/S)\)-warfarin (specific activity, 2.11 GBq/mmol) was purchased from Amersham (Buckinghamshire, UK) and separated into each enantiomer according to the method developed by Takanashi et al. (1997). All other chemicals were obtained from Wako (Osaka, Japan).

**In Vivo Study for Warfarin-Bucolome Interaction.** Patients. Fifty-five Japanese cardiac patients [24 males and 31 females; mean age, 59 years (range: 30–76 years); mean weight, 53.0 kg (range: 37.5–75 kg)] were recruited at the Department of Cardiovascular Surgery, International Medical Center of Japan; the First Department of Surgery, Showa University Hospital; the Second Department of Surgery, Sapporo Medical University Hospital; and the Department of Internal Medicine, National Yokohama Hospital. Thirty-four patients were given only warfarin (the control group) and 21 patients were given warfarin and bucolome (300 mg/day; the bucolome group) for the anticoagulant therapy after prosthetic valve replacement. They were not given any concomitant medications that could have interfered with pharmacokinetics or pharmacodynamics of warfarin (e.g., simvastatin, clofibrate, allopurinol, benz bromarone, tolbutamide, cimetidine, loxoprofen, lansoprazole, diltiazem, quinidine, and levethyroxine) according to previous studies (for review, see Harder and Thürmann, 1996). Each patient received a constant maintenance dose of racemic warfarin for at least 1 month before the study and demonstrated a stable anticoagulation status throughout the three preceding international normalized ratio (INR) measurements (i.e., <15% of variation). Biochemical and hematological tests performed before the study revealed no evidence of hepatic impairment but indicated renal impairment in three patients (i.e., creatinine clearances: 20–30 ml/min). Informed consent was obtained from each patient after explaining the purpose of the study thoroughly before the study began. The study protocol had been approved by institutional review boards at the respective medical institutes.

**Study protocol.** According to the criteria for patient selection described above, we assumed that plasma concentrations of warfarin were obtained at steady state. Blood samples (ca. 10 ml) were taken at the time of a routine visit from each patient approximately 16 h after oral administration. Separated plasma was divided into two portions: one for determining INR or Thrombotest (TT%), depending on the selection of an anticoagulant test in each institution and the other for measuring \(C_u\) of warfarin enantiomers. To calculate the \(C_{m,u}\) additional urine samples were obtained from 33 and 10 patients in the control and the bucolome groups, respectively; urine was collected for approximately 2 h after voiding bladder completely and the blood was withdrawn at around the midpoint of the urine collection period. Total urine volume and collection period were recorded and a portion (ca. 10 ml) of the sample was retained for 7-hydroxywarfarin assay. Plasma and urine samples were stored at –70°C until analyzed.

**Determination of concentrations and unbound fraction of warfarin enantiomers in plasma and \((S)\)-7-hydroxywarfarin in urine.** Detailed assay procedures for determining \(C_u\) and \(f_u\) of warfarin enantiomers and \((S)\)-7-hydroxywarfarin concentrations in urine were reported elsewhere (Takahashi et al., 1997). Briefly, enantiomers of warfarin in plasma and \((S)\)-7-hydroxywarfarin in urine were determined by the chiral HPLC-UV and HPLC-fluorescence methods, respectively, using a cellulose derivative column (Chiralcel OD, 10 μm, 250 × 4.6 mm i.d.; Daicel Chemical Industries, Tokyo, Japan). The \(f_u\) of warfarin enantiomers was determined by an ultrafiltration method with Centricon MPS-3 (Amicon Co., Beverly, MA), adding small amounts of \(^{14}\text{C}\)warfarin enantiomers to plasma samples.

**In vivo pharmacokinetic analysis.** \(C_{po}\) and \(C_{po,u}\) for the respective warfarin enantiomers were obtained according to the following equations:

\[
C_{po} = (D/2\tau)/C_{po,u}
\]

(1)

\[
C_{po,u} = (D/2\tau)/C_{m,u}
\]

(2)

where \(D/2\tau\) is the daily dose of respective warfarin enantiomers, \(\tau\) is the dosing interval (i.e., 24 h), and \(C_{po,u}\) and \(C_{m,u}\) (calculated as \(C_{po}\), \(f_u\)) are the average total and unbound concentrations of warfarin enantiomers at steady state, respectively.

Assuming that \((S)\)-7-hydroxywarfarin is formed exclusively from \((S)\)-warfarin and undergoes urinary excretion without additional metabolism, \(C_{m,u}\) was calculated by the following equation:

\[
C_{m,u} = \text{the urinary excretion rate of } (S)\text{-7-hydroxywarfarin/}
C_{po,u}\text{ of } (S)\text{-warfarin}
\]

(3)
different concentrations (2.5, 5, and 10 mM potassium phosphate buffer (pH 7.4), and (S)-warfarin through a membrane (0.45 μm), 100 mM was centrifuged at 21,800 g. It was prepared by differential centrifugation and stored at -80°C until used. Microsomes containing recombinant human CYP2C9*1 (wild-type) were prepared from fission yeast (Schizosaccharomyces pombe) according to the methods of Yasumori (1997) and Giga-Hama et al. (1994). For simplicity, CYP2C9 instead of CYP2C9*1 is used henceforth for designating the wild-type genotype of this isoform. Protein concentration, CYP content, and NADPH-cytochrome c reductase activity of microsomes obtained from the recombinant CYP2C9 were determined to be 29.2 mg/ml, 67 pmol P-450/mg protein, and 420 nmol/min/mg protein, respectively.

In vitro inhibition study. To assess the effect of bucolome on the in vitro (S)-warfarin 7-hydroxylation activity, (S)-warfarin was incubated under the conditions described below with use of the microsomes prepared from human liver and the recombinant CYP2C9, respectively. Reaction mixtures (250 μl) containing 0.1 mg/ml of human liver microsomes or CYP2C9 protein, and 100 mM potassium phosphate buffer (pH 7.4), and (S)-warfarin at three different concentrations (2.5, 5, and 10 μM for human liver and CYP2C9 microsomes) were incubated at 37°C for 30 min. The reaction was stopped by adding cold acetonitrile (100 μl); then 0.5 mg/ml naproxen in methanol (25 μl) was added into the reaction mixture as the internal standard. Then the mixture was centrifuged at 21,800 g for 20 min at 4°C. After filtration of the supernatant through a membrane (0.45 μm), 100 μl of the filtrate was injected onto an HPLC apparatus. Three separate sets of the experiment were also performed in the presence of bucolome at concentrations of 49.2, 98.4, and 196.8 μM for human liver microsomes and 100, 200, and 400 μM for CYP2C9 microsomes, respectively. All experiments described above were performed in triplicate. To avoid any inadvertent interference in the reaction mixture, two sets of negative control experiments were performed by eliminating either enzyme sources (i.e., human liver microsomes or the recombinant CYP2C9) or (S)-warfarin from the incubation mixture.

Determination of (S)-7-hydroxywarfarin concentrations in microsomes. 7-Hydroxywarfarin formed in the reaction mixture was determined according to the method reported elsewhere (Takahashi et al., 1997) with minor modifications. Briefly, 7-hydroxywarfarin and the internal standard was separated by a reversed-phase chiral HPLC column (Chiralcel OD-RH, 5 μm, 150 X 4.6 mm i.d.; Daicel Chemical Industries, Tokyo, Japan) at 25°C and detected with a fluorescent detector set at excitation and emission wavelengths of 315 and 400 nm, respectively. The mobile phase consisted of a 59:41 (v/v) mixture of 0.2 M phosphate buffer (pH 2.0) and acetonitrile was delivered at a flow rate of 0.5 ml/min.

In vitro enzyme kinetic analysis for warfarin-bucolome interaction. In a preliminary experiment we confirmed that the in vitro metabolism of (S)-warfarin performed under the current experimental conditions was linear with regard to incubation time and microsomal protein concentration (data not shown). In addition, the above reaction was considered to be described by a single component enzyme kinetic model over the substrate concentrations used herein (data not shown). To assess the inhibitory effect of bucolome on the microsomal metabolism of (S)-warfarin, the velocity of (S)-7-hydroxywarfarin formation (V) was measured in the presence and absence of bucolome in the incubation mixture. In the absence of bucolome:

\[
V = V_{max} \cdot \frac{[S]}{[S] + K_m}
\]

where \([S]\) is the concentration of (S)-warfarin in the reaction mixture, \(V_{max}\) is the maximum velocity of the catalytic reaction, and \(K_m\) is the dissociation constant of (S)-warfarin-enzyme complex. Enzyme kinetic analysis on the in vitro interaction between (S)-warfarin and bucolome with both Lineweaver-Burk (Fig. 1) and Eadie-Scatchard (not shown) plots indicated that bucolome would be a mixed-type inhibitor. Thus, we considered that the velocity of the reaction in the presence of bucolome \([V(B)]\) would be described as follows according to Segel (1993):

\[
V(B) = V_{max} \cdot \frac{[S]}{[S] + K_m} \cdot \left(1 + \frac{[I]}{K_i}\right)
\]

where \([I]\) is the unbound concentration of bucolome used for the inhibition study (i.e., 49.2, 98.4, and 196.8 μM for human liver microsomes and 100, 200, and 400 μM for recombinant CYP2C9 microsomes, respectively), \(K_i\) is the inhibition constant of bucolome for warfarin-enzyme complex, and \(\alpha (≥ 1)\) is the dissociation constant of the (S)-warfarin-enzyme-bucolome complex to the (S)-warfarin-enzyme and bucolome-enzyme complexes. \(V_{max}\), \(K_m\), \(K_i\), and \(\alpha\) were estimated by fitting a set of data obtained without the inhibitor to eq. 4 and three sets of experimental data obtained in the presence of the different concentrations of the inhibitor to eq. 5 simultaneously, using a nonlinear least-squares regression method assuming uniform error for the dependent variables \([V]\) and \([V(B)]\) and percent uniform error for the independent variable \([S]\) and the normal distribution for enzymatic parameters (\(V_{max}\), \(K_m\), \(K_i\), and \(\alpha\)). The kinetic parameters estimated by the Lineweaver-Burk plots were used as the initial estimate for the nonlinear regression analysis.

Prediction of the In Vivo (S)-Warfarin-Bucolome Interaction from In Vitro Data. Prediction of in vivo \(Cl_{int}, Cl_{out}\), and \(Cl_{max}\) in the presence or absence of bucolome was made according to the methods of Shaw and
patients given warfarin alone (i.e., the control group) and those given warfarin and bucolome, cant differences in age, sex distribution, and body weight between the

during the coadministration of bucolome,

presence over those in the absence of bucolome in patients (e.g., Cl(B)/Cl in

test. The 95% confidence intervals for the ratios of the Cl values in the

receiving warfarin with and without bucolome were performed by unpaired

study. Comparisons for clinical and pharmacokinetic data between the patients

context,

avoid falsely negative prediction rather than falsely positive prediction. In this

inhibitor into the hepatocytes as follows:

\[ L = A \cdot L_{\max} = A \cdot [C_{f}(I)_{\max} \cdot R_b + (K_s \cdot D \cdot F_u/Q)] \cdot f_j/R_b \]  

where A is a factor for assumed active transport of inhibitors into the hepa-
tocytes, \( C_{f}(I)_{\max} \) is the maximum plasma concentration of the inhibitor (in this case, bucolome), \( R_b \), \( K_s \), \( D \), and \( F_u \) are the ratio of inhibitor concentration in blood to that in plasma, the absorption rate constant, the oral dose and the bioavailability of bucolome through the gastrointestinal tract, respectively. Applicability of the above equation to the bucolome and (S)-warfarin interaction will be described in detail in Results.

To further validate the current approach to predict the magnitude of war-
farin-bucolome interaction in vivo from in vitro data, we applied this method for another metabolic inhibition for (S)-warfarin by fluconazole.

Effect of Bucolome on the Relationship between Unbound (S)-Warfarin Concentration Versus Anticoagulation Response. Relationships between \( C_u \) of (S)-warfarin and INR were examined in 12 of 21 patients given warfarin with bucolome and in all patients \( n = 34 \) given warfarin alone. The data were analyzed using the orthogonal least-squares linear regression method (Wagner and Ayres, 1977).

Statistical Analysis. All data are expressed as means \( \pm \) S.D. throughout the study. Comparisons for clinical and pharmacokinetic data between the patients receiving warfarin with and without bucolome were performed by unpaired t

test. The 95% confidence intervals for the ratios of the Cl values in the presence over those in the absence of bucolome in patients (e.g., Cl(B)/Cl in Table 4) were obtained by the Aspin-Welch test. A p value of < .05 was considered statistically significant throughout the study.

Results

In Vivo Warfarin-Bucolome Interaction. There were no significant differences in age, sex distribution, and body weight between the patients given warfarin alone (i.e., the control group) and those given warfarin and bucolome (i.e., the bucolome group): 61 \( \pm \) 8 (mean \( \pm \) S.D.) versus 56 \( \pm \) 10 years, 16/18 versus 8/13 (males/females), and 54.1 \( \pm \) 10.2 versus 51.3 \( \pm \) 9.1 kg for the control and bucolome


<table>
<thead>
<tr>
<th>TABLE 1</th>
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<tr>
<td><strong>Oral doses of racemic warfarin, ( f_u ), ( C_u ), and ( C_p ) values of warfarin enantiomers and anticoagulant responses in patients given warfarin alone (Control) and those given warfarin and bucolome (With Bucolome)</strong></td>
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<tr>
<td><strong>Dosage of racemic warfarin (mg/day)</strong></td>
</tr>
<tr>
<td>Control ( n = 34 )</td>
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<tr>
<td>INR</td>
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<tr>
<td>Dose-adjusted INR (mg(^{-1}))</td>
</tr>
<tr>
<td>( (S)-)warfarin ( C_u ) (( \mu )g/ml)</td>
</tr>
<tr>
<td>( (R)-)warfarin ( C_u ) (( \mu )g/ml)</td>
</tr>
<tr>
<td>( (S/R)-)warfarin ( C_u ) (( \mu )g/ml)</td>
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<tr>
<td>( (S/R)-)ratio</td>
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<tr>
<td>( C_p ) (ng/ml)</td>
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<tr>
<td>( C_u ) (ng/ml)</td>
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<tr>
<td>( (S)-)warfarin ( K_a ) (( \mu )g/ml)</td>
</tr>
<tr>
<td>( (R)-)warfarin ( K_a ) (( \mu )g/ml)</td>
</tr>
<tr>
<td>( (S/R)-)ratio</td>
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</table>

Data are means \( \pm \) S.D. ** \( p < .01 \) as compared with Control group.
control group. Therefore, an increase in the (S)/(R) ratios would be associated not only with the pharmacogenetic polymorphism of CYP2C9*3 mutation (Takahashi et al., 1998) but also with a metabolic inhibition of warfarin preferentially to its (S)-enantiomer.

Pharmacokinetic analysis using the above described plasma concentration data and oral doses of individual patients revealed that the mechanism responsible for the in vivo bucolome-warfarin interaction would be attributable largely to the bucolome-induced metabolic inhibition preferentially against the pharmacologically more active (S)-warfarin (Table 2). Specifically, the mean Cl po,u and Cl m,u obtained from the bucolome group were reduced by about 85 and 90% (p < .01), respectively, as compared with the corresponding values obtained from the control group. Similarly, the mean Cl po for (S)-warfarin obtained from the bucolome group was reduced by 70% (p < .01) as compared with that obtained from the control group. As described earlier, Cl po of warfarin can be expressed as \( f_a \cdot Cl_{po,u} \), and Cl po was decreased but increased \( f_a \) of (S)-warfarin. Therefore, the inhibitory effect of bucolome assessed by Cl po tended to underestimate that assessed by Cl po,u (Tables 1 and 2). Regarding the (R)-warfarin-bucolome interaction Cl po,u for (R)-warfarin obtained from the bucolome group was reduced by 25% (p < .05) as compared with that obtained from the control group. Because bucolome increased \( f_a \) of (R)-warfarin by approximately 2-fold, Cl po,u tended to underestimate that assessed by Cl po,u (Tables 1 and 2). Therefore, the inhibitory effect of bucolome was assessed by Cl po,u for pharmacologically more active (S)-warfarin so that the inhibition of the nonradioactive warfarin was so strong that INR obtained from the bucolome group was significantly greater than that obtained from the control group despite the fact that the ratio of racemic warfarin of the former was reduced by about 50% as compared with that obtained from the latter (Table 1).

In Vitro Warfarin-Bucolome Interaction. Eadie-Scatchard plots for the in vitro formation of (S)-7-hydroxywarfarin from (S)-warfarin with microsomes obtained either from human liver or CYP2C9 in the absence of bucolome appeared to consist of a single component within the substrate concentrations studied herein by visual inspection (data not shown). Thus, each set of the data were fitted to Eadie-Scatchard plots (data not shown) indicated that bucolome by Dixon plots would be a mixed-type, rather than a pure competitive, inhibitor for (S)-warfarin-7-hydroxylation. Based on these considerations, we used \( K_i \) values calculated by assuming the mixed-type inhibition for the warfarin-bucolome interaction for estimating quantitative changes in vivo pharmacokinetics of warfarin enantiomers as described below. However, when the data were fitted to the mixed-type inhibition model as shown in the eq. 5, extremely large \( K_i \) values were obtained (Table 3), indicating that the contribution of the noncompetitive component to V(B) or the overall (S)-warfarin-bucolome interaction would be minimal under the in vitro experimental conditions used herein and possibly in vivo conditions. In addition, \( K_i \) values calculated by assuming the mixed-type inhibition for (S)-warfarin-bucolome interaction for the data obtained from human liver microsomes and the recombinant CYP2C9 (i.e., 20.2 and 8.2 \( \mu M \), respectively) were largely similar to the corresponding values calculated by assuming the pure competitive inhibition model (14.2 and 6.6 \( \mu M \), respectively) for the reaction.

Predictability of In Vivo Reduction for (S)-Warfarin Cl from In Vitro Data. In the present study, \( f_a \) for (S)-warfarin was assumed to be equal to the fraction that was not mediated by the reaction forming warfarin alcohol with or without subsequent conjugation reaction. It was also assumed that bucolome would possess largely similar \( K_i \) values not only for a major metabolic pathway of (S)-warfarin (i.e., 7-hydroxylation) but also other minor pathway(s) (e.g., 7-hydroxylation). Furthermore, because the value in eq. 5 was revealed to be extremely large (Table 3), the contribution of noncompetitive component (i.e., \( f_{na}/\alpha \cdot K_i \)) to overall V(B) value was considered insignificant and thus was neglected in eqs. 6 to 8. Taking these assumptions, the previously reported data (Kunze and Trager, 1996) and bucolome-induced changes in \( f_a \) in patients (Table 2) into account, \( f_{na} \), \( f_{n}(B) \), and \( f_{na}(B) \) for the interaction between (S)-warfarin and bucolome were considered to be 0.87, 0.98, and 2.1, respectively. Because the oral bioavailability for warfarin was shown to be almost unity (Porter and Sawyer, 1992), we assumed that the bioavailability of the drug through the gastrointestinal tract (\( f_a \)) and the liver would be unaffected by the coadministration of bucolome. Table 4 shows the predictibility of the present in vitro approach for bucolome-induced changes in in vivo kinetic parameters assessed as the percentage relative to the respective control values (i.e., Cl po,u(B)/Cl po,u,Cl po,u(B)/Cl po,u,Cl po(B)/Cl po, and Cl po(B)/Cl po). For this attempt, we used in vitro \( K_i \) values obtained from both human liver and cDNA-expressed human liver microsomes.

### Table 2

<table>
<thead>
<tr>
<th>Condition</th>
<th>Cl po,u (ml/min)</th>
<th>Cl m,u (ml/min)</th>
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<tbody>
<tr>
<td>Control</td>
<td>238.7 ± 342.6</td>
<td>26.1 ± 13.7</td>
</tr>
<tr>
<td>With Bucolome</td>
<td>674 ± 308</td>
<td>107 ± 68</td>
</tr>
<tr>
<td>(S)-warfarin</td>
<td>255 ± 99</td>
<td>189 ± 26</td>
</tr>
<tr>
<td>(R)-warfarin</td>
<td>5.59 ± 0.34</td>
<td>1.70 ± 0.21</td>
</tr>
<tr>
<td>(S)-warfarin</td>
<td>2.15 ± 0.76</td>
<td>2.85 ± 1.45</td>
</tr>
</tbody>
</table>

Data are means ± S.D. *p < .05 and **p < .01 as compared with Control group. Urinary samples for calculating Cl po and plasma samples for \( f_a \) were available in 33\(^\text{rd}\) and 7\(^\text{th}\) of 21 patients for the Control and Bucolome groups, respectively.

### Table 3

<table>
<thead>
<tr>
<th>Kinetic Parameters</th>
<th>Human Liver Microsomes</th>
<th>CYP2C9 Microsomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>( V_{max} ) (pmol/mg protein/min)</td>
<td>4.33 ± 0.15</td>
<td>18.9 ± 0.57</td>
</tr>
<tr>
<td>( K_m ) (( \mu M ))</td>
<td>1.50 ± 0.23</td>
<td>1.86 ± 0.23</td>
</tr>
<tr>
<td>( K_i ) (( \mu M ))</td>
<td>20.2 ± 3.62</td>
<td>8.24 ± 1.48</td>
</tr>
<tr>
<td>( \alpha )</td>
<td>8696 ± 349</td>
<td>1293 ± 527</td>
</tr>
</tbody>
</table>

Data are means ± S.D., which are asymptotic S.D.

* Equivalent to 282 pmol/nmol P-450/min.

The analysis of in vitro interaction between (S)-warfarin and bucolome by Dixon plots (data not shown) indicated that bucolome would be either a competitive or mixed-type inhibitor. However, the analysis of the data by Lineweaver-Burk plots (Fig. 1) revealed that the regression lines drawn for each set of data converged at a point in the second quadrant for both human liver and CYP2C9 microsomes. In addition, Eadie-Scatchard plots (data not shown) showed that the regression lines drawn for each data set converged under the x-axis for both human liver and CYP2C9 microsomes. Both of the findings suggested that bucolome would be a mixed-type, rather than a pure competitive, inhibitor for (S)-warfarin-7-hydroxylation. Based on these considerations, we used \( K_i \) values calculated by assuming the mixed-type inhibition for the warfarin-bucolome interaction for estimating quantitative changes in vivo pharmacokinetics of warfarin enantiomers as described below. However, when the data were fitted to the mixed-type inhibition model as shown in the eq. 5, extremely large \( K_i \) values were obtained (Table 3), indicating that the contribution of the noncompetitive component to V(B) or the overall (S)-warfarin-bucolome interaction would be minimal under the in vitro experimental conditions used herein and possibly in vivo conditions. In addition, \( K_i \) values calculated by assuming the mixed-type inhibition for (S)-warfarin-bucolome interaction for the data obtained from human liver microsomes and the recombinant CYP2C9 (i.e., 20.2 and 8.2 \( \mu M \), respectively) were largely similar to the corresponding values calculated by assuming the pure competitive inhibition model (14.2 and 6.6 \( \mu M \), respectively) for the reaction.

### Table 4

<table>
<thead>
<tr>
<th>Condition</th>
<th>Cl po,u Reduction %</th>
<th>Cl m,u Reduction %</th>
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<tbody>
<tr>
<td>Control</td>
<td></td>
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<tr>
<td>With Bucolome</td>
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<tr>
<td>(S)-warfarin</td>
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<tr>
<td>(R)-warfarin</td>
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For this attempt, we used in vitro \( K_i \) values obtained from both human liver and cDNA-expressed human liver microsomes.
Collectively, R predictions for the changes observed in Cl po,u and Cl po are largely within the range of 10.9 (1.8–23.2) obtained from patients. Data in parenthesis are the 95% confidence intervals for the respective ratios obtained from patients. Detailed description for the equations used for calculating the ratios for the respective clearance values is given in the text.

CYP2C9 microsomes. Iu was estimated by substituting the following parameters into eq. 9. Regarding A in eq. 9, we substituted both 10 and 1 for A, assuming the presence or absence of active transport of the metabolic inhibitor into the hepatocytes, respectively, because it remains unknown whether bucolome would be actively transported into the hepatocytes or not. A steady-state C(I)max during chronic oral administration of bucolome was estimated to be approximately 100 µg/ml based on the data obtained by healthy volunteers (n = 5; Yashiki, 1971); the average maximum plasma concentration after a single oral administration of the drug at a dose of 300 mg was 50 µg/ml, the accumulation factor (i.e., 1/1 – e−Kt) calculated by the elimination rate constant (K) of 0.028 h−1 and the dosing interval of 24 h was 2.04. Kp calculated based on their data was approximately 0.018 min−1. In addition, Fp for bucolome and Q were assumed to be unity and 1,610 ml/min (Ito et al., 1998), respectively. According to the report of Kakemi et al. (1966) fpo for bucolome was considered to be 0.1. Mf for bucolome was estimated to be 0.7, because Cm for bucolome determined with an HPLC-UV method in plasma that was separated from blank blood spiked with the drug to make a concentration of 50 µg/ml was 69.3 ± 5.7 µg/ml (n = 3; H.T., M. Sakurai and H.E., unpublished data). Collectively, Iu for bucolome at the steady-state oral dosing of 300 mg was estimated to be 394 and 39.4 µM when the active transport factor in eq. 9 (i.e., A) was set to 10 and 1 for A, assuming the presence or absence of active transport, respectively. As for bucolome, approximately 95% and 5% of Iu were considered to be derived from the hepatic artery and portal vein, respectively. To further validate eq. 9, we compared the Iu value calculated by eq. 9 with that estimated by eq. 6 for which Iu for bucolome and Fm, Kp, and Mm of bucolome were reported to be 0.9, 0.89, and 0.013 min−1, respectively (Blum et al., 1991). Based on these data, the steady-state maximum Cm for the drug given orally (400 mg) once a day was estimated to be 15.2 µg/ml. Kp values used for estimation were obtained from an in vitro inhibition study performed with human liver microsomes (7.5 µM) and from an in vivo study performed with healthy subjects (22 µM; Kunze and Trager, 1996). Because Ervine and Houston (1994) reported no active transport for the drug into the hepatocytes, A in eq. 9 was considered unity. Results indicated that this comprehensive approach predicted the reduction in Clm to be 71 and 88%, respectively, using in vivo and in vitro Kp values. Our result was largely similar to the observed reduction in in vivo patients (73%; Kunze and Trager, 1996).

When active transport was assumed (i.e., A = 10 in eq. 9), the in vitro approach somewhat overestimated the changes observed in Clpo,u regardless of the sources of Kp values but it gave largely correct predictions for the changes observed in Clpo,u and Clpo. In contrast, when the predictions were made by assuming no active transport of the inhibitor (i.e., A = 1), the present in vitro approach underestimated the changes for all the kinetic parameters as compared with those observed in patients (Table 4). In addition, the accuracy of the predictions made by using the Kp value derived from CYP2C9 seemed better than those made with that derived from human liver microsomes. Nonetheless, almost all predicted values lay within the 95% confidence intervals for those observed in patients except for those obtained by using Kp from human liver microsomes and assuming no active transport.

An attempt for applying the present in vitro approach to another case of metabolic interaction between (S)-warfarin and fluconazole revealed the following results. Because the elimination rate constant (k) of fluconazole is known to be 0.025 h−1, an accumulation ratio for the drug during chronic dosing given once a day (τ = 24 h) was assumed to be 2.22. In addition, the peak drug concentration after a single oral dose (400 mg) was reported to be 6.86 µg/ml and Fm, Fp, and Kp of fluconazole were reported to be 0.9, 0.89, and 0.013 min−1, respectively (Blum et al., 1991). Based on these data, the steady-state maximum Cm for the drug given orally (400 mg) once a day was estimated to be 15.2 µg/ml. Kp values used for estimation were obtained from an in vitro inhibition study performed with human liver microsomes (7.5 µM) and from an in vivo study performed with healthy subjects (22 µM; Kunze and Trager, 1996). Because Ervine and Houston (1994) reported no active transport for the drug into the hepatocytes, A in eq. 9 was considered unity. Results indicated that this comprehensive approach predicted the reduction in Clm to be 71 and 88%, respectively, using in vivo and in vitro Kp values. Our result was largely similar to the observed reduction in in vivo patients (73%; Kunze and Trager, 1996).

**Effect of Bucolome on the Relationship between Unbound (S)-Warfarin Concentration versus Anticoagulation Response.** There was a significant correlation (p < .05) between Cm of (S)-warfarin and INR in the patients given warfarin alone [INR = 0.267 · Cm (ng/ml) + 1.06] (Fig. 2). When the data obtained from patients given warfarin alone and those obtained from patients given warfarin and bucolome were combined and analyzed, there was also a significant correlation (p < .01) between Cm of (S)-warfarin and INR [INR = 0.235 · Cm (ng/ml) + 1.17] (Fig. 2). The two regression lines were almost superimposable because their slopes and y-intercepts were similar. Based on these findings, we considered that bucolome elicited no significant influence on the sensitivity of patients to anticoagulation.
effect of (S)-warfarin. As shown in Fig. 2, most patients receiving warfarin and buclocome showed higher $C_L$ for (S)-warfarin and INR than those receiving warfarin alone, despite that they were on lower doses of warfarin than those given warfarin alone (Table 1).

Discussion

The present study is the first to demonstrate that the mechanism of an anecdotaly recognized potentiation of the anticoagulant response to warfarin by coadministration of a uricosuric agent, buclocome, can be attributed to a metabolically based inhibition on the pharmacologically more active (S)-warfarin rather than to a pharmacodynamically based event (Tables 1 and 2). In addition, the magnitude of inhibition for (S)-warfarin metabolism observed in vivo may be predictable based on an in vitro metabolic model using human liver or CYP2C9 microsomes (Table 4).

We have revealed that buclocome given at a therapeutic dose (300 mg orally once a day) elicited a potent and enantioselective inhibition for both $C_{\text{po, u}}$ of (S)-warfarin and $C_{\text{m, u}}$ by 85 and 90%, respectively, in Japanese patients (Table 2). According to the well-stirred model, $C_{\text{po, u}}$ for (S)-warfarin represents the overall catalytic activity of the liver toward the enantiomer (i.e., $C_{\text{int, u}}$) and $C_{\text{m, u}}$ represents the portion of $C_{\text{int, u}}$ associated with the 7-hydroxylation of the enantiomer. Previous studies have shown that approximately 40 to 90% of (S)-warfarin is metabolized to (S)-7-hydroxywarfarin (Toon et al., 1986; Heimark et al., 1992; O’Reilly et al., 1992) and that the formation of these metabolites is catalyzed almost exclusively by a single CYP isofrom, CYP2C9 (Rettie et al., 1992). Thus, our data strongly indicate that the buclocome-induced reduction in in vivo metabolism of (S)-warfarin would most likely account for its inhibitory effect on CYP2C9. The finding that buclocome elicited a significant ($p < .05$) but much less potent inhibitory effect on $C_{\text{po, u}}$ for (R)-warfarin than that for (S)-warfarin (Table 2) may be explained by the fact the metabolism of (R)-warfarin involves the net results of catalysis by multiple isoforms of CYP (e.g., CYP1A2, CYP2C19, and CYP3A4) and ketoreductases (Rettie et al., 1992). Our data are in accordance with the findings that certain pyrazolone derivatives with anti-inflammatory and/or uricosuric property (e.g., phenylbutazone and sulfinpyrazone) caused an enantioselective inhibition of $C_{\text{po, u}}$ of (S)-warfarin, thereby potentiating anticoagulant response to warfarin (Toon et al., 1986; Chan et al., 1994). In contrast with the buclocome-induced changes in the pharmacokinetics of (S)-warfarin, there were no appreciable changes in the pharmacodynamics of the pharmacologically more active (S)-warfarin (Fig. 2). Collectively, the coadministration of buclocome with warfarin might lead to major bleeding unless the dose of warfarin is appropriately reduced. We wish to emphasize that the correct mechanism for the buclocome-warfarin interaction would not have been clarified if only plasma total or unbound concentrations for racemic warfarin, rather than the respective enantiomers, had been determined (Table 1).

Certain acidic drugs (e.g., warfarin and buclocome) not only bind extensively to albumin but also tend to be substrates for CYP2C9. Recent UV-visible spectroscopy/NMR experiments coupled with molecular modeling (Poli-Scaife et al., 1997) has suggested that common structural characteristics for selective CYP2C9 substrates are the presence of an anionic site that is able to establish an ionic bond with a putative cationic residue of the protein. The $K_f$ value of buclocome for warfarin binding on human albumin was approximately 7 μM (Y. Kurihara, Eisai Co. Ltd., personal communication) and $K_f$ values of buclocome for (S)-warfarin 7-hydroxylation mediated by human liver microsomes and recombinant CYP2C9 were 8 to 20 μM (Table 3). In addition, our pharmacokinetic simulation based on the reported kinetic parameters for buclocome indicated that its plasma unbound concentration during the repeated oral dosing (37.5 μM) would likely exceed the $K_i$ values for both plasma protein binding and metabolic inhibition for (S)-warfarin. This is the reason why coadministration of buclocome with warfarin gave rise to both plasma binding displacement and CYP2C9-mediated metabolic inhibition simultaneously. Such a complex drug interaction has also been reported for the warfarin-phenylbutazone interaction (Chan et al., 1994).

Numerous attempts have been made to establish an experimental paradigm that allows quantitative prediction of in vivo drug interaction from in vitro data (von Moltke et al., 1998). In the present study we have demonstrated that using an in vitro approach combining those reported by Shaw and Houston (1987), Sugiyama et al. (1996), and Ito et al. (1998) it may be possible to predict the reduction in in vivo $C_{\text{po, u}}$ for (S)-warfarin in a quantitative manner based on five pharmacokinetic parameters (i.e., $f_{\text{pu}}, f_{\text{mu}}, f_{\text{mu}}/f_{\text{pu}}$, $I_{\text{u}, \text{max}}$, and $K_i$). These parameters can be retrieved from literature ($f_{\text{pu}}, f_{\text{mu}}$, and $f_{\text{hu}}$), estimated according to Sugiyama’s equation (i.e., $I_{\text{u}, \text{max}}$), and obtained from in vitro experiments (i.e., $K_i$). In previous studies, little attention has been paid to both $f_{\text{hu}}$ and $f_{\text{pu}}$ parameters (von Moltke et al., 1998). However, neglect of these two parameters may cause serious misleading in the extrapolation of in vitro data into in vivo situations, because hepatic metabolism and the metabolic pathway inhibited by concomitant drug(s) are not always the major route of overall drug elimination, respectively. Regarding the buclocome-warfarin interaction, $f_{\text{hu}}$ of warfarin would be least variable because a previous study (Porter and Sawyer, 1992) demonstrated that $f_{\text{hu}}$ for subjects given warfarin alone is almost i.e., (0.98) and did not change appreciably by the coadministration of a potent (S)-warfarin inhibitor, sulfinpyrazone (Toon et al., 1986). In the present study, $f_{\text{hu}}$ for (S)-warfarin was assumed to be equal to the fraction that was not mediated by alcohol formation with or without subsequent conjugation reaction ($f_{\text{hu}} = 0.87$). However, this parameter might have been more variable than $f_{\text{hu}}$ due to variable levels of expression of distinct CYP isoforms. Indeed, $C_{\text{po, u}}$ and $C_{\text{m, u}}$ obtained from patients given warfarin alone exhibited more than 5- to 10-fold intersubject variability (Table 2) and comparable levels of intersubject variability were also reported in in vitro CYP2C9 activity determined with human liver microsomes (Hall et al., 1994).

To our knowledge, one of the major obstacles to traversing the gap between in vitro and in vivo potency of a certain metabolic inhibitor is a lack of an appropriate method for estimating effective concentrations of an inhibitor in the vicinity of microsomal enzymes in vivo. It is generally believed that unbound concentrations of inhibitors in plasma may be equilibrated with those in the hepatocytes. Kunze and Trager (1996) adopted this assumption for their study on the metabolic interaction between warfarin and fluconazole, because hepatic tissue concentrations of the inhibitor were shown to be only slightly greater than those in plasma (Ervine and Houston, 1994). As for buclocome, however, the drug was found to be accumulated substantially in the liver compared with total plasma concentrations in rabbits (Yashiki, 1971). In this context, we predicted in vivo changes in pharmacokinetic parameters of warfarin by assuming both the presence and absence of an active transport of buclocome from plasma into the hepatocytes. Results indicated that the predictions made by assuming a possible active transport of buclocome in the liver would be better than those made by assuming a simple equilibration of the unbound drug between plasma and hepatocytes (Table 4). Because Sugiyama’s equation allows us to estimate a theoretically maximum inhibitor concentration after oral dosing based on fundamental pharmacokinetic parameters of the inhibitor, it may serve as a useful tool in various clinical situations. However, additional studies are definitely required to determine whether buclocome would undergo active transport from plasma into the hepatocyte. Sugiyama et al. (1996) and
Ito et al. (1998) suggested that the ratio of $K_{in}$ or $K_{out}$ obtained from cultured hepatocytes to those obtained from microsomes would represent the ratio of unbound drug concentration in hepatocytes to medium, thereby an accumulation by active transport.

Another possible limitation of the present study was that we did not take the presence of enantiomers of bucolome and its major alcohol metabolites into account in predicting in vivo changes in (S)-warfarin. Although no data are available as to whether bucolome enantiomers would undergo enantioselective pharmacokinetics and/or any of their metabolites possess significant inhibitory effects on CYP2C9, this information is definitely important for improving the accuracy of our approach.

When we apply the present method for predicting in vivo changes in $Cl_{ua}$ of (S)-warfarin during coadministration of fluconazole based on $K_{in}$ obtained in vitro and in vivo, the predicted reductions (by 88 and 71%, respectively) showed a reasonable concordance with that observed (by 73%) in patients (Kunze and Trager, 1996). These findings appear to support an idea that our approach may also be applicable to (S)-warfarin.

Mechanistically based in vivo interaction between (S)-warfarin and bucolome was predictable with reasonable accuracy using the equation incorporating $K_{in}$ obtained from in vitro experiments with recombinant CYP2C9 and $F_{un,max}$ of the inhibitor calculated by Sugiyama’s equation (Sugiyama et al., 1996). The present approach allowing a prediction for in vivo inhibition of warfarin from in vitro data might also be applicable for other metabolically based interactions of warfarin with therapeutically important drugs in clinical situations.

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References


In conclusion, coadministration of bucolome with racemic warfarin elicited not only a displacement of both warfarin enantiomers from their plasma protein binding site but also an enantioselective inhibition of CYP2C9-mediated 7-hydroxylation of pharmacologically more potent (S)-warfarin, thereby potentiating the anticoagulation response of warfarin. Metabolically based in vivo interaction between (S)-warfarin and bucolome was predictable with reasonable accuracy using the equation incorporating $K_{in}$ obtained from in vitro experiments with recombinant CYP2C9 and $F_{un,max}$ of the inhibitor calculated by Sugiyama’s equation (Sugiyama et al., 1996). The present approach allowing a prediction for in vivo inhibition of warfarin from in vitro data might also be applicable for other metabolically based interactions of warfarin with therapeutically important drugs in clinical situations.