IN VITRO STIMULATION OF WARFARIN METABOLISM BY QUINIDINE: INCREASES IN THE FORMATION OF 4′- AND 10-HYDROXYWARFARIN

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ABSTRACT:

It has been demonstrated that the activity of cytochrome P450 (CYP3A4 in certain cases is stimulated by quinidine (positive heterotropic cooperativity). We report herein that the 4′- and 10-hydroxylation of S- and R-warfarin are enhanced in human liver microsomal incubations containing quinidine. These reactions were catalyzed by CYP3A4, based on data derived from immunoinhibitory studies, with 4′-hydroxylation being preferentially associated with S-warfarin and 10-hydroxylation with R-warfarin. The 4′-hydroxylation of S-warfarin and 10-hydroxylation of R-warfarin increased with increasing quinidine concentrations and maximized at ~3- and 5-fold the values of controls, respectively. Stimulatory effects of quinidine also were observed with recombinant CYP3A4, suggesting that increases in warfarin metabolism were due to quinidine-mediated enhancement of CYP3A4 activity. This positive cooperativity of CYP3A4 was characterized by a 2.5-fold increase in \( V_{\text{max}} \) for the 4′-hydroxylation of S-warfarin and a 5-fold increase in \( V_{\text{max}} \) for the 10-hydroxylation of R-warfarin, with little change in \( K_{\text{m}} \) values. Conversely, \( V_{\text{max}} \) for the 3-hydroxylation of quinidine was not influenced by the presence of warfarin. These results are consistent with previous findings suggesting the existence of more than one binding site in CYP3A4 through which interactions may occur between substrate and effector at the active site of the enzyme. Such interactions were subsequently illustrated by a kinetic model containing two binding domains, and a good regression fit was obtained for the experimental data. Finally, stimulation of warfarin metabolism by quinidine was investigated in suspensions of human hepatocytes, and increases in the formation of 4′- and 10-hydroxywarfarin again were observed in the presence of quinidine, indicating that this type of drug-drug interaction occurs in intact cells.

Introduced a half-century ago as a rodenticide, warfarin has become a mainstay for prevention of thromboembolic complications in patients with atrial fibrillation (Majerus et al., 1990; Fihn, 1995). The drug, however, has a narrow therapeutic index and therefore is prone to serious drug-drug interactions when coadministered with other agents that alter warfarin clearance (Wells et al., 1994; Harder and Thurmann, 1996). Numerous such interactions have been reported, some of which were attributed to induction or inhibition of hepatic cytochrome P450 (CYP) enzyme(s) (Wells et al., 1994; Harder and Thurmann, 1996). In this regard, the metabolism of warfarin in humans has been shown to be catalyzed by CYP1A1/2, 2C9, and 3A4, resulting in the formation of 4′-, 6-, 7-, 8- and 10-hydroxy derivatives (Fig. 1; Rettie et al., 1992; Kaminsky and Zhang, 1997). Coadministration of CYP inhibitors such as fluconazole or miconazole was associated with elevated plasma concentrations of warfarin and prolonged prothrombin times (O’Reilly et al., 1992; Black et al., 1996). Conversely, diminished anticoagulant effects of warfarin were observed when the drug was administered together with rifampin or barbiturates and were attributed to induction of CYP enzyme(s) and a subsequent increase in warfarin metabolism (Koch-Weser and Sellers, 1971; Heimark et al., 1987). This type of drug-drug interaction, however, would become evident only after chronic dosing of enzyme inducers (Conney, 1967; O’Reilly, 1975).

Modulation of warfarin therapy also was reported with quinidine; both agents may be used together for the management of atrial fibrillation (Harder and Thurmann, 1996). The outcome of this drug-drug interaction was either hypoprothrombinemic hemorrhage or a need for an increase in the anticoagulant dosage (Koch-Weser, 1968; Sylven and Anderson, 1983). While the decrease in prothrombin levels during coadministration of warfarin and quinidine had been speculated to be due to a synergistic depression of vitamin K-dependent clotting factors by the two drugs, no explanation was given for the reduced anticoagulant effect of warfarin in the presence of quinidine (Koch-Weser, 1968; Sylven and Anderson, 1983).

Quinidine is a naturally occurring cinchona alkaloid, which has been used clinically for cardioversion and is one of the most frequently prescribed antiarrhythmic drugs (Grace and Camm, 1998). The metabolism of quinidine in humans is mediated mainly by hepatic CYP3A4, resulting in 3-hydroxy and N-oxide derivatives (Fig. 1; Guengerich et al., 1986). Quinidine is known to cause drug interactions at the level of CYP3A4, although recent studies have shown that these interactions are more complex than would be expected on the basis of a simple relationship between a substrate and enzyme. For
example, the drug has been suggested to inhibit CYP3A4 in a non-
competitive manner (Schellens et al., 1991; Bowles et al., 1993).
Quinidine also has been demonstrated to be an effector of the enzyme,
since the CYP3A4-catalyzed metabolism of diclofenac, meloxicam,
and phenanthrene can be stimulated by the presence of quinidine
(Ludwig et al., 1999; Ngui et al., 2000; Sai et al., 2000). In this report,
we describe that the CYP3A4-mediated 4'- and 10-hydroxylation
of warfarin are enhanced by quinidine in incubations with human liver
microsomes as well as with human hepatocytes. Investigation of this
in vitro drug-drug interaction was carried out in the context of the
influence of quinidine on the $K_n$ and $V_{\text{max}}$ values of warfarin meta-
bolism and vice versa.

Experimental Procedures

Materials. 7,8-Benzoflavone, cinchonine, NADPH, quinidine, quinine, and
racemic warfarin were purchased from Sigma Chemical Co. (St. Louis, MO).
S- and R-Warfarin, 4', 6-, 7-, 8-, and 10-hydroxywarfarin, [phenyl-$^3$H]-[7-
hydroxywarfarin, and 3-hydroxyquinidine were purchased from GENTEST
Co. (Woburn, MA). 9-Epiquinidine and 9-epiquinine were from Buchler
GmbH (Braunschweig, Germany). BondElut C18 extraction cartridge columns
were obtained from Varian Chromatography Systems (Walnut Creek, CA), and
Oasis MCX extraction plates were from Waters Co. (Milford, MA). All other
chemicals were obtained from Fisher Scientific (Fair Lawn, NJ).

Quinidine N-oxide was synthesized through oxidation of quinidine with 6% hydrogen peroxide (Guentert et al., 1982). Liquid chromatography/mass spec-
trometry (LC/MS), 341 (MH$^+$), 7.9 (d, 1H), 8.7 (d, 1H).

LC/MS experiments were performed using either a Heated Nebulizer interface
or a Turbo IonSpray interface with positive ion detection. With the Heated
Nebulizer interface, the source temperature was set at 500°C, corona discharge
at 3.0 $\mu$A, orifice potential at 39 V, and collision energy at 40 eV. The collision
gas was nitrogen. With the Turbo IonSpray interface, the source temperature
was set at 150°C, ionization voltage at 5 kV, orifice potential at 50 V, and
collision energy at 35 eV. The collision gas again was nitrogen.

Incubations with Human Liver Microsomes or Recombinant CYP3A4.
Human liver samples from three male and two female donors were obtained
from the Pennsylvania Regional Tissue Bank (Exton, PA). An agreement was
made between the tissue bank and Merck & Co. for research use of the samples.
Liver microsomes were isolated from individual livers by differential
centrifugation (Raucy and Lasker, 1991). The activity of CYP3A4 in these
microsomal preparations was estimated based on the 6β-hydroxylation
of testosterone (Table 1). Aliquots from each preparation then were pooled on the
basis of equivalent protein concentrations to yield a representative pool of
human liver microsomes.

Human liver microsomes or recombinant CYP3A4 was suspended in phos-
phate buffer (0.1 M, pH 7.4) containing EDTA (1 mM). The final concentra-
tion of CYP enzymes was 0.24 nmol/ml for human liver microsomes and 0.07
nmol/ml for CYP3A4. Warfarin (R-, S-, or racemic) in methanol and quinidine
in water (or quinidine analogs in methanol) then were added to the incubations.
The final concentration of warfarin ranged from 0 to 1 mM and of quinidine
from 0 to 0.1 mM. The final concentration of methanol in incubation media
was 0.2% (v/v). Controls lacked quinidine (or quinidine analogs) but contained
the same amount of methanol. The reaction mixture was incubated at 37°C for
5 min, and NADPH in phosphate buffer was added to a final concentration of
1 mg/ml. After incubation for an additional period of 20 min, the reaction was
quenched with 10% aqueous trifluoroacetic acid.

Immunoinhibition experiments followed a protocol similar to that described
above. Briefly, monoclonal antibodies against CYP3A4 or CYP2C9 (5–40 mg
of IgG/mol of CYP) were preincubated with human liver microsomes for 15
min at room temperature. Control incubations contained ascites from untreated
animals. Warfarin, quinidine, and NADPH were added and incubated for an
additional 20 min. All experiments were performed in duplicate.
Incubations with Human Hepatocytes. Liver samples from three human donors were obtained from the Pennsylvania Regional Tissue Bank. The death of one donor, a 26-year-old female, was caused by drug overdose; the second donor, a 47-year-old female, died from subarachnoid hemorrhage; the third donor was a 56-year-old male who died from head trauma. Hepatocytes were isolated based on a two-step perfusion procedure (Pang et al., 1997) and exhibited a viability of greater than 80% as determined by the trypan blue exclusion test. The cells were suspended in Krebs-bicarbonate buffer. Warfarin in dimethyl sulfoxide was added to the suspension to provide a final concentration of 25 μM, while quinidine in aqueous solution was added to afford final concentrations ranging from 5 to 100 μM. The final concentration of dimethyl sulfoxide was 0.1% (v/v). Controls lacked quinidine. After incubation at 37°C for 2 h, reactions were quenched with 10% trifluoroacetic acid. These experiments were performed in duplicate.

Quantification of Metabolites. Aliquots (0.1 ml) from incubations with human liver microsomes were mixed with [phenyl-1H]-7-hydroxywarfarin and cinchonine (50 ng, internal standards) and 4 M urea (1 ml) and applied to an Oasis MCX extraction plate, which was washed with methanol and water. The plate then was washed with water (1 ml) and eluted with 70% aqueous acetonitrile (0.3 ml) containing 0.1% trifluoroacetic acid and 1.0 mM ammomium acetate. The eluates were analyzed by LC/MS/MS (Heated Nebulizer interface) with multiple reaction monitoring as described above for analyses of microsomal samples.

Kinetic Calculations. Apparent $K_{m}$ and $V_{max}$ values were calculated according to the Michaelis-Menten equation.

A kinetic model was proposed to illustrate the interaction of R-warfarin, quinidine, and CYP3A4. The model contains two distinct binding domains in the CYP active site, which is defined as the area wherein substrates interact with the ferric-oxygen complex. Basic assumptions include rapid equilibrium, fast release of product(s), and two independent substrate binding sites, one for warfarin and another for quinidine. The velocity equations derived from the model are as follows:

$$v_{p1} = \frac{V_{max}\cdot[S_1](aK_{S1} + q[S_2])}{K_{S1} + aK_{S1}}$$

(1)

$$v_{p2} = \frac{V_{max}\cdot[S_2](aK_{S2} + \beta[S_1])}{K_{S2} + aK_{S2}}$$

(2)

$$V_{max} = [E]\cdot[k_1] + \frac{V_{max} + V_{max}}{[E]\cdot[k_2]}$$

(3)

where $v_{p1}$ and $v_{p2}$ are the initial velocities, $V_{max}$ and $V_{max}$ are the maximum velocities, and $k_1$ and $k_2$ are the rate constants for the formation of 10-hydroxywarfarin and 3-hydroxyquinidine, respectively; $[S_1]$ and $[S_2]$ are the concentrations of warfarin and quinidine; $K_{S1}$ and $K_{S2}$ are the dissociation constants for $E \leftrightarrow S_1$ and $E \leftrightarrow S_2$, respectively; $\delta$, $\alpha$, and $\beta$ are the factors by which $K_{S1}$ ($K_{S2}$), $k_1$, and $k_2$ are influenced, respectively, upon binding of the second substrate (effector) (Fig. 2). The equations were solved by using the Marquardt-Levenberg nonlinear least-squares algorithm (Marquardt, 1963).

Results

Warfarin Metabolism in Incubations with Human Liver Microsomes. Hydroxylated derivatives of warfarin were identified by LC/MS/MS based on coincidence of high performance liquid chromatography retention times and product ion spectra with those of authentic standards. In incubations with human liver microsomes, the formation of warfarin metabolites was linear over a period of 30 min, and all studies were performed following 20 min incubations.

The metabolism of R- and S-warfarin in incubations with human liver microsomes resulted in the formation of 4'- and 10-hydroxywarfarin. The rate of these reactions appeared to correlate well with the activity of CYP3A4, assessed by the rate of testosterone 6β-hydroxylation in individual liver microsomal preparations (Table 1). Kinetic studies indicated that the intrinsic clearance, expressed as the ratio of $V_{max}/K_{m}$ for the 10-hydroxylation of R-warfarin was

![Table 1](https://example.com/table1.png)

**Table 1**: Effects of quinidine on the 4'- and 10-hydroxylation of warfarin in incubations with human liver microsomes or hepatocytes.
16-fold higher than that of the 4′-hydroxylation pathway. The intrinsic clearance for the 10-hydroxylation of S-warfarin, however, was ~70% that of the 4′-hydroxylation of this enantiomer (Table 2). In comparing the corresponding pathway for the two enantiomers of warfarin, it was found that the intrinsic clearance for the 10-hydroxylation of R-warfarin was approximately 10-fold that for the same reaction from S-warfarin, whereas the intrinsic clearance for the 4′-hydroxylation of R-warfarin was 50% that for S-warfarin. These differences in the 4′- and 10-hydroxylation pathways of R- and S-warfarin were due to changes in $V_{\text{max}}$ values, with the $K_m$ being nearly equal for the two antipodes of the substrate (Table 2).

The apparent $K_m$ and $V_{\text{max}}$ values for the 4′- and 10-hydroxylation of racemic warfarin also were determined. In this case, the $K_m$ was higher and the $V_{\text{max}}$ was lower than the corresponding values obtained when single enantiomers were used as substrate (Table 2). These may be due to the fact that the R- or S-enantiomer consists only 50% of racemic warfarin plus a possibility that the metabolism of one enantiomer is inhibited by its antipode. It was observed that the 4′-hydroxylation of S-warfarin was inhibited by R-warfarin, while 10-hydroxylation of R-warfarin was inhibited by the S-isomer (Table 3). The nature of this inhibition was difficult to evaluate because the absolute stereochemistry of the products was not determined.

Both the 4′- and 10-hydroxylation of warfarin in incubations with human liver microsomes were inhibited ~90% or more by a monoclonal antibody against CYP3A4, whereas these biotransformation pathways were not influenced by a monoclonal antibody against CYP2C9 (Table 4). The formation of 7-hydroxywarfarin, on the other hand, was not affected by the anti-CYP3A4 IgG but was inhibited ~80% by the anti-CYP2C9 IgG (data not shown). These data are consistent with the notion that the metabolism of warfarin to its 4′- and 10-hydroxy derivatives is catalyzed mainly by CYP3A4, while the 7-hydroxylation is mediated by CYP2C9 (Rettie et al., 1992; Yamazaki and Shimada, 1997).

When incubations with human liver microsomes were performed in the presence of quinidine, the formation of 4′- and 10-hydroxywarfarin from warfarin increased with increasing quinidine concentrations (Fig. 3). Based on these profiles, the concentration of quinidine required to produce 50% of the maximum stimulatory effect (EC$_{50}$) was estimated to be 15 μM for the 10-hydroxylation of R-warfarin and 8 μM for the 4′-hydroxylation of S-warfarin. Similar increases in the magnitude of the 4′- or 10-hydroxylation were observed for R- and S-warfarin, despite the fact that the $V_{\text{max}}$ values differ severalfold between the two enantiomers for the same metabolic pathway. Stimulation of warfarin metabolism also was observed with quinine, epiquinidine, epiquinine, and 3-hydroxyquinidine, while the use of quinidine N-oxide led to inhibition of warfarin metabolism (Table 3). Quinidine and quinine represent a pair of erythro diastereoisomers and epiquinidine and epiquinine are the corresponding threo epimers. 3-Hydroxyquinidine and quinidine N-oxide are metabolites of quinidine, resulting from CYP3A4-catalyzed reactions (Guengerich et al., 1986). The formation of 4′- and 10-hydroxywarfarin, however, was inhibited 75 to 95% by 7,8-benzoflavone (Table 3). This flavone derivative was reported to stimulate a number of CYP3A4-mediated reactions (Buening et al., 1981; Kerr et al., 1994). The formation of 6-, 7-, and 8-hydroxywarfarin was not affected by the presence of quinidine (data not shown). The structures of the CYP3A4 modulators are shown in Fig. 4.

Stimulation of the 10-hydroxylation of warfarin by quinidine was observed with liver microsomes prepared from five individual donors; the magnitude of increases in metabolite formation ranged from 5- to 9-fold (Table 1; Fig. 5). The stimulatory effect of quinidine on the 4′-hydroxylation of warfarin, however, was not evident in one liver microsomal preparation (Table 1). The magnitude of increases in the formation of 4′-hydroxywarfarin was 2- to 4-fold for the other four microsomal preparations (Table 1).

To simplify interpretation of data, the influence of quinidine on the kinetics of warfarin metabolism was investigated in incubations of a single warfarin enantiomer with pooled human liver microsomes. The $V_{\text{max}}$ value for the 10-hydroxylation of R-warfarin increased with increasing quinidine concentrations and maximized at ~650 pmol/min/mg of protein, which represented a 5-fold elevation over controls (Table 5). The $V_{\text{max}}$ value for the 4′-hydroxylation of S-warfarin also increased and maximized at ~35 pmol/min/mg of protein, a ~2.5-fold increase over controls (Table 5). The $K_m$ values for these reactions changed little, ranging from 0.11 to 0.14 mM for the 4′-hydroxylation and from 0.20 to 0.26 mM for the 10-hydroxylation.

### Table 2

<table>
<thead>
<tr>
<th>Warfarin</th>
<th>Formed of 10-Hydroxywarfarin</th>
<th>Formed of 4′-Hydroxywarfarin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$ ($\mu$M)</td>
<td>$V_{\text{max}}$ (pmol/min/mg)</td>
</tr>
<tr>
<td>$R$</td>
<td>0.24 ± 0.04</td>
<td>176 ± 12</td>
</tr>
<tr>
<td>$S$</td>
<td>0.24 ± 0.05</td>
<td>18 ± 2</td>
</tr>
<tr>
<td>Racemic</td>
<td>0.59 ± 0.09</td>
<td>46 ± 4</td>
</tr>
</tbody>
</table>

*Warfarin in methanol was added to human liver microsomes suspended in phosphate buffer (0.1 M, pH 7.4) containing EDTA.

*The kinetic parameters were determined based on the Michaelis-Menten equation. Data are presented as mean ± S.E.
pathway (Table 5). It is of interest to note that similar phenomena were observed with racemic warfarin, the $V_{\text{max}}$ value for 4'- and 10-hydroxylation of which increased while the $K_m$ values remained relatively constant, when incubations were performed in the presence of quinidine (data not shown).

The stimulatory effects of quinidine on warfarin metabolism were attenuated when human liver microsomes were pretreated with anti-CYP3A4 IgG (Table 4). The amounts of 4'- and 10-hydroxywarfarin generated in incubations with liver microsomes pretreated with the antibody plus quinidine were essentially the same as those formed in incubations that lacked quinidine.

**Warfarin Metabolism in Incubations with Recombinant CYP3A4.** Incubations of warfarin with recombinant CYP3A4 coexpressed with NADPH-CYP oxidoreductase resulted in the formation of 4'- and 10-hydroxywarfarin. Once again, these biotransformation pathways were stimulated by the presence of quinidine, such that the formation of 4'- and 10-hydroxywarfarin increased approximately 4-fold (Table 3).

**Quinidine Metabolism in Incubations with Human Liver Microsomes.** The formation of 3-hydroxyquinidine and quinidine N-oxide was linear over a period of 20-min incubation with either the individual or pooled human liver microsomes. The $K_m$ and $V_{\text{max}}$ values were estimated at 24 $\mu$M and 150 pmol/min/mg of protein for the 3-hydroxylation and 89 $\mu$M and 76 pmol/min/mg of protein for the N-oxidation in incubations with the pooled liver microsomes. Therefore, the N-oxidation reaction represented a minor pathway for quinidine metabolism as compared with 3-hydroxylation. This assessment is consistent with previously published data (Guengerich et al., 1986).

Further investigation of the effect of warfarin on quinidine metabolism was carried out by examining only the formation of 3-hydroxyquinidine in incubations with the pooled human liver microsomes. In contrast to the stimulatory effect of quinidine on warfarin metabolism, R-warfarin had little or no effect on the 3-hydroxylation of quinidine. Neither $K_m$ (17–24 $\mu$M) nor $V_{\text{max}}$ ($\sim$140 pmol/min/mg of protein) changed significantly relative to controls (Table 6). A similar result was obtained for S-warfarin (data not shown).

### Kinetic Model for the Interaction of Warfarin and Quinidine with CYP3A4.

The proposed kinetic scheme for the interaction of R-warfarin and quinidine with CYP3A4 was based on assumptions of a rapid equilibrium between the enzyme and substrates (Fig. 2). Experimental data were fit to the equations derived from the model, and resulting velocities (z-axis) were plotted against both substrate (x-axis) and effector (y-axis) concentrations (Fig. 6). The dissociation constants were estimated at $\sim$250 $\mu$M for the equilibrium between warfarin and CYP3A4 and 18 to 25 $\mu$M for the equilibrium between quinidine and the enzyme (Table 5). These values decreased slightly upon binding of a second substrate or an effector (ES, or ES2) (Figs. 2 and 6). The formation of 10-hydroxywarfarin increased with increasing effector (quinidine) concentrations (Fig. 6A). The maximal velocity for the formation of 10-hydroxywarfarin from the warfarin-CYP3A4-quinidine complex was 6-fold higher than that from the warfarin-CYP3A4 complex (Table 7). The velocity for the formation of 3-hydroxyquinidine from the quinidine-CYP3A4-warfarin complex was similar to that from the quinidine-CYP3A4 complex; in other words, the metabolism of quinidine was negligibly influenced by the presence of R-warfarin (Fig. 6B and Table 7).

### Stimulation of Warfarin Metabolism in Human Hepatocyte Suspensions.

Five monohydroxylated derivatives of warfarin, namely 4'-, 6-, 7-, 8-, and 10-hydroxywarfarin, were identified by LC/MS/MS in incubations of racemic warfarin with human hepatocytes. The

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**TABLE 3**

Effects of chemical modulators on the 4'- and 10-hydroxylation of warfarina

<table>
<thead>
<tr>
<th>Modulator</th>
<th>Concentration</th>
<th>Enzyme Sources</th>
<th>4'-Hydroxylation (S-Warfarin)b</th>
<th>10-Hydroxylation (R-Warfarin)b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol/mmol CYP</td>
<td></td>
<td>% of Control</td>
<td>% of Control</td>
</tr>
<tr>
<td>Quinidine</td>
<td>10–50 HzM</td>
<td>3A4</td>
<td>150–200</td>
<td>200–350</td>
</tr>
<tr>
<td>Quinidine</td>
<td>50 HzM</td>
<td>HLM</td>
<td>380</td>
<td>500</td>
</tr>
<tr>
<td>Quinidine</td>
<td>50 HzM</td>
<td>HLM</td>
<td>230</td>
<td>300</td>
</tr>
<tr>
<td>Epiquinidine</td>
<td>50 HzM</td>
<td>HLM</td>
<td>310</td>
<td>300</td>
</tr>
<tr>
<td>Epiquinidine</td>
<td>50 HzM</td>
<td>HLM</td>
<td>230</td>
<td>300</td>
</tr>
<tr>
<td>3-Hydroxyquinidine</td>
<td>50 HzM</td>
<td>HLM</td>
<td>110</td>
<td>280</td>
</tr>
<tr>
<td>Quinidine N-oxide</td>
<td>60 HzM</td>
<td>HLM</td>
<td>60</td>
<td>80</td>
</tr>
<tr>
<td>7,8-Benzoflavone</td>
<td>20–100 HzM</td>
<td>HLM</td>
<td>25–10</td>
<td>25–5</td>
</tr>
<tr>
<td>S-Warfarin</td>
<td>10–300 HzM</td>
<td>HLM</td>
<td>90–60</td>
<td></td>
</tr>
<tr>
<td>R-Warfarin</td>
<td>5–250 HzM</td>
<td>HLM</td>
<td>90–70</td>
<td></td>
</tr>
</tbody>
</table>

HLM, pooled human liver microsomes; 3A4, recombinant CYP3A4.

a Warfarin and chemical modulators in methanol were added to human liver microsomes or recombinant CYP3A4 suspended in phosphate buffer (0.1 M, pH 7.4) containing EDTA. The concentration of warfarin was 50 $\mu$M. Controls contained no modulator but the same amount of methanol. Incubations were performed in duplicates.

b % Control was based on the formation of hydroxylated warfarin derivatives in test incubations relative to the values in control experiments that lacked modulator.

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**TABLE 4**

Inhibition of warfarin metabolism by monoclonal antibodies in incubations with human liver microsoma

<table>
<thead>
<tr>
<th>Monoclonal Antibody</th>
<th>Warfarin</th>
<th>10-Hydroxywarfarinb</th>
<th>4'-Hydroxywarfarinb</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP3A4</td>
<td>10–40</td>
<td>R</td>
<td>&lt;1</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>10–40</td>
<td>S</td>
<td>0–10</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>10–40</td>
<td>Racemic</td>
<td>&lt;2</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>40</td>
<td>Racemic + quinidine</td>
<td>~5</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>10–40</td>
<td>R, S, or racemic</td>
<td>~100</td>
</tr>
</tbody>
</table>

a Warfarin in methanol was added to human liver microsomes suspended in phosphate buffer (0.1 M, pH 7.4) containing EDTA. The final concentration of warfarin ranged from 50 to 1000 $\mu$M. In cases where quinidine was included in incubations, the concentration of quinidine was 50 $\mu$M. Human liver microsomes were preincubated with monoclonal anti-CYP IgG for 15 min at room temperature. Reactions were initiated by adding NADPH and proceeded for an additional 30 min.

b Test incubations were compared with their respective controls containing control ascites.
formation of 4'- and 10-hydroxywarfarin increased when incubations were performed in the presence of quinidine, although intersubject difference in the increases were evident (Table 1). The 6-, 7-, and 8-hydroxylation pathways of warfarin metabolism in human hepatocyte suspensions were not affected by the presence of quinidine (data not shown).

**Discussion**

Among CYP enzymes, CYP3A4 is the most abundant in human liver and is involved in the metabolism of numerous therapeutic agents (Wrighton and Stevens, 1992). While activity of the enzyme
STIMULATION OF WARFARIN METABOLISM BY QUINIDINE

TABLE 5

Influence of quinidine on warfarin metabolism in human liver microsomes

<table>
<thead>
<tr>
<th>Quinidine (µM)</th>
<th>10-Hydroxylation of R-Warfarin</th>
<th>4'-Hydroxylation of S-Warfarin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kₘᵇ</td>
<td>Vₘₜₐₓᵇ</td>
</tr>
<tr>
<td>0</td>
<td>0.24 ± 0.04</td>
<td>133 ± 12</td>
</tr>
<tr>
<td>1</td>
<td>0.25 ± 0.07</td>
<td>147 ± 17</td>
</tr>
<tr>
<td>5</td>
<td>0.26 ± 0.05</td>
<td>282 ± 22</td>
</tr>
<tr>
<td>10</td>
<td>0.22 ± 0.04</td>
<td>359 ± 27</td>
</tr>
<tr>
<td>25</td>
<td>0.23 ± 0.04</td>
<td>499 ± 34</td>
</tr>
<tr>
<td>50</td>
<td>0.21 ± 0.03</td>
<td>568 ± 31</td>
</tr>
<tr>
<td>100</td>
<td>0.20 ± 0.03</td>
<td>658 ± 39</td>
</tr>
<tr>
<td>200</td>
<td>0.22 ± 0.04</td>
<td>654 ± 44</td>
</tr>
</tbody>
</table>

*Warfarin in methanol and quinidine in distilled water were added to human liver microsomes suspended in phosphate buffer (0.1 M, pH 7.4) containing EDTA.

TABLE 6

Influence of R-warfarin on quinidine metabolism in human liver microsomes

<table>
<thead>
<tr>
<th>Warfarin (µM)</th>
<th>Formation of 3-Hydroxyquinidine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kₘᵇ</td>
</tr>
<tr>
<td>0</td>
<td>23.7 ± 3.1</td>
</tr>
<tr>
<td>25</td>
<td>24.2 ± 2.5</td>
</tr>
<tr>
<td>100</td>
<td>26.4 ± 3.0</td>
</tr>
<tr>
<td>200</td>
<td>22.9 ± 5.0</td>
</tr>
<tr>
<td>300</td>
<td>17.6 ± 3.2</td>
</tr>
<tr>
<td>350</td>
<td>18.8 ± 4.5</td>
</tr>
<tr>
<td>400</td>
<td>15.6 ± 0.9</td>
</tr>
<tr>
<td>450</td>
<td>15.7 ± 0.8</td>
</tr>
<tr>
<td>500</td>
<td>16.4 ± 2.0</td>
</tr>
<tr>
<td>550</td>
<td>17.3 ± 4.5</td>
</tr>
<tr>
<td>600</td>
<td>14.1 ± 1.3</td>
</tr>
<tr>
<td>700</td>
<td>17.0 ± 3.5</td>
</tr>
</tbody>
</table>

*R-Warfarin in methanol and quinidine in distilled water were added to human liver microsomes suspended in phosphate buffer (0.1 M, pH 7.4) containing EDTA.

The kinetic parameters were determined based on the Michaelis-Menten equation. Data are presented as mean ± S.E.

with recombinant enzymes (Kaminsky and Zhang, 1997), while a separate study indicates that the 4'-hydroxylation of S-warfarin is catalyzed by both recombinant CYP3A4 and 2C9, with the rate of metabolite formation being 6-fold higher in incubations with CYP3A4 (Rettie et al., 1992). The 4'-hydroxylation of R-warfarin was shown to be catalyzed by CYP3A4 (Rettie et al., 1992). In the present study, the formation of 4'- and 10-hydroxywarfarin, from either R- or S-warfarin, in incubations with human liver microsomes was inhibited 90% or more by a monoclonal antibody against CYP3A4. These data are consistent with earlier findings and appear to suggest that CYP3A4 plays a dominant role in the 4'- and 10-hydroxylation of warfarin.

The 4'- and 10-hydroxylation of R- or S-warfarin in incubations with human liver microsomes were enhanced by the presence of quinidine. Thus, under the conditions used, the formation of 4'-hydroxywarfarin from S-warfarin increased approximately 3-fold, while the formation of 10-hydroxywarfarin from R-warfarin increased 5-fold. The enhancement of warfarin metabolism most likely is due to interactions of quinidine with CYP3A4 because the stimulatory effect of quinidine was diminished when human liver microsomes were pretreated with an inhibitory directed antibody against CYP3A4. This interpretation also is supported by the fact that the formation of 4'- and 10-hydroxywarfarin in incubations with recombinant CYP3A4 increased in the presence of quinidine.

Stimulation of the CYP3A4-catalyzed metabolism of warfarin also was observed with diastereoisomers of quinidine, namely quinine and the threo epimers, indicating that the stereochemistry of the effectors is not critical in this type of interaction. On the other hand, limited data generated in this study with five different preparations of human liver microsomes indicate that there may be variability among individuals with respect to cooperativity of CYP3A4. For example, stimulation of the 4'-hydroxylation of S-warfarin was not observed with one liver microsomal preparation. The magnitude of increases in the 10-hydroxylation of R-warfarin appeared to be similar within the five liver microsomal preparations, despite large variations in the CYP3A4 activities in these livers.

The quinidine-mediated stimulation of warfarin 4'- and 10-hydroxylation in incubations with pooled human liver microsomes was characterized by increases in Vₘₜₐₓ values with minimal changes in Kₘᵇ values. Conversely, the Vₘₜₐₓ for the 3-hydroxylation of quinidine was not influenced by the presence of warfarin. These phenomena are similar to those observed in studies of interactions between diclofenac and quinidine, wherein the formation of 5-hydroxydiclofenac in incubations with recombinant CYP3A4 was stimulated by quinidine, but quinidine metabolism was not affected by diclofenac (Ngui et al., 2000). On the basis of these results, it was proposed that the CYP3A4 active site contained two distinct binding domains, one for diclofenac
FIG. 6. A, CYP3A4-mediated 10-hydroxylation of R-warfarin in the presence of quinidine, and B, CYP3A4-mediated 3-hydroxylation of quinidine in the presence of warfarin. The surfaces represent the theoretical fits to eqs. 1 and 2 (see Experimental Procedures), respectively.
and a second for quinidine (Ngui et al., 2000). However, interactions involving warfarin, quinidine, and CYP3A4 appear to be more complex, taking into account the results with the two enantiomers of warfarin. Thus, the 10-hydroxylation of R-warfarin and 4’-hydroxylation of S-warfarin exhibited different $K_d$ values and were subject to inhibition by the respective antipodes. The $EC_{50}$ of quinidine was different for stimulation of the 4’- versus the 10-hydroxylation of warfarin. These findings may imply the existence of two or even three binding domains in the active site of CYP3A4, one for each enantiomer of warfarin and the third for quinidine. It also is conceivable that there may be an allosteric binding site for quinidine that may, or may not, be identical to that for quinidine metabolism.

Scenarios involving multiple ligands binding in the active site of CYP3A4, or substrate bindings plus a distinct allosteric binding site, have been hypothesized to describe the cooperative properties of CYP3A4. These proposals are based on studies involving site-directed mutagenesis (Harlow and Halpert, 1998; Domanski et al., 2000), enzyme kinetics (Shou et al., 1994; Ueng et al., 1997; Korzewska et al., 1998), computational docking modeling (Szklarz and Halpert, 1997; Ekins et al., 1999), and ligand binding determinations (Hosea et al., 2000). In most of these studies, 7,8-benzoflavone was described as an effector of CYP3A4. However, in the present studies, this flavonoid was inhibitory toward the metabolism of warfarin. The distinction between 7,8-benzoflavone and quinidine in terms of CYP stimulation may reside in differences in effector-mediated changes of enzyme conformations. In this regard, based on measurements of the rate of reformation of the ferroheme-carbon monoxide complex, it was proposed that CYP3A4 may exist in two or more subpopulations or conformations (Koley et al., 1995, 1997). Hence, the stimulation of warfarin metabolism by quinidine could be explained in the context that the subpopulation (conformation) of CYP3A4 responsible for hydroxylation of warfarin increased upon binding of quinidine to the enzyme. This perturbation of the equilibrium between different CYP3A4 conformations in favor of warfarin metabolism would be reflected by an enhancement of substrate (warfarin) turnover without impact on binding affinity, consistent with the observed increases in $V_{max}$ but minimal changes in the $K_m$ values. Conversely, the lack of effect of warfarin on the 3-hydroxylation of quinidine could be interpreted as indicating that binding of warfarin to the enzyme does not perturb the equilibrium among CYP conformations.

The interactions of warfarin, quinidine, and CYP3A4 also were considered in the context of a kinetic model containing two binding domains in the active site of CYP3A4. In this model, only the $R$-antipode of warfarin was considered, and relationships among binding sites were simplified by assuming that these sites were independent from each other. The rate of formation of 10-hydroxywarfarin was therefore described to increase 6-fold upon binding of quinidine to the enzyme, whereas the 3-hydroxylation of quinidine was affected minimally by the presence of warfarin. A kinetic model appears to have the advantage of demonstrating the cooperative properties of CYP3A4 with mathematic equations.

The stimulation of the 4’- and 10-hydroxylation of warfarin by quinidine in incubations with human liver microsomes is unlikely to be an in vitro artifact because a similar enhancement of warfarin metabolism was observed in human hepatocyte suspensions. In this regard, it may be expected that this type of drug-drug interaction will occur in vivo, the effect of which would be to alter the pharmacokinetics of a therapeutic agent. Data generated in animal species supporting this hypothesis include increases in zoxazolamine metabolism in rats treated concurrently with flavone and elevation of the hepatic clearance of diclofenac in rhesus monkeys following coadministration of quinidine (Lasker et al., 1982; Tang et al., 1999). In a controlled human trial, the clearance of warfarin was found to be increased slightly, but to a statistically significant extent, following coadministration of 3-hydroxy-10,11-dihydroquinidine (Trenk et al., 1993). In the present study, 3-hydroxyquinidine, a close analog of 3-hydroxy-10,11-dihydroquinidine, was observed to stimulate the metabolism of warfarin.

While warfarin is used therapeutically as a racemic mixture, $S$-warfarin has been shown to be 2- to 5-fold more potent as an anticoagulant than its $R$ counterpart (Harder and Thurmann, 1996). The clearance of warfarin in humans is due mainly to hepatic metabolism involving multiple CYP enzymes, although the duration of the anticoagulant effect is determined primarily by the rate of 7-hydroxylation of $S$-warfarin catalyzed by CYP2C9 (Rettie et al., 1992). Evidence also is available to indicate that the metabolism of $S$-warfarin is inhibited by the presence of $R$-warfarin and vice versa. Warfarin and quinidine often are used together for the treatment of atrial fibrillation, and drug-drug interactions between these two agents have been reported, the outcome of which may require an increase in the warfarin dose (Sylven and Anderson, 1983). While it is tempting to speculate in light of current findings that the diminished effect of warfarin under these circumstances is due to stimulation of its metabolism by quinidine, it may be argued that the 4’-hydroxylation of warfarin is a minor pathway for the clearance of $S$-isomer, and increases in the 10-hydroxylation of $R$-warfarin represent at best a secondary effect in altering the disposition of $S$-warfarin by removing the inhibitory $R$-enantiomer.

In summary, the present investigation has demonstrated that the 4’- and 10-hydroxylation of warfarin in incubations with human liver microsomes or hepatocytes are enhanced by the presence of quinidine. While this drug-drug interaction is mediated by CYP3A4, its clinical significance remains unclear in light of the complexities associated with intersubject variability, the racemic nature of warfarin, and pharmacological side effects elicited by quinidine. Nevertheless, the findings of this in vitro study reinforce emerging views on the existence of multiple binding domains in CYP3A4 that underlie the

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**TABLE 7**

Parameters generated from a kinetic model for the interaction of diclofenac and quinidine with CYP3A4

<table>
<thead>
<tr>
<th>Reaction</th>
<th>$K_d^{b}$</th>
<th>$K_d^{c}$</th>
<th>Factor$^b$</th>
<th>$V_{max}^{b}$</th>
<th>Factor$^b$</th>
<th>Equation$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-Warfarin 10-hydroxylation</td>
<td>0.3 ± 0.1</td>
<td>18.2 ± 3.5</td>
<td>0.8 ± 0.2</td>
<td>119 ± 13</td>
<td>5.9 ± 0.8</td>
<td>1</td>
</tr>
<tr>
<td>Quinidine 3-hydroxylation</td>
<td>0.3 ± 0.1</td>
<td>25.3 ± 2.8</td>
<td>0.5 ± 0.2</td>
<td>156 ± 6</td>
<td>0.7 ± 0.2</td>
<td>2</td>
</tr>
</tbody>
</table>

$^a$The kinetic model is shown in Fig. 2 and equations are described under Experimental Procedures.

$^b$ $V_{max,W}$ and $V_{max,Q}$ are the maximum velocities for the 10-hydroxylation of R-warfarin and 3-hydroxylation of quinidine, respectively; $K_d$ is the dissociation constant for $E := ES$ and $K_d$ for $E := ES_2$. $\delta$, $\alpha$, and $\beta$ are the factors by which $K_d$ (or $K_d$), $k_1$, and $k_2$ are influenced upon binding of a second substrate to the enzyme. Data are presented as mean ± standard error.
complex kinetics and drug-drug interaction characteristics of this important enzyme.

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References