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Hydroxywarfarin Metabolites Potently Inhibit CYP2C9 Metabolism of *S*-Warfarin

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Abstract

Coumadin (*R/S*-warfarin) anticoagulant therapy poses a risk to over 50 million Americans, in part due to interpersonal variation in drug metabolism. Consequently, it is important to understand how metabolic capacity is influenced among patients. Cytochrome P450s (P450 or CYP for a specific isoform) catalyze the first major step in warfarin metabolism to generate five hydroxywarfarins for each drug enantiomer. These primary metabolites are thought to reach at least 5-fold higher levels in plasma than warfarin. We hypothesized that hydroxywarfarins inhibit the hydroxylation of warfarin by CYP2C9, thereby limiting enzymatic capacity toward *S*-warfarin. To test this hypothesis, we investigated the ability of all five racemic hydroxywarfarins to block CYP2C9 activity toward *S*-warfarin using recombinant enzyme and human liver microsomes. We initially screened for the inhibition of CYP2C9 by hydroxywarfarins using a P450-Glo assay to determine IC₅₀ values for each hydroxywarfarin. Compared to the substrate, CYP2C9 bound its hydroxywarfarin products with less affinity but retained high affinity for 10- and 4'-hydroxywarfarins, products from CYP3A4 reactions. *S*-Warfarin steady-state inhibition studies with recombinant CYP2C9 and pooled human liver microsomes confirmed that hydroxywarfarin products from CYP reactions possess the capacity to competitively inhibit CYP2C9 with biologically relevant inhibition constants. Inhibition of CYP2C9 by 7-hydroxywarfarin may be significant given its abundance in human plasma, despite its weak affinity for the enzyme. 10-Hydroxywarfarin, which has been reported as the second most abundant plasma metabolite, was the most potent inhibitor of CYP2C9, displaying approximately 3-fold higher affinity than *S*-warfarin. These results indicate that hydroxywarfarin metabolites produced by CYP2C9 and other CYPs may limit metabolic capacity toward *S*-warfarin through competitive inhibition. Subsequent

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Supporting Information Available: All raw data, an example Dynafit script file for the determination of the inhibition mechanism and *K_i* values, Dynafit model discrimination analyses, the uninhibited steady-state kinetic profile of human liver microsomal metabolism of *S*-warfarin, and inhibition by sulfaphenazole. This material is available free of charge via the Internet at <http://pubs.acs.org>.

processing of hydroxywarfarins to secondary metabolites, such as hydroxywarfarin glucuronides, could suppress product feedback inhibition, and therefore could play an important role in the modulation of metabolic pathways governing warfarin inactivation and elimination.

Introduction

Coumadin (*R/S*-warfarin) is a highly efficacious anticoagulant for the prevention of thromboembolic events associated with atrial fibrillation and stroke. However, warfarin is widely underutilized due to the fear of hemorrhage (1). Clinical use of the drug requires frequent monitoring due to its narrow therapeutic range and adverse consequences from supratherapeutic and subtherapeutic dosing. Warfarin is subject to drug–drug interactions, drug–diet interactions, and interpersonal variability due to metabolism (2). Efficient warfarin metabolism eliminates the active drug and decreases anticoagulant activity, while inefficient drug metabolism leads to toxic warfarin levels. Consequently, an understanding of processes that affect metabolic activity will improve our ability to explain variations in drug response and potentially avoid adverse effects during therapy due to metabolism.

Cytochrome P450s (CYP for specific isoforms) catalyze the first major step in warfarin metabolism by generating an array of hydroxywarfarins (Figure 1). Although multiple CYPs metabolize warfarin, each enzyme exhibits regioselectivity and enantiospecificity for their respective reactions (3). CYP2C9 efficiently metabolizes *S*-warfarin, the more active form of the drug (4), into *S*-6- and *S*-7-hydroxywarfarin (5), such that *S*-7-hydroxywarfarin is the most abundant metabolite in humans (6–8). Through a minor pathway, CYP3A4 hydroxylates *S*-warfarin to *S*-4'-hydroxywarfarin (5). Unlike *S*-warfarin, no single CYP dominates *R*-warfarin metabolism. CYP1A2 and CYP2C19 both generate *R*-6- and *R*-8-hydroxywarfarin, but with differing regioselectivity (9, 10). CYP3A4 also metabolizes *R*-warfarin to *R*-10-hydroxywarfarin (11, 5), which is possibly the second most abundant plasma metabolite in humans (8). Clearly, warfarin hydroxylation involves a complex array of pathways involving different enzymes; nevertheless, these studies have focused mainly on the individual CYP reactions and not the combined effects of all reactions.

Similar to the reported warfarin-drug/nutrient interactions (12), we hypothesize that hydroxywarfarins from CYP reactions compete with warfarin for CYP active sites and thereby suppress drug metabolism. The recognition of hydroxywarfarins by CYPs is plausible given the access to multiple sites for oxidation and overlapping specificities for warfarin among the enzymes regardless of whether oxidation occurs. Although *R*-warfarin is not metabolized by CYP2C9, this enantiomer binds and inhibits the enzyme with a K_i of $\sim 8 \mu\text{M}$ (13, 14). Furthermore, warfarin alcohols are known to competitively inhibit CYP2C9 yet are not metabolized (15). Hydroxywarfarins may similarly retain affinity for the CYP2C9 active site. Hydroxywarfarin plasma levels will also play an important role in driving these inhibitory interactions. Racemic hydroxywarfarins associate with plasma proteins with approximately 4-fold less affinity than warfarin (16, 17) suggesting that higher levels of unbound and hence inhibitory hydroxywarfarins are possible. The biological impact of these inhibitory interactions may also depend on variations in patient excretion of hydroxywarfarins or further metabolism to secondary glucuronide metabolites (5, 18).

We tested our hypothesis by assessing the inhibitory potential of 6-, 7-, 8-, 10-, and 4'-hydroxywarfarin on *S*-warfarin metabolism by CYP2C9. We carried out these studies with racemic hydroxywarfarins because patients are dosed with racemic warfarin and produce mixtures of hydroxywarfarins (6). We employed the P450-Glo marker reaction to screen for potential inhibitors based on IC₅₀ values. Surprisingly, all hydroxywarfarins inhibited CYP2C9 activity, and thus, we further assessed the inhibitory potential of all racemic hydroxywarfarins through subsequent steady-state kinetic studies with recombinant CYP2C9 and human liver microsomes. We avoided contributions of other CYPs to *S*-warfarin metabolism by initially determining the inhibition mechanism and parameters for recombinant CYP2C9. We subsequently studied the effect of the hydroxywarfarins on *S*-warfarin metabolism using pooled human liver microsomes. Control studies with sulfaphenazole, a CYP2C9-specific inhibitor (19), validated the predominance of that enzyme in *S*-warfarin metabolism. We were then able to attribute the inhibitory effect of hydroxywarfarins to CYP2C9 present in the human liver microsomes.

Experimental Procedures

Materials

All chemicals used in this study were of at least reagent grade. Unless otherwise specified, all chemicals and reagents were purchased from either Sigma-Aldrich (St. Louis, MO) or Thermo Fisher Scientific (Waltham, MA). In particular, the substrate (*S*-warfarin), internal standard (7-hydroxycoumarin), and all inhibitors (6-hydroxywarfarin, 7-hydroxywarfarin, 8-hydroxywarfarin, 10-hydroxywarfarin, and 4'-hydroxywarfarin) were purchased from Sigma-Aldrich. Ethyl alcohol (100%) was purchased from AAPER (Shelbyville, KY). Pooled human liver microsomes, human recombinant CYP2C9*1, expressed in baculovirus-infected insect cells (supersomes), and sulfaphenazole were purchased from BD Biosciences (San Jose, CA).

IC₅₀ Studies on Hydroxywarfarin Inhibition of Recombinant CYP2C9 Activity

Hydroxywarfarins were screened for inhibitory potential toward recombinant CYP2C9 using Promega (Madison, WI) CY2C9 P450-Glo Assay following the manufacturer's protocol. These IC₅₀ studies were analyzed on a PerkinElmer Victor³V 1420 Multilabel Counter. Recombinant CYP2C9, 50 nM, was incubated with 50 μ M Luciferin H and six concentrations of each hydroxywarfarin from approximately 10 nM to 4 mM final concentration, as optimized for each compound. The appropriate volume of hydroxywarfarin stocks in 100% ethanol was added to each well prior to the addition of the substrate and enzyme, and the ethanol was allowed to evaporate at room temperature to minimize solvent effects on CYP2C9 activity. A final DMSO concentration of 1% was present in the reactions due to P450-Glo Luciferin H. The reaction was initiated upon the addition of an NADPH regenerating solution (BD Biosciences) and quenched with a luciferase quench (Promega) at 30 min. The resulting luminescence was measured at 25 min after quenching, and the IC₅₀ value was calculated with GraphPad Prism 5 (GraphPad Software Inc.) after scaling the data to 100% activity.

Steady-State Metabolism of *S*-Warfarin

The inhibition of *S*-warfarin metabolism by recombinant CYP2C9*1 and human liver microsomes was carried out in the presence of each of the five racemic hydroxywarfarins. For recombinant CYP2C9, 25 nM enzyme was incubated with 0.5 to 64 μM *S*-warfarin in 50 mM potassium phosphate at pH 7.4, at 37 °C. Instead of the recombinant enzyme, the human liver microsomal reactions contained 2.0 mg/mL protein. In both cases, reactions were initiated upon the addition of an NADPH-regenerating system (2 μU μL^{-1} glucose 6-phosphate dehydrogenase, 10 mM glucose 6-phosphate, 2 mM MgCl_2 , and 1 mM NADP+) and quenched with an equal volume of ethanol containing the internal standard (7-hydroxycoumarin) at 45 min. In most cases, hydroxywarfarin concentrations were varied 5-fold above and below the IC_{50} value observed in the initial screen to optimize the analysis of inhibitor data. In some cases, additional inhibitor concentrations were used to explore potential secondary binding sites suggested by uncompetitive and mixed inhibition mechanisms. For each hydroxywarfarin, we performed at least 4 independent experimental replicates. The quenched reactions were centrifuged, and the resulting supernatant containing substrate and products resolved and quantified by LC-MS/MS.

LC-MS/MS Analysis of Kinetic Reactions

The levels of 6- and 7-hydroxywarfarin in the reactions were analyzed with an Agilent 1100 HPLC system coupled to an API4000 Triple Quadupole (MS/MS) mass spectrometer (Applied Biosystems) using a 4.6×150 mm Zorbax Eclipse 5 μm XDB-C18 column (Agilent) at 40 °C. A gradient method developed in our laboratory (6) effectively separated all hydroxywarfarins and warfarin at a flow rate of 1 mL/min. The mobile phase consisted of (A) H_2O (0.1% acetic acid) and (B) methanol (0.1% acetic acid) with 50% A and 50% B held for the first 30 s, followed by a linear gradient from 50% B to 90% B (0.5–5.5 min), 90% B to 100% B (5.5–5.6 min), 100% B maintained for 2.5 min, and 100% B to 50% B (7.9–8.0 min) with 50% B maintained for 2 min. We employed MS/MS experimental conditions as described previously (6).

Identification of the Inhibitory Mechanism and Corresponding Parameters

We identified the most probable inhibition mechanism for recombinant CYP2C9 and human liver microsome reactions using DynaFit version 3.28 (Biokin, Ltd.) (20). Data from each set of hydroxywarfarin experiments were fit to traditional reversible inhibition mechanisms (competitive, uncompetitive, noncompetitive, or mixed). The respective kinetic parameters (K_i and K_i') were set as variables, while the kinetic parameters (V_{max} and K_m) were constants based on values obtained from uninhibited reactions. The most probable mechanism was then identified using the Akaike Information Criterion (AICc) for model discrimination (21). The raw data from each set of experiments and an example of the DynaFit script file used for the model discrimination analyses are included in Supporting Information files 1 and 2, respectively.

Results

IC₅₀ Values for Racemic Hydroxywarfarins Support Inhibition of CYP2C9 Activity

On the basis of P450-Glo assays, the initial inhibition screen for CYP2C9 activity demonstrated varying capacities for hydroxywarfarins to block enzyme activity (Table 1). Recombinant CYP2C9 was inhibited by racemic warfarin with an IC₅₀ value comparable to the reported K_m of 2.6 μM (22). IC₅₀ values for the hydroxywarfarins ranged from approximately 3-fold below to 10-fold above that measured for warfarin. 10-Hydroxywarfarin had the lowest IC₅₀ toward CYP2C9, while 6-hydroxywarfarin had the highest. Intermediate affinity inhibitors were 8-hydroxywarfarin and 4'-hydroxywarfarin. Racemic 7-hydroxywarfarin yielded an IC₅₀ approximately 8-fold higher than that observed for warfarin.

Inhibition Kinetics of *S*-Warfarin with Recombinant CYP2C9

Steady-state inhibition experiments with recombinant enzyme validated the inhibitory potential of hydroxywarfarins on CYP2C9-mediated *S*-warfarin metabolism. We initially determined the uninhibited steady-state parameters for CYP2C9 toward *S*-warfarin. For *S*-6-hydroxywarfarin, the K_m and V_{max} parameters were 3.3 μM (2.6 to 4.0, 95% confidence interval) and 46 pmol/min/nmol P450 (43 to 49, 95% confidence interval), respectively, while the parameters for *S*-7-hydroxywarfarin were 2.3 μM (1.9 to 2.7, 95% confidence interval) and 68 pmol/min/nmol P450 (65 to 71, 95% confidence interval) for K_m and V_{max} , respectively. These parameters were then used for subsequent inhibition studies. For 6-, 8-, 10-, or 4'-hydroxywarfarin studies, we analyzed the effects of inhibition on formation of the major product, 7-hydroxywarfarin, as the marker of CYP2C9 activity. Because of the interference between the product and inhibitor, we studied 7-hydroxywarfarin inhibition on the basis of 6-hydroxywarfarin formation. The concentration of inhibitors did not change significantly over the course of the reaction indicating minimal, if any, secondary metabolism. The most probable inhibition mechanism for each hydroxywarfarin was identified and listed in Table 2 based on model discrimination analysis between reversible inhibition mechanisms using DynaFit software. Mechanisms with high Akaike weights but open confidence intervals were excluded from being the most likely mechanism of inhibition if another mechanism had sufficient support and closed confidence intervals. Model discrimination analyses for each set of inhibition data are included in Supporting Information file 3.

These inhibition studies with recombinant CYP2C9 generated results similar to those observed for the IC₅₀ studies. Although kinetic analysis was performed with DynaFit, the data are shown as Hanes-Woolf plots to visually demonstrate the mechanism of inhibition. The Hanes-Woolf plots of 7- and 10-hydroxywarfarin (Figure 2) support the competitive mode of action as indicated by the parallel lines for inhibited and uninhibited reactions. 10-Hydroxywarfarin was the most potent inhibitor of CYP2C9 with a K_i approximately 2.5-fold lower than the K_m for *S*-warfarin (Table 2). The second most potent inhibitor was 7-hydroxywarfarin demonstrating a K_i 4.5-fold higher than the marker K_m , while no inhibition was observed with 6-hydroxywarfarin up to 170 μM inhibitor as indicated by the overlapping lines. Mixed inhibition was observed with both 4'- and 8-hydroxywarfarin,

indicating that these metabolites inhibited CYP2C9 primarily through an intermediate affinity interaction but also through a second low affinity ($K_i' > 100 \mu\text{M}$) interaction, which causes an increase in the slope of the Hanes-Woolf line (Figure 2 and Table 2).

Inhibition Kinetics of *S*-Warfarin with Human Liver Microsomes

CYP2C9 was the dominant CYP present in human liver microsomes that metabolized *S*-warfarin as reported by others (5). The steady-state kinetic profile was hyperbolic indicating the presence of a single enzyme capable of hydroxylating *S*-warfarin (Supporting Information, Figure S1). The K_m and V_{max} kinetic parameters for 7-hydroxywarfarin from these studies were $5.2 \mu\text{M}$ (4.3 to 6.1, 95% confidence interval) and 173 pmol/min/mg protein (165 to 182, 95% confidence interval) respectively, which are comparable to those reported in the literature (3, 5). For *S*-6-hydroxywarfarin formation, the K_m and V_{max} kinetic parameters were $7.5 \mu\text{M}$ (6.4 to 8.6, 95% confidence interval) and 90 pmol/min/mg protein (86 to 94, 95% confidence interval), respectively. In the presence of sulfaphenazole, activity toward *S*-warfarin was potently inhibited (Supporting Information, Figure S2), demonstrating a competitive inhibition constant of $2.5 \mu\text{M}$ (1.3 to 5.3, 95% confidence interval). Sulfaphenazole is reported to be specific to CYP2C9 at concentrations up to 20-fold higher than those used in this study (23), and the kinetic parameters found here are in agreement with previous reported values (24). These results confirm that *S*-warfarin is metabolized by a single microsomal enzyme, CYP2C9, under these experimental conditions.

The results from the human liver microsomes were very similar to those observed for the recombinant CYP2C9 studies (Table 3). For each hydroxywarfarin, the mechanism of inhibition was competitive, as demonstrated by the parallel lines in the Hanes-Woolf plots (Figure 3). The most potent inhibitor of microsomal metabolism of *S*-warfarin was 10-hydroxywarfarin, the second most abundant metabolite in plasma (8). The corresponding K_i was 2.4-fold lower than the observed microsomal K_m for *S*-warfarin ($5.2 \mu\text{M}$). Both 4'- and 8-hydroxywarfarin (Figure 2) yielded intermediate affinity K_i values that were approximately 3-fold above the K_m for *S*-warfarin. The K_i for 7-hydroxywarfarin was 8.5-fold higher than the marker K_m , which is in close agreement with the IC_{50} results. 6-Hydroxywarfarin was a very weak competitive inhibitor of human liver microsomal activity, with an estimated K_i approximately 32-fold higher than the K_m for *S*-7-hydroxywarfarin (Table 3). Unlike the studies with recombinant CYP2C9, the apparent mechanism of inhibition for each hydroxywarfarin was competitive.

Discussion

This study expands our initial discovery that hydroxywarfarins inhibit CYP2C9 (18) and stresses the potential impact of hydroxywarfarins on metabolic capacity toward *S*-warfarin. These studies provide evidence that all hydroxywarfarins associate with CYP2C9 to block activity toward substrates. More importantly, hydroxywarfarins inhibited *S*-warfarin metabolism by CYP2C9 with both recombinant enzyme and human liver microsomes. As products of CYP2C9 and other CYPs, hydroxywarfarins are feedback inhibitors which suppress CYP2C9 activity toward warfarin and consequently link multiple CYP metabolic pathways to the overall efficiency of warfarin metabolism. These complex interactions could

potentially lead to the biological accumulation of toxic levels of *S*-warfarin, the most active form of the drug (4). The use of racemic hydroxywarfarins, as opposed to pure enantiomers, in this study potentially underestimates the potency of these inhibitors, but provides upper bounds for the inhibition constants of the individual enantiomers. Further studies will be required to determine the enantiospecificity of these inhibitory interactions.

The broad specificity of CYP2C9 for four of the racemic hydroxywarfarins indicates that these metabolites inhibit CYP2C9 metabolism of *S*-warfarin through cis and trans interactions (Scheme 1). 7-Hydroxywarfarin is the most abundant metabolite of warfarin in human urine and plasma (6, 8) and retains intermediate affinity for the enzyme, indicating that traditional product (cis) inhibition is possible. By contrast, 6-hydroxywarfarin did not significantly inhibit CYP2C9. The remaining hydroxywarfarins were effective trans-acting inhibitors on *S*-warfarin metabolism by CYP2C9. The most potent inhibitor in these studies was 10-hydroxywarfarin, which is the second most abundant metabolite in patient plasma (8). CYP3A4 generates this metabolite by metabolism of *R*-warfarin, thereby linking CYP3A4- and CYP2C9-mediated pathways to the overall efficiency of warfarin metabolism. Although 4'- and 8-hydroxywarfarin are usually minor plasma metabolites from CYP1A2 and CYP2C19 metabolism of *R*-warfarin (9, 10), these metabolites achieve high abundance in some patients (6, 8), and thus, these metabolites could further contribute to the trans inhibition of *S*-warfarin metabolism by CYP2C9. For patients, the hydroxywarfarins may collectively compete for the CYP2C9 active site, thereby suppressing *S*-warfarin metabolism and potentiating supra-therapeutic dosing and possibly bleeding complications.

Feedback inhibition of CYP2C9 becomes physiologically relevant when hydroxywarfarins out compete *S*-warfarin. The degree of competition is a function of hydroxywarfarin concentration and binding affinity. Although the binding affinity of hydroxywarfarins for CYP2C9 is constant, the potential for in vivo inhibition of CYP2C9 is augmented by the differing plasma protein binding properties of warfarin and its metabolites. *S*-Warfarin has high affinity for plasma proteins (~ 99% bound) (16), while 6-, 7-, and 8-hydroxywarfarin bind plasma proteins with 3- to 4-fold lower affinity (17). 4'-Hydroxywarfarin is thought to bind human plasma albumin with affinity comparable to that of warfarin (16), but the affinity of 10-hydroxywarfarin is unknown. The weaker affinity of hydroxywarfarin metabolites for plasma albumin increases their bioavailability, allowing hydroxywarfarins to potentially achieve higher unbound levels than warfarin. The level of CYP2C9 inhibition in vivo would therefore be a function of the ratio of *S*-warfarin to total hydroxywarfarin.

Locatelli et al. (8) determined the levels of total hydroxywarfarins as well as *S*-warfarin in the plasma of long-term warfarin therapy patients. The levels of unbound hydroxywarfarin enantiomers have not been reported in human plasma, but we can use the known affinities of warfarin and its metabolites for albumin (16, 17), and the Locatelli et al. data to estimate the levels of unbound warfarin and hydroxywarfarins in plasma. The unbound level of *S*-warfarin has been reported at 0.54% (25), which is in agreement with other similar reports (26). We therefore estimate the mean level of unbound *S*-warfarin in human plasma to be 2.7 ng/mL (8). We estimate the mean unbound concentration of 7- and 10-hydroxywarfarin to be 6.2 and 5.8 ng/mL, respectively, given that ~4% of the hydroxywarfarins are unbound (17). We expect the inhibitory effect of each of these metabolites to be additive; therefore, 7-, and

10-hydroxywarfarin have a combined concentration approximately 4.5 times higher than that of *S*-warfarin. Both 6- and 8-hydroxywarfarin are significant metabolites in some patients (6, 8), and these metabolites would also contribute to the inhibition of CYP2C9 activity, but no data were reported from those studies; therefore, we cannot estimate the unbound concentrations. These estimations are based on mean plasma values, but the extensive variation in individual patient levels for *S*-warfarin suggests that some populations are more prone to CYP2C9 inhibition by hydroxywarfarins, while others may be resistant (8).

In addition to CYP activities toward *R*- and *S*-warfarin, the hydroxywarfarin levels will depend on the efficiency of elimination through excretion and further metabolism. Little is known about hydroxywarfarin transport. Nevertheless, patients with chronic kidney disease may have reduced ability to clear hydroxywarfarins, which would lead to product accumulation. Dreisbach et al. (27) observed a 50% increase in the *S/R*-warfarin ratio in end stage renal disease patients with the CYP2C9*1/*1 (wild type) genotype. This observation was attributed to a selective decrease in hepatic CYP2C9 activity, albeit with an unknown mechanism. Limdi et al. (28) further demonstrated that such patients require significantly lower doses to maintain therapeutic anticoagulation and are at increased risk for supratherapeutic dosing and major hemorrhage even after adjustment for the CYP2C9 and vitamin K epoxide reductase (VKOR) genotype as well as other clinical factors. Feedback inhibition due to the accumulation of hydroxywarfarins would provide a mechanistic explanation for these observations. The processing of hydroxywarfarins into secondary metabolites such as glucuronides by UDP-glucuronosyltransferases (UGTs) would suppress the feedback inhibition and effectively create an extended metabolic pathway for warfarin. We have demonstrated the potential significance of hydroxywarfarin conjugation by UGTs in human urine samples (6) and identified the enzymes responsible for those reactions (18). Although potentially significant (29), the combined effect of CYP and UGT activity on warfarin metabolism remains unresolved at this time.

For 8- and 4'-hydroxywarfarin, we observed mixed inhibition toward CYP2C9, which could derive from two alternate mechanisms. The binding of *S*-warfarin to CYP2C9 may create a second binding site for other molecules as suggested by recent computational studies of the CYP2C9 active site (30). Hydroxywarfarins could bind to this secondary site and alter *S*-warfarin metabolism. Alternatively, the apparent mixed inhibition may be a consequence of the use of racemic hydroxywarfarin inhibitors. If the enantiomers of the hydroxywarfarins possess differing affinities toward CYP2C9, the resulting inhibition plot could appear biphasic and thus resemble mixed inhibition. Nevertheless, the current studies cannot distinguish between these possibilities. Further work is clearly needed to establish the mode of interaction between these molecules and CYP2C9.

CYP2C9 has long been recognized by metabolic and genetic studies as an important determinant of warfarin drug response (5, 31, 32). These studies provide a novel mechanistic explanation for reduced CYP2C9 metabolic capacity toward *S*-warfarin through a feedback inhibition mechanism. Hydroxywarfarins from multiple CYP pathways bind or rebinding CYP2C9, linking together the different oxidative pathways to the efficiency of *S*-warfarin metabolism (Scheme 1) and the potential toxic accumulation of the drug. The biological relevance of this mechanism requires further investigation to (1) establish the level of free

metabolites driving inhibitory interactions, (2) determine the significance of conjugative reactions on hydroxywarfarin levels, and (3) characterize the enantiospecificity of interactions between CYPs and hydroxywarfarins.

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Figure 1.
Chemical structure of warfarin. Numbers indicate sites for cytochrome P450 hydroxylations.

Figure 2.

Hanes-Woolf plots of hydroxywarfarin inhibition of *S*-warfarin metabolism by recombinant CYP2C9. Linear plots represent the Hanes-Woolf transformation of the DynaFit nonlinear regression for the reported model of inhibition for each hydroxywarfarin inhibitor (panels A–E). Individual tracings represent different concentrations of hydroxywarfarin inhibitors as shown in each legend. Reactions were performed at least four times at 37 °C and pH 7.4. Further reaction conditions are described in Experimental Procedures.

Figure 3.

Hanes-Woolf plots of hydroxywarfarin inhibition of *S*-warfarin metabolism by human liver microsomes. Linear plots represent the Hanes-Woolf transformation of the DynaFit nonlinear regression for the competitive model of inhibition for each hydroxywarfarin inhibitor (panels A–E). Individual tracings represent different concentrations of hydroxywarfarin inhibitors as shown in each legend. Reactions were performed at least four times at 37 °C and pH 7.4. Further reaction conditions are described in Experimental Procedures.

Scheme 1. cis and trans Feedback Inhibition of CYP2C9 by Hydroxywarfarins^a

^aCYP2C9 metabolizes *S*-warfarin (SWAR) to *S*-6- and *S*-7-hydroxywarfarin (OHWAR), while CYP3A4 metabolizes *R*-warfarin (RWAR) to *R*-10-hydroxywarfarin. 7-Hydroxywarfarin and 10-hydroxywarfarin then inhibit CYP2C9 through cis and trans interactions, respectively. Bold arrows indicate the major products of the reaction.

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Table 1
IC₅₀ Values for Racemic Hydroxywarfarins toward Recombinant CYP2C9 Activity^a

	CYP2C9 inhibitors					
	WAR	4'OHWAR	10OHWAR	6OHWAR	7OHWAR	8OHWAR
IC ₅₀ (μ M)	4.5 (3.2 – 5.8)	9.3 (6.7 – 12)	1.6 (1.3 – 1.8)	47 (28 – 65)	35 (27 – 42)	16 (9.2 – 23)

^aOHWAR = hydroxywarfarin; 95% confidence intervals are shown in parentheses.

Table 2Racemic Hydroxywarfarin Inhibition of Recombinant CYP2C9 Metabolism of *S*-Warfarin^a

inhibitor	mechanism	K_i (μ M)	K_i' (μ M)
4'OHWAR	mixed	17 (10 – 33)	133 (75 – 413)
10OHWAR	competitive	0.94 (0.74 – 1.2)	
6OHWAR	none	>170	
7OHWAR ^b	competitive	10 (7.5 – 14)	
8OHWAR	mixed	21 (11 – 54)	103 (59 – 271)

^a OHWAR = hydroxywarfarin; 95% confidence intervals are shown in parentheses.^b Results are based on *S*-6-hydroxywarfarin formation.

Table 3Racemic Hydroxywarfarin Inhibition of Pooled Human Liver Microsome Metabolism of *S*-Warfarin^a

inhibitor	mechanism	K_i (μM)	K_i' (μM)
4'OHWAR	competitive	17 (13 – 24)	
10OHWAR	competitive	2.2 (1.7 – 3.0)	
6OHWAR	competitive	167 (117 – 257)	
7OHWAR ^b	competitive	64 (55 – 74)	
8OHWAR	competitive	13 (11 – 16)	

^aOHWAR = hydroxywarfarin; 95% confidence intervals are shown in parentheses.^bResults are based on *S*-6-hydroxywarfarin formation.