The Impact of R10-Hydroxywarfarin on CYP2C9-Mediated S-Warfarin Metabolism

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Abstract

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Warfarin therapy is highly effective in treating both atrial fibrillation and deep vein thrombosis. Currently, more than 20 million Americans are receiving warfarin therapy. However, increased risk of adverse events are associated with both under and overdosing the drug. Sub-therapeutic plasma levels may be insufficient to prevent the recurrence of a life-threatening clot. Alternatively, if warfarin levels are too high, the risk of dangerous bleeding events is greatly increased. Inter-individual variability in warfarin sensitivity underlies much of the difficulty in managing treatment by influencing dose requirements in a largely unpredictable manner. For example, one clinical study involving 185 subjects reported a 32-fold difference in daily warfarin doses, from 0.5-16 mg/day, needed to achieve a safe, therapeutic degree of anticoagulation. Many major contributors to this variability have been identified, such as age, race, sex, genetics, as well as several clinical factors. It is estimated that roughly 12% of this variability can be attributed to inherited variants of the Cytochrome P450 2C9 (CYP2C9) isoform. This enzyme is critical for the elimination of the more potent S-enantiomer of warfarin.

Another 24-30% can be attributable to different variants of the gene *VKORC1*, which codes for the target enzyme that warfarin inhibits. However, about 40% of the variability in the dose required to achieve a therapeutic level of anticoagulation remains unknown.

One additional source of variability in warfarin dose-response could arise from mutual pharmacokinetic interactions between R- and S- warfarin enantiomers. An interaction of this kind would be more significant if it primarily impacted S-warfarin clearance, because this enantiomer is 3-5 fold more potent a vitamin K antagonist than R-warfarin. This has been investigated previously. For example, S-warfarin was found to be a weak inhibitor of R-warfarin metabolism, whereas R-warfarin inhibited the production of S-6- and S-7-hydroxywarfarin in human liver microsomes with K_i ranges of 7.0-8.4 μM and 6.0-6.9 μM respectively. In addition, recently, the racemic 4'-, 6-, 7-, 8-, & 10-hydroxyl metabolites of warfarin have been shown to inhibit CYP2C9 mediated hydroxylation of S-warfarin, both in recombinant enzyme incubations and human liver microsomal incubations. In these experiments, the 10-hydroxywarfarin metabolite was reported to be the most potent inhibitor of CYP2C9-dependent activity towards S-warfarin. Because 10-hydroxywarfarin is a major circulating metabolite of warfarin following multiple dosing, we propose that it may reach high enough plasma concentrations at steady-state to produce a significant impact on the disposition and pharmacological response of S-warfarin.

To test this hypothesis, we determined the ability of racemic 10-hydroxywarfarin and its four stereoisomers to inhibit S-warfarin 7-hydroxylation using human liver microsomes. We determined the unbound fraction of the 10-hydroxywarfarin stereoisomers in human plasma in order to establish the relevant unbound

concentration that, based on the free-drug hypothesis, determines the potential for inhibition. Pharmacokinetic data were obtained from an ongoing pharmacogenetic warfarin study wherein subjects were randomized to receive warfarin alone or warfarin in the presence of rifampin (one week pretreatment; three weeks total duration).

We found that 10-hydroxywarfarin was highly bound to human plasma proteins, with an unbound fraction of 0.98 ± 0.22%. Racemic 10-hydroxywarfarin and its stereoisomers inhibited human liver microsomal S-warfarin 7-hydroxylation with apparent IC_{50} values ranging from 4.4 to 30 μM. The 9R,10S-hydroxywarfarin metabolite was the most potent inhibitor with an estimated K_i of 3.7 μM. Based on calculated steady-state R-10-hydroxywarfarin stereoisomer concentrations predicted from the single dose clinical study and the estimated K_i values, we concluded that only limited inhibition of CYP2C9-mediated S-warfarin metabolism by R-10-hydroxywarfarin will occur when warfarin is dosed alone or with rifampin (increase in the S-warfarin AUC ratio = 16% and 23%, respectively).

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Chapter 1

Introduction

1.1 Clinical Use of Warfarin

Coumadin (R/S-warfarin) is an anticoagulant used therapeutically in the prevention and treatment of thromboembolic diseases and for the prevention of ischemic stroke in patients with atrial fibrillation. Currently, nearly 20 million Americans are receiving warfarin treatment and, while efficacious, there are significant limitations to its safe administration (1). Warfarin therapy requires that a clinician closely monitor and maintain the degree of anticoagulation (as assessed by INR, International Normalized Ratio of prothrombin times) and adjust the dose to achieve the desired therapeutic effect. The INR should be maintained within a narrow therapeutic window, due to increased risk of serious adverse events from both over and under dosing the drug. The risk of severe bleeding events is associated with a high INR, while a low INR may fail to adequately prevent the recurrence of dangerous clots. Although the pharmacologic response to warfarin depends in part on blood concentrations of the drug, the effect also depends on the intrinsic sensitivity of the drug target – vitamin K oxidoreductase (VKOR). Moreover, the pharmacological effect of warfarin is an indirect one and thus blood or plasma concentrations of the drug are not routinely measured. Nonetheless, there are wide inter-individual differences in the intrinsic response of the body that are the result of differences in the systemic exposure to warfarin and the pharmacodynamic response to the drug. Understanding the factors underlying this variation is critical to improving the safety and efficacy of warfarin therapy.

As mentioned above, the safety and effectiveness of warfarin therapy is monitored using INR testing. Normal lab values of INR range from 0.8 to 1.2, reflecting a properly functioning vitamin K cycle and adequate production of biologically active

clotting factors. The objective of warfarin therapy is to decrease vitamin K cycle activity, which increases the prothrombin time, such that the INR is shifted upwards to a range of 2 to 3. This is the goal for most clinical indications, including atrial fibrillation, deep vein thrombosis and pulmonary embolism. For certain other cases, such as the management of prosthetic heart valves, higher intensity anticoagulation is needed and a target INR of 3 to 3.5 is desirable. INR values higher than 4 are associated with increased risk of a major bleeding event, whereas values < 2 will provide reduced protection against clot formation (2, 3).

Prior to initiating warfarin therapy, clinicians will have incomplete information with which to determine the appropriate initial dose needed to achieve a target INR for a given patient. Attempts have been made to develop predictive models utilizing factors such as: age, sex, height, weight, underlying medical conditions, and other health status factors to better determine effective initial dosing (4). A patient's genetic profile may also provide key information about their ability to metabolically eliminate warfarin from the body, which defines steady-state exposure following a fixed dose, as well as their biochemical response to the drug (5, 6). Although initially promising, prospective evaluation of these genomic prediction methods has yet to validate them as clinically useful (7).

1.2 Warfarin Mechanism of Action

Warfarin inhibits the vitamin K cycle, which is responsible for the maturation of blood clotting factors produced in the liver (Figure 1.1) (8). In the normal functioning cycle, the enzyme γ-glutamyl carboxylase (GGCX) utilizes the cofactor vitamin KH_2 to

catalyze the γ-carboxylation of glutamyl residues within the N-terminal region of certain clotting factors (e.g., Factors II, VII, IX and X and anticoagulant proteins C and S; also referred to as Gla-proteins). An oxidized form of vitamin K (vitamin K epoxide) is produced in the process and must be reduced (recycled) back to vitamin KH_2, through the action of VKOR, for the production of γ-carboxylated clotting factors to continue at an optimal rate. Warfarin inhibits the function of VKOR, resulting in depletion of vitamin KH_2 that limits hepatic γ-carboxylation activity and subsequent activation of the vitamin K-dependent pro-coagulant proteins. Decreased plasma concentrations of the four vitamin K-dependent coagulation factors (factors II, VII, IX and X) results in decreased prothrombin levels and a decrease in the amount of thrombin generated and bound to fibrin. Overall, this reduces the thrombogenicity of clot promoting stimuli.

Its indirect mechanism of action makes warfarin ideal for long-term management of thromboembolic and hemostatic disorders in patients with prosthetic heart valves, atrial fibrillation, deep vein thrombosis, and pulmonary embolism, because therapy is less sensitive to minor, episodic variations in dosing schedules. The half-life of pharmacological effect is determined by the half-life of the Gla-clotting factors and to some extent the half-life of the drug, which is relatively long (~ 40 hrs). Importantly, the effect of warfarin therapy can be reversed with a bolus dose of vitamin K_1 (phylloquinone) that transiently accelerates the synthesis of the Gla-clotting factors, an important factor that makes the use of warfarin safer in overdose and more desirable than other anticoagulants such as heparin, and the new Factor II and Factor X inhibitors.

Warfarin is administered as a racemic mixture of R- and S-enantiomers. However, R-warfarin and S-warfarin have distinct pharmacodynamic and pharmacokinetic properties. The disposition of warfarin is a critical determinant of systemic drug exposure and hence pharmacological response. The drug is rapidly absorbed in the gastrointestinal tract and exhibits a high oral bioavailability. Warfarin circulates in the blood mainly bound to the protein albumin, and accumulates in the liver where it exerts its pharmacological effect (VKOR inhibition). The metabolic clearance of warfarin also occurs in this organ (8).

Warfarin is cleared from the body primarily by metabolism, with less than 5% of the total dose excreted from the body unchanged (9). There is considerable inter-individual variability in the efficiency of warfarin metabolism (i.e., its metabolic clearance) that arises from differences in the expression and function of hepatic oxidative enzymes (10). Understanding the enzymology behind warfarin clearance can translate into a useful measure of individual metabolic capacity, and thereby improve clinical strategies during anticoagulant therapy.

1.3 Principal Genes Involved in Variable Warfarin Response; *CYP2C9, CYP4F2, VKORC1*

There are three major genetic determinants of warfarin response in humans (11-13). Variation in *VRORC1* variation (-1639 G>A) affects the rate of synthesis of vitamin K oxidoreductase in the liver and its ability to convert vitamin K epoxide to vitamin KH_2 (13). Variation in the cytochrome P450 gene *CYP4F2* (i.e., the **3* allele) affects the accumulation of its gene product in the liver and the metabolic clearance of vitamin K

(14, 15). With regard to warfarin itself, *CYP2C9* variation *(*2, *3, *5* alleles, among others) affects the metabolic clearance of the more active S-enantiomer (16). Thus, variation in *VKORC1* and *CYP4F2* genes can influence the basal efficiency of the vitamin K cycle and apparently its response to warfarin inhibition, whereas *CYP2C9* variation affects the accumulation of warfarin in the blood and liver under steady-state dosing conditions.

As stated above, warfarin undergoes almost complete (~95%) conversion to metabolites that are excreted in the urine and bile. Metabolism occurs through reductive, oxidative, and conjugative pathways. Each of these pathways is independent of one another except the conjugative pathway, which requires an initial oxidation of warfarin, because conjugation of parent drug is minimal in humans (17). Due to their dominance in warfarin metabolism, the oxidative pathways have received the most attention while the reductive and conjugative pathways remain to be fully characterized.

1.4 Isomeric Composition and Metabolic Clearance of Warfarin

Whereas warfarin is dosed as a racemic mixture, the S-enantiomer possesses up to five-fold greater pharmacological activity than does the R-enantiomer. Cytochromes P450 (CYPs) are primarily responsible for the metabolic clearance of both warfarin enantiomers. CYPs 1A2, 2C19, and 3A4 all contribute to the metabolic clearance of R-warfarin while, in the case of S-warfarin, CYP2C9 plays the dominant role. The other major metabolic pathway of warfarin elimination involves reduction of the ketone at C-11, generating diastereomeric warfarin alcohols (18). CYP enzymes metabolize warfarin into many different hydroxywarfarin metabolites by regio-selectively introducing a

hydroxyl group at one of five different positions (4´, 6, 7, 8, or 10) on the molecule (Figure 1.2).

CYP2C9 dominates (>80%) the oxidative metabolism of S-warfarin in a high affinity reaction, generating both S-6-hydroxywarfarin (K_m: 7.5 μM, V_{max}: 90 pmol/min/nmol P450) and S-7-hydroxywarfarin (K_m: 5.2 μM, V_{max}: 173 pmol/min/nmol P450) at a 1:3.5 ratio (10, 19). Consequently, CYP2C9 expression and activity most directly influences plasma levels of S-warfarin and thus can greatly impact dose requirements. Previously, it was demonstrated that the CYP2C9 poor metabolizer (PM) genotypes are associated both with increased warfarin sensitivity as well as an increased ratio of S- to R-warfarin levels in plasma (20). Other enzymes make minor contributions to S-warfarin metabolism including: CYP2C8 for S-6- and 4´-hydroxywarfarin formation; CYP2C19 for S-6- and 7-hydroxywarfarin formation; and CYP3A4 for S-4´-hydroxywarfarin (10). However, S-7-hydroxywarfarin may be used a biomarker for CYP2C9 activity, because its formation is relatively enzyme-specific and it is one of the most abundant oxidation products in plasma and urine.

R-warfarin can be metabolized by multiple CYPs, including those in the CYP3A subfamily (CYP3A4, CYP3A5, CYP3A7), CYP1A2, and CYP2C19 (10). These enzymes typically generate multiple R-hydroxywarfarin metabolites. The low affinity activity of CYP1A2 produces both R-6 (K_m: 1.6 mM, V_{max}: 1455 pmol/min/nmol P450) and R-8-hydroxywarfarin (K_m: 1.4 mM, V_{max}: 291 pmol/min/nmol P450) (21). CYP1A2 generates R-6- and 8-hydroxywarfarin in a 5:1 ratio. Similarly, CYP2C19 possesses intermediate affinity toward R-warfarin, producing primarily 8-hydroxywarfarin with a K_m of 0.3-0.4 mM and a V_{max} of between 3.5-183 pmol/min/nmol P450 (22). CYP3A4 converts R-

warfarin primarily to R-10-hydroxywarfarin and S-warfarin to S-4'-hydroxywarfarin. Despite intermediate affinity for both substrates, CYP3A4 generates five-fold more R-10-hydroxywarfarin than S-4´-hydroxywarfarin (10). Interestingly, like side-chain alcohol reduction at C-11, hydroxylation at the C-10 position creates a second chiral center such that there are actually four possible isomeric products of warfarin (two diastereomeric pairs), although the literature generally only refers to the "R" and "S" enantiomers, as defined by the stereochemistry at C-9. The importance of CYP3A in overall warfarin metabolism is evident from the consistently high plasma levels of 10-hydroxywarfarin. This metabolite is often reported to be the second most abundant metabolite in human plasma after S-7-hydroxywarfarin, and in some cases, is the most abundant metabolite (23).

1.5 Inhibition of S-warfarin Metabolism by R-warfarin and Hydroxywarfarin Metabolites

Besides the factors discussed above, variability in the warfarin dose-response relationship could arise from mutual pharmacokinetic interactions between R- and S-warfarin enantiomers. An interaction of this kind would be more significant if it primarily impacted S-warfarin clearance, because this enantiomer is 3-5 fold more potent a vitamin K antagonist than R-warfarin. This has been investigated previously. For example, S-warfarin was found to be a weak inhibitor of R-warfarin metabolism, whereas R-warfarin inhibited the production of S-6- and S-7-hydroxywarfarin in human liver microsomes with K_i ranges of 7.0-8.4 μM and 6.0-6.9 μM respectively (24). The potency of warfarin alcohol metabolites as inhibitors of CYP2C9 has also been

previously demonstrated (25). Recently, racemic mixtures of 4'-, 6-, 7-, 8-, & 10-hydroxy metabolites of warfarin have been shown to inhibit CYP2C9-mediated hydroxylation of S-warfarin, both in recombinant enzyme and human liver microsomal incubations (19). In these experiments, 'racemic' 10-hydroxywarfarin was the most potent inhibitor of CYP2C9 mediated metabolic activity towards S-warfarin, with a K_i of 0.9-2.2 μM. However, in vivo, each hydroxywarfarin metabolite is formed in a stereospecifically preferred manner, dependent on the hepatic composition of the different CYP isoforms. This results in dissimilar plasma concentration-time profiles for the respective parent enantiomers and metabolite stereoisomers (26). Thus, each individual stereoisomer metabolite, rather than the racemic pair or quartet, must be assessed individually for its ability to inhibit CYP2C9.

10-Hydroxywarfarin is reported to be the second most abundant plasma monohydroxy metabolite (behind 7-hydroxywarfarin), displays the most potent inhibition of CYP2C9, and has approximately a three-fold higher affinity for CYP2C9 compared to S-warfarin (19). Therefore, the use of racemic hydroxywarfarin metabolites in this prior in vitro study, as opposed to the individual stereoisomers, potentially underestimates the potency of these inhibitors, although it helps provide the maximum estimates for the inhibition constants of the individual enantiomers. Characterizing the effects of 10-hydroxywarfarin stereoisomers on the CYP2C9-mediated metabolism of S-warfarin, in conjunction with rigorous characterization of the in vivo circulating concentrations of metabolite stereoisomers, will be essential in determining if variation in hydroxywarfarin metabolite formation significantly influences inter-individual variability in warfarin clearance and associated dose requirement (27).

1.6 Hypothesis and Specific Aims

The overall goal of this research was to determine if the concentrations of circulating 10-hydroxywarfarin metabolite(s) of R-warfarin significantly impact warfarin dose requirements by inhibiting CYP2C9-mediated hydroxylation of the more pharmacologically active S-warfarin enantiomer. To assess this possibility, two aims were proposed. The first aim was to isolate and characterize the effect of individual 10-hydroxywarfarin stereoisomers on the CYP2C9-catalyzed S-warfarin 7-hydroxylation reaction in human liver microsomes. The second aim was to predict, based on the in vitro CYP2C9 inhibition parameters and circulating plasma concentrations of R-10-hydroxywarfarin at steady-state, the capacity of R-10-hydroxywarfarin to inhibit S-warfarin clearance in vivo.

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Figure 1.1 A schematic of the vitamin K cycle showing warfarin's mechanism of action as inhibiting the enzyme Vitamin K epoxide reductase complex subunit 1. Image kindly provided by Dr. Allan Rettie, Department of Medicinal Chemistry, University of Washington.

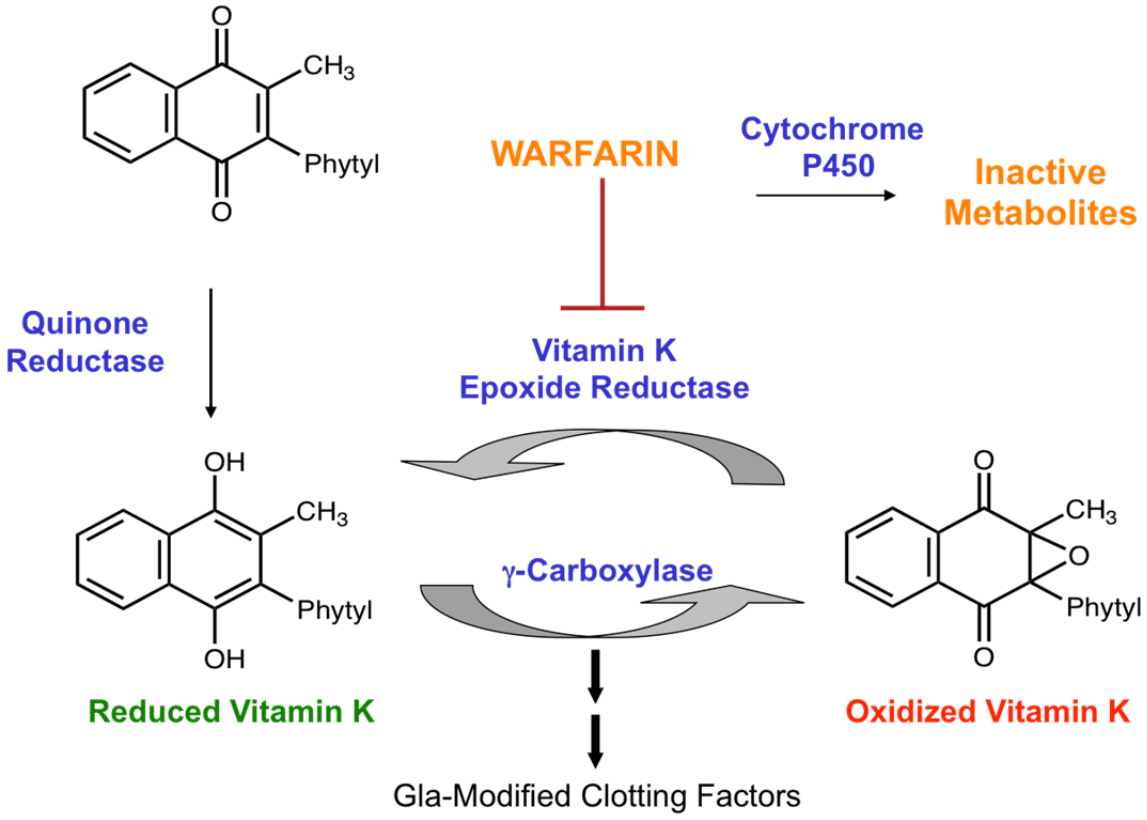
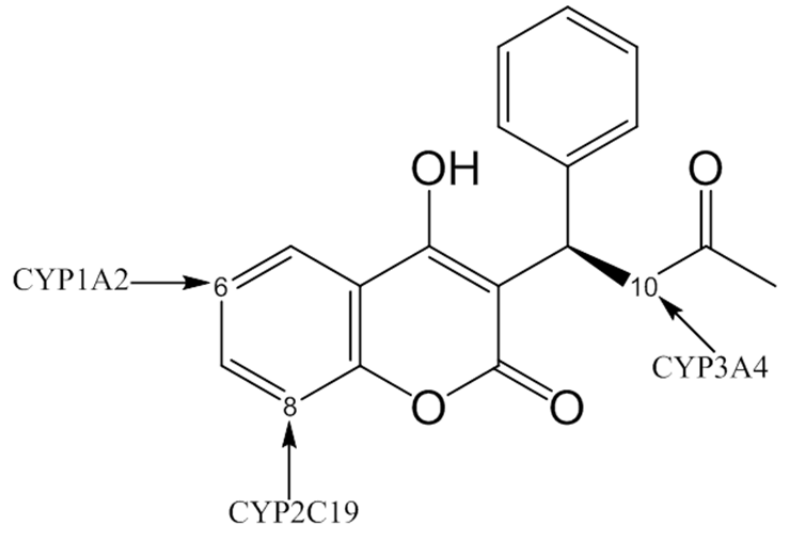
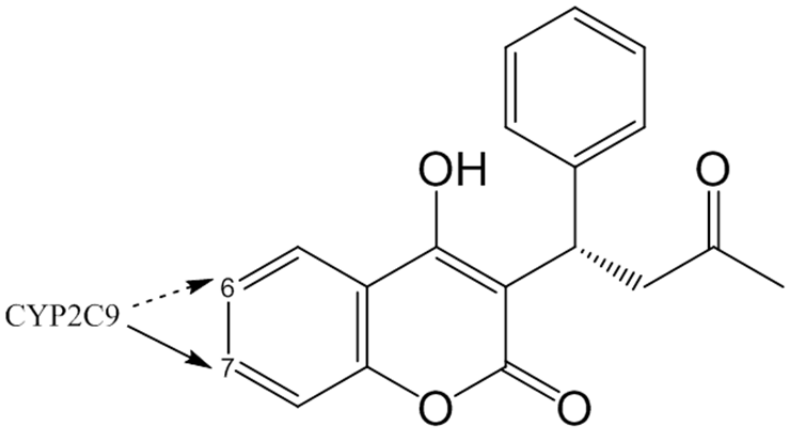


Figure 1.2 Structures of R-warfarin and S-warfarin indicating key locations of hydroxylation by the CYP enzymes responsible for the metabolic elimination of each enantiomer.



R-warfarin



S-warfarin

Chapter 2

R10-Hydroxywarfarin is an Inhibitor of CYP2C9-Mediated Hydroxylation of S-Warfarin

2.1 Introduction

Coumadin (R/S-warfarin) is a highly efficacious anticoagulant for the prevention of thromboembolic events associated with atrial fibrillation, deep vein thrombosis, and stroke (1). However, determining an effective initial warfarin dose for a patient is complicated by a narrow therapeutic window and variability in dose response between patients (2). Clinical use of the drug requires frequent monitoring to avoid adverse consequences from supratherapeutic and subtherapeutic blood concentrations. A balance must be achieved such that active drug levels are sufficient for the desired anticoagulant activity while remaining below toxic levels that may lead to life-threatening bleeding episodes. Choosing an appropriate starting dose can be complicated by drug-drug interactions, drug-diet interactions and interindividual variation in warfarin metabolism and pharmacological response (2). Consequently, an understanding of processes that affect metabolic activity may improve our ability to explain variations in drug response and potentially avoid adverse events during warfarin therapy.

Cytochrome P450s catalyze the first step in warfarin metabolism, generating five hydroxywarfarin metabolites (4', 6, 7, 8, and10-hydroxywarfarin) (Figure 2.1) (3). Although multiple CYPs metabolize warfarin, each enzyme exhibits regioselectivity and stereospecificity for their respective reactions. S-warfarin is most extensively metabolized by CYP2C9 (>80%) into S-6- and S-7-hydroxywarfarin, at a ratio of 1:3.5 and S-7-hydroxywarfarin is the most abundant hydroxywarfarin in plasma and in urine (3-5). CYP3A4 forms S-4´-hydroxywarfarin, though this is a minor pathway of elimination (3). Unlike S-warfarin, no single CYP dominates R-warfarin metabolism. However, CYP3A4 dominates the conversion of R-warfarin to R-10-hydroxywarfarin,

and R-10-hydroxywarfarin is the second most abundant plasma metabolite in humans (3, 6). Racemic 10-hydroxywarfarin has been shown to potently inhibit CYP2C9 mediated S-warfarin hydroxylation with a K_i of 2.2 μM (7). This suggests that accumulation of 10-hydroxywarfarin in vivo, especially under conditions where CYP3A4 is induced, might be a contributing factor to interindividual variability in patients' response to warfarin.

Our goal in this investigation was to determine whether steady-state plasma levels of 10-hydroxywarfarin following chronic warfarin dosing may be sufficient to decrease CYP2C9-mediated 7-hydroxylation of S-warfarin in vivo. We measured the unbound fraction of 10-hydroxywarfarin stereoisomers in human plasma and assessed the inhibitory capacity of 10-hydroxywarfarin and each of its stereoisomers towards CYP2C9-mediated S-warfarin metabolism in human liver microsomes. Based on pre-existing data from a two-phase, randomized clinical study in which subjects were given warfarin alone or in the presence of rifampin co-treatment, we estimated the in vivo potential of 10-hydroxywarfarin to inhibit S-warfarin metabolism.

2.3 Materials and Methods

2.3.1 Reagents and Chemicals

All chemicals used in this study were of reagent grade or better. Unless otherwise specified, all chemicals and reagents were purchased from either Sigma-Aldrich (St. Louis, MO) or Thermo Fisher Scientific (Waltham, MA). In particular, S-warfarin, 7-ethoxycoumarin (internal standard), and racemic R-10-hydroxywarfarin were purchased from Sigma-Aldrich. Specific 10-hydroxywarfarin stereoisomers (9R,10R-

hydroxywarfarin, 9R,10S-hydroxywarfarin, 9S,10R-hydroxywarfarin, 9S,10S-hydroxywarfarin, and d_5-7-hydroxywarfarin) were provided by Drs. Matthew McDonald and Allan Rettie, Department of Medicinal Chemistry, University of Washington, Seattle WA. Blank plasma (outdated) was obtained from the Puget Sound Blood Center

2.3.2 Determination of Warfarin Unbound Fraction (f_u) in Plasma

The binding of 10-hydroxywarfarin to human plasma proteins was measured by ultracentrifugation using a Sorvall micro-ultracentrifuge Discovery M150 SE with a Thermo Scientific S100-AT3 rotor (Waltham, MA). R-10-hydroxywarfarin (prepared as a 10X stock of 50 μM in 50% methanol: 50% PBS) was spiked into blank human plasma to achieve target final concentrations. Aliquots of 230 μL were taken for either ultracentrifugation (unbound drug concentration determination) or incubation (total drug concentration determination). Samples for ultracentrifugation were placed into polycarbonate ultracentrifugation tubes (Beckman-Coulter, 7x20 mm, cat. No. 343775) and centrifuged at 435,630 x *g* at 37˚C for 140 min. After ultracentrifugation, a 50 μL aliquot was removed from the clear, top layer of the tube and added to 250 μL of ice-cold acetonitrile containing 20 ng/mL of 7-ethoxycoumarin as an internal standard. For incubated samples, the plasma samples were incubated without centrifugation at 37˚C. At the end of 140 min, samples were vortexed and a 50 μL aliquot was removed and added to 250 μL of ice-cold acetonitrile containing internal standard. Following the addition of acetonitrile, all samples were vortexed and centrifuged (16,100 x *g* for 5 min at 10˚C). The supernatants were transferred to clean Eppendorf tubes and centrifuged again under the same conditions. The final supernatants were transferred to glass

HPLC inserts, and evaporated under a gentle stream of N_2 gas and reconstituted in 40 µL of mobile phase. Samples were analyzed as described in the LC-MS assay for determination of unbound fraction in plasma. All the samples were prepared in triplicate or quadruplicate. Standard curves were determined for both centrifuged and non-centrifuged samples over a range from 0.30 to 15 µM. The unbound fraction of 10-hydroxywarfarin in plasma was calculated as the ratio of the respective unbound drug concentration (centrifuged samples) to the total drug concentration (incubated samples).

2.3.3 LC-MS Analysis of 10-hydroxywarfarin for Unbound Fraction Determination

LC-MS analyses were conducted using an Agilent Technology 1100 HPLC coupled to a G1946B Mass Spectrometer (Agilent, Santa Clara, CA). The system was run in the ESI positive mode, with a source temperature of 350˚C and a fragmentor voltage of 50V. The following masses were monitored: *m/z* 325 (10-hydroxywarfarin), 309 (warfarin – when necessary), and 191 (7-ethoxycoumarin – internal standard). The compounds were separated using an Agilent Eclipse XDB 100 x 4.6 mm 5 u C18 column with a flow rate of 0.3 ml/min with solvent A: 0.1% acetic acid in water and solvent B: 0.1% acetic acid in acetonitrile. The gradient was set to 50% B for 0.5 min and increased linearly to 90% B over 5 min. The solvent composition was maintained for 0.1 minutes before re-equilibration. The total run time was 18 min (Figure 2.2).

2.3.4 Incubation of S-Warfarin with Racemic, and Individual Stereoisomers of, 10-hydroxywarfarin

Metabolic incubations were carried out using a pool of human liver microsomes (HLMs) prepared from 39 different human liver samples obtained from the University of Washington Human Liver Bank. This is an anonymous tissue bank and, as such, its use is considered “non-human research”, as determined previously by the UW Human Subjects Internal Review Board. Incubations were carried out in potassium phosphate buffer, pH 7.4. All incubation mixtures contained 0.45 mg/mL liver microsomal protein,, 1 mM NADPH, 20X concentrated inhibitor stock (in methanol) and 1 μM S-warfarin added as substrate. The inhibitors were racemic 10-hydroxywarfarin, 9R,10R-hydroxywarfarin, 9R,10S-hydroxywarfarin, 9S,10R-hydroxywarfarin, and 9S,10S-hydroxywarfarin. The final concentration of inhibitors ranged from 0 to 25 μM in a total volume of 500 μL. Incubations were carried out in triplicate.

HLMs were preincubated with S-warfarin and inhibitor at 37°C in a shaking waterbath for 5 min prior to the initiation of the reaction with the addition of NADPH. After 30 min, the reactions were quenched by the addition of 500 μL of acetonitrile containing 50 ng of d_5-7-hydroxywarfarin internal standard. Samples were analyzed as described in the LC-MS/MS method for kinetic reactions.

2.3.5 LC-MS/MS Analysis of Kinetic Reactions

Samples were centrifuged (16,100 x *g* for 5 min at 10˚C). A 100 μL aliquot of the supernatant was transferred to a 96-well plate to quantify 7-hydroxywarfarin formation by LC-MS/MS. LC-MS/MS analyses were conducted using an Agilent Technology 1290 HPLC coupled to a G6410B Mass Spectrometer. The system was run in ESI positive mode with a gas temperature of 350˚C. The capillary and CE voltages were set to 3500

V and 12 V, respectively. The fragmentation voltage was set to 85 V for S-7-hydroxywarfarin and 80 V for d_5-7-hydroxywarfarin. The following masses were monitored: *m/z* 325.01 > 179 (S-7-hydroxywarfarin), and 330.01 > 272.2 (d_5-7-hydroxywarfarin). The compounds were separated using a Phenomenex Luna 150 x 2 mm 3 µm Phenyl-hexyl column with a flow rate of 0.3 ml/min with solvent A: 0.05% formic acid in water and solvent B: 0.05% formic acid in acetonitrile. The column was held at 50˚C. The gradient was set to 25% B until 2 min and increased linearly to 60% B at 15 min, and held at 95% for 2 minutes, until re-equilibration to 25% for 5 min. The total run time was 22 min. The formation of S-7-hydroxywarfarin was determined and the percent of S-7-hydroxywarfarin inhibition was plotted against the inhibitor concentration of the various 10-hydroxywarfarin species. IC50s were estimated using Phoenix WinNonlin (Cetera, St. Louis, MO). The K_i was calculated using the following equation: $IC_{50} = K_i \cdot (1 + [S]/K_m)$, where [S] was 1 µM S-warfarin and K_m was assumed to be 5.2 µM for the formation of 7-hydroxywarfarin based on literature values (8).

2.3.6 Clinical Study

A clinical study was conducted at the University of Minnesota to determine the magnitude of warfarin-rifampin or fluconazole drug interactions and the impact of genetic variation on the interaction response. Healthy human volunteers were recruited into a randomized three-phase study (warfarin alone, warfarin + fluconazole, and warfarin + rifampin). Only results from the warfarin alone and warfarin-rifampin arms were used for this secondary analysis. Eligible subjects could not have any evidence of abnormal renal function (serum creatinine > 2 mg/dL) or clinical evidence of cirrhosis.

Subjects were non-smokers and not on any other drug therapies, with the exception of oral contraceptives. Healthy subjects were identified using medical history, physical examination, and biochemistry and urine test results. Whole blood samples were used to determine *CYP2C9* genotypes. The study was approved by the Institutional Review Board at the University of Minnesota.

For the warfarin-alone arm, subjects were given a single 10 mg oral dose of racemic warfarin. For the warfarin + rifampin arm, subjects were given daily 300 mg oral doses of rifampin for 1 week prior to the dose of warfarin (single 10 mg oral dose) and rifampin was continued through the end of the study period (15 days). Washout consisted of one week between study periods. Plasma samples were collected for warfarin pharmacokinetics at pre-dose, 2, 6, 24, 48, 72, 96, 120, 144, 148, 216, and 264 hours.

2.3.7 Clinical Study Assay Methods

At blood collection prior to isolation of plasma, racemic deuterated warfarin (5 µg/mL) and the corresponding racemic deuterated metabolites were added to whole blood samples. Plasma was isolated and stored at -20˚C until analysis. Warfarin and hydroxywarfarin metabolites were analyzed using a chiral LCMS assay as described previously (9). Pharmacokinetic data analysis was performed on the thirteen subjects that did not possess either the *CYP2C9*2* or *CYP2C9*3* alleles.

2.4 Results

2.4.1 10-Hydroxywarfarin Fraction Unbound in Human Plasma

For quantification of the unbound fraction of 10-hydroxywarfarin in human plasma, two separate calibration curves were used. A lower concentration calibration curve (range of 30 - 500 nM) was used to quantify 10-hydroxywarfarin in those samples which underwent ultracentrifugation to determine free drug concentrations. These curves were linear with correlation coefficients greater than 0.98. A higher concentration calibration curve (range of 0.50 - 15 μM) was used to quantify 10-hydroxywarfarin in the non-centrifuged samples for determination of total drug concentration. Standard curves were linear with correlation coefficients of greater than 0.9. The fraction unbound was calculated by dividing the free drug concentration by the total drug concentration determined in each experiment. The results of three separate experiments are summarized in Table 2.1. The mean 10-hydroxywarfarin plasma unbound fraction was 0.98 ± 0.22%.

2.4.2 10-hydroxywarfarin Inhibition of CYP2C9 Metabolism

Kinetic studies were carried out using pooled HLMs to determine the IC_{50} for the inhibition of (S)-warfarin metabolism by racemic 10-hydroxywarfarin and the individual, purified 10-hydroxywarfarin stereoisomers (Figure 2.3). These compounds inhibited human liver microsomal S-warfarin 7-hydroxylation with IC_{50} values ranging from 4.4 to 30 μM. The 9R,10S-hydroxywarfarin metabolite was the most potent CYP2C9 inhibitor (Table 2.2).

2.4.3 Clinical Study

In total, thirteen healthy subjects completed the study (Table 2.3). Among these subjects, 7 were male and 6 were female (54% and 46%, respectively), the mean age was 26 years and the mean weight was 65.7 kg. The majority of subjects were White (62%). Of the thirteen subjects, five subjects were *CYP2C9*1/*1*, three subjects were *CYP2C9*1/*1B*, and five subjects were *CYP2C9*1B/*1B.*

Following warfarin dosing, plasma concentrations of R-warfarin, S-warfarin, S-7-hydroxywarfarin and R-10-hydroxywarfarin were measured (Figure 2.4). Pharmacokinetic parameters estimated by non-compartmental modeling are summarized in Table 2.4. For R- and S-warfarin, the observed C_{max} and T_{max} were not altered by rifampin treatment. However, the apparent oral clearance and terminal half-life were significantly different following rifampin treatment compared to warfarin alone. The apparent oral clearance of R-warfarin was increased by 149% and that of S-warfarin was increased by 87%. For R-warfarin, half-life decreased by 19 hours from a control value of 71 ± 10 hours. Conversely, the half-life of S-warfarin increased by 20 hours from a control value of 69.4 ± 8.6 hours.

When warfarin was given alone, the mean peak concentration of R-10-hydroxywarfarin was 18 ± 6.2 ng/mL and Tmax occurred at 72 ± 28 hours. With rifampin treatment, the peak of R-10-hydroxywarfarin increased to 43 ± 18 ng/mL (2.4-fold increase) and occurred earlier at 33 ± 12 hours. This change in peak metabolite concentrations was accompanied by a 40% increase in R-10-hydroxywarfarin AUC (4.7 ± 2.0 and 6.6 ± 4.8 μg·hr/mL for warfarin alone and rifampin treatment, respectively; $p<0.05$). The half-life of R-10-hydroxywarfarin decreased from 136 ± 32 hours to 77 ±

28 hours following rifampin treatment. In addition, the estimated mean steady-state concentration for R-10-hydroxywarfarin was 0.28 ± 0.20 μg/mL (0.85 ± 0.62 μM) in the presence of rifampin compared to 0.20 ± 0.09 μg/mL (0.61 ± 0.27 μM) following warfarin alone.

2.4.4 Determination of [I]/Ki for R10-hydroxywarfarin

The predicted inhibitory ratio (1 + [I]/Ki) is defined as the steady-state plasma concentration of R10-hydroxywarfarin divided by the in vitro K_i (apparent) value measured for the inhibition of S-warfarin 7-hydroxylation in HLMs. The values of the 9R,10R-hydroxywarfarin and 9R,10S-hydroxywarfarin inhibitory constants, K_i, were calculated to be 25.3 ± 3.6 and 3.7 ± 0.5 μM , respectively (see Table 2.5). Although we did not quantitate the individual stereoisomers of R-10-hydroxywarfarin in plasma, we assumed that the steady-state R-10-hydroxywarfarin plasma concentrations would represent either 9R,10R-hydroxywarfarin or 9R,10S-hydroxywarfarin to calculate the greatest inhibitory potential for each stereoisomer. The predicted inhibitory ratios for warfarin alone were negligible. The predicted inhibitory ratios for 9R,10R-hydroxywarfarin and 9R,10S-hydroxywarfarin were 1.02 and 1.16, respectively. Following rifampin treatment, the predicted inhibitory ratios increased slightly for 9R,10R-hydroxywarfarin and 9R,10S-hydroxywarfarin to 1.03 and 1.23, respectively. It is most likely that the R-10-hydroxywarfarin plasma concentrations are predominantly (>95%) the 9R,10S-hydroxywarfarin diastereomer (10). Thus, the increase in S-warfarin AUC caused by R-10-hydroxywarfarin at steady-state is predicted to be less than 25%.

2.5 Discussion

We estimated the unbound fraction of racemic 10-hydroxywarfarin to be 0.98 ± 0.22%. This is lower than reported values of 3.24-4.49% for the individual enantiomers of 6- and 7-hydroxywarfarin, two of the significant circulating hydroxywarfarin metabolites (11). The unbound fractions for the individual R- and S-enantiomers of warfarin have been reported by both Chan (0.54% and 0.53%, respectively), and by Takahashi (1.2% and 0.7%, respectively) (12, 13). The free drug hypothesis states that only the unbound concentration of a compound is available to interact/complex with an enzyme. Therefore, determining the free fraction value of 10-hydroxywarfarin is critical for predicting the magnitude of its interaction with CYP2C9 in vivo.

Our study expands on previous findings concerning the inhibitory effects of hydroxywarfarins on CYP2C9-mediated hydroxylation of S-warfarin. Previous investigators demonstrated that racemic hydroxywarfarin metabolites (4', 6, 7, 8, and 10) acted as competitive inhibitors of S-warfarin metabolism in HLMs with K_i values ranging from 2.2 to 167 μM. (7) The most potent inhibitor was 10-hydroxywarfarin with a K_i value of 2.2 μM in HLMs and a K_i of 0.94 μM in recombinant CYP2C9 incubations (7). However, this racemic 10-hydroxywarfarin is a combination of the 9R,10S- and 9S,10R-hydroxywarfarin stereoisomers. We tested the inhibition of CYP2C9-mediated S-warfarin metabolism in HLMs by the racemate and each of the four individual stereoisomers of 10-hydroxywarfarin. Our results for racemic 10-hydroxywarfarin were consistent with previous reports (within 2.3-fold). When the individual stereoisomers were tested, 9R,10S-hydroxywarfarin was the most potent inhibitor with a calculated K_i of 3.7 μM.

A clinical study was conducted by colleagues at the University of Minnesota, whereby healthy volunteers were administered a single dose of racemic warfarin alone and again following one week pretreatment with rifampin. We evaluated the pharmacokinetics of R-warfarin, S-warfarin, and the two highest circulating hydroxywarfarin metabolites (R-10-hydroxywarfarin and S-7-hydroxywarfarin). In general, the pharmacokinetics of R- and S-warfarin differed when compared to those reported in other studies. The clearance of R- and S-warfarin were moderately lower than reported by Frymoyer and Robertson (14, 15). Interestingly, the terminal half-lives for R- and S-warfarin were also substantially longer than reported in these studies. It is possible this is a result of the study design employed by our UM colleagues, where samples were collected for 11 days compared to 5 or 7 days for the other studies. The longer study might better capture the true terminal elimination phase. For the metabolites, the pharmacokinetics were comparable to the values determined both by Frymoyer et al., and by Uno et al. (14, 16).

Based on the observed R-10-hydroxywarfarin concentrations, we determined the steady-state metabolite concentrations based on daily 10 mg dosing of racemic warfarin. We believe that 9R,10S-hydroxywarfarin is the predominant diastereomer of 10-hydroxywarfarin in plasma following racemic warfarin administration (10). We calculated ratios of 1.16 and 1.23 for inhibition by 9R,10S-hydroxywarfarin of the CYP2C9-mediated metabolism of S-warfarin under basal and enzyme-induced conditions in vivo, respectively. To verify whether these inhibitory ratios are correct, we would need to dose with S-warfarin alone to be able to estimate the S-warfarin AUC in the absence of the metabolite inhibitor. To date, only three studies have administered

the S- and R-warfarin enantiomers separately (17-19). None of them measured S-7-hydroxywarfarin or R-10-hydroxywarfarin concentrations. The ideal study would involve administering S-warfarin in the presence of various doses of R-10-hydroxywarfarin to allow for a more rigorous examination of the impact of R-10-hydroxywarfarin inhibition on S-warfarin metabolism.

Some limitations of our study should be noted. Our K_i values were estimated using IC_{50} measurements. While this may be a valid method of estimating a K_i, other methods, such as an experimental determination of K_i based on varying substrate and inhibitor concentrations, may produce more reliable estimates. Additionally, the in vivo circulating concentrations of the 10-hydroxywarfarin stereoisomers were not determined in the pharmacokinetic samples that were available for this investigation. However, based on literature reports, we believe that the major circulating 10-hydroxywarfarin species is the 9R,10S diastereomer (17). Nonetheless, the quantitation of the four 10-hydroxywarfarin stereoisomers would be preferable for an in vitro-in vivo prediction. Finally, the contribution of CYP3A5 to the formation of 10-hydroxywarfarin and the subsequent downstream fate of this metabolite are unknown. It is known that 10-hydroxywarfarin is not significantly glucuronidated and its conjugates have not been detected in urine (8, 20). The determination of genotypic data regarding the status of other relevant CYP may be desirable (e.g. *CYP3A5*).

Overall, this is the first in vitro study to show the differential inhibitory potencies of 10-hydroxywarfarin stereoisomers on of CYP2C9-mediated 7-hydroxylation of S-warfarin and we found that 9R,10S-hydroxywarfarin is the most potent of the 10-hydroxywarfarin metabolites. We determined that the unbound fraction in plasma was

similar to values reported in the literature. Based on our determination of the pharmacokinetics of R- and S-warfarin and its major metabolites, studies need to be conducted for extended periods of time due to the long terminal half-lives. Further experiments are needed to more clearly determine the potential importance of 10-hydroxywarfarin in influencing inter-patient variability in response to warfarin treatment.

2.6 References

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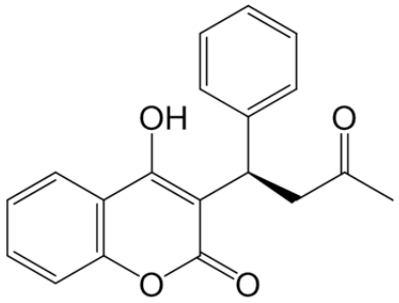
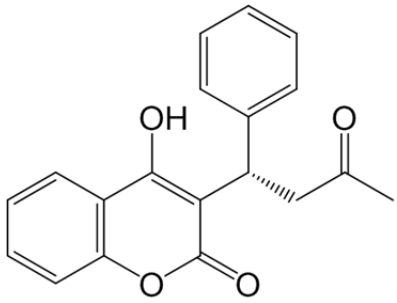
Figure 2.1. Warfarin enantiomers and major circulating hydroxywarfarin metabolites.

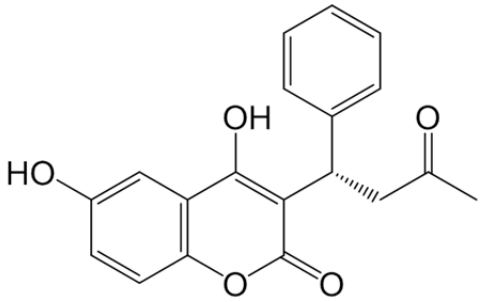
R-warfarin



S-warfarin



S-6-hydroxywarfarin



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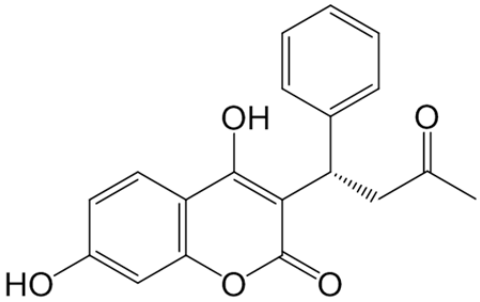
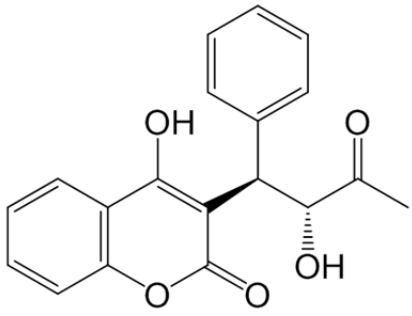
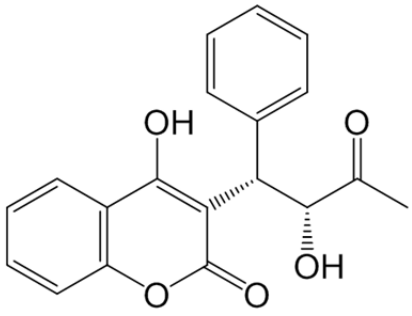


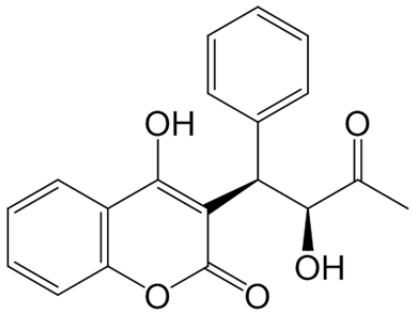
9R,10R-hydroxywarfarin



9S,10R-hydroxywarfarin



9R,10S-hydroxywarfarin



9S,10S-hydroxywarfarin

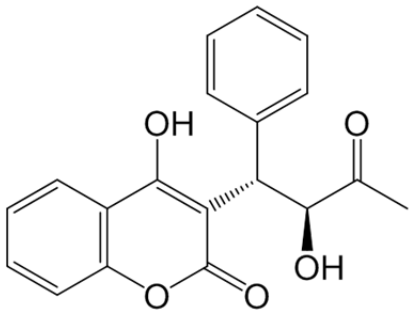


Figure 2.2. Liquid chromatography-electrospray ionization mass spectrometry analysis of chemical standards spiked into plasma. The following masses were monitored: *m/z* 325 (10-OHWAR), 309 (WAR), and 191 (7-ETH). 10-OHWAR, 10-hydroxywarfarin; WAR, warfarin; 7-ETH, 7-ethoxycoumarin (internal standard).

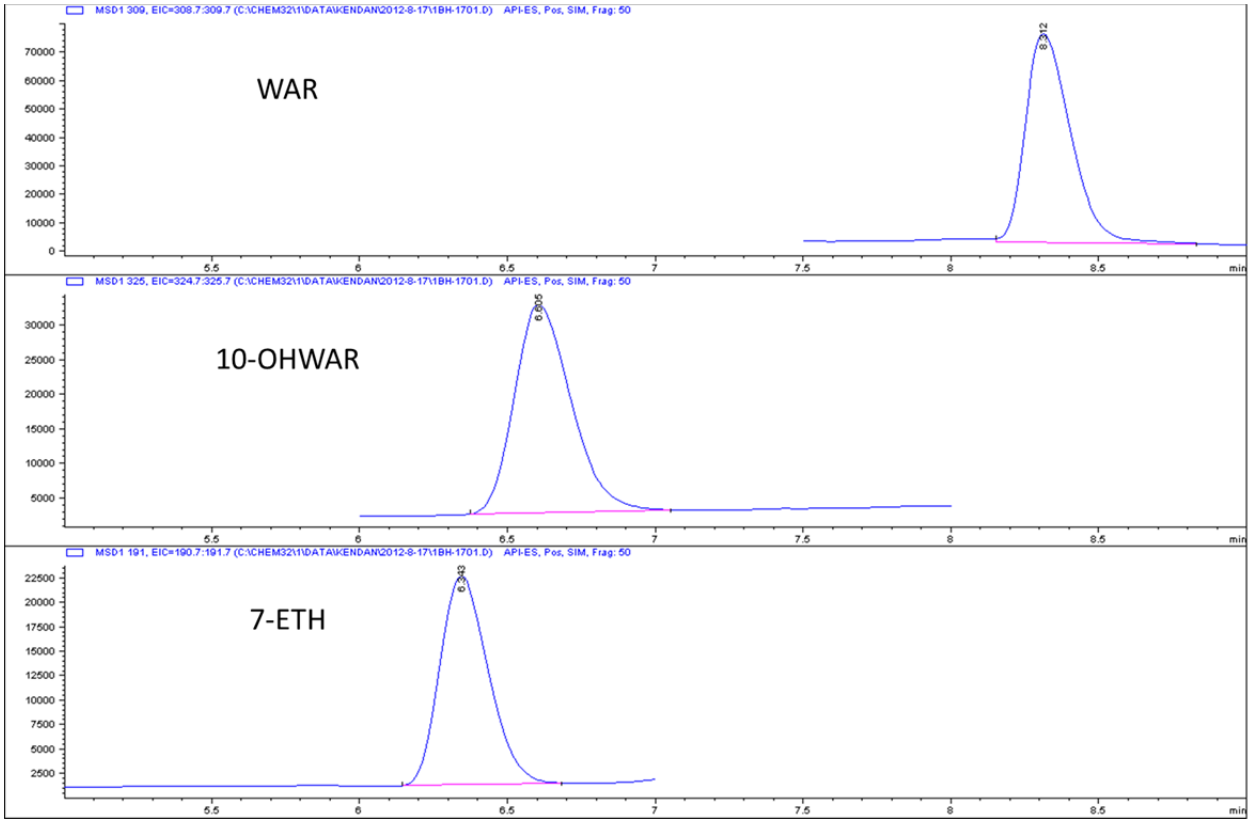
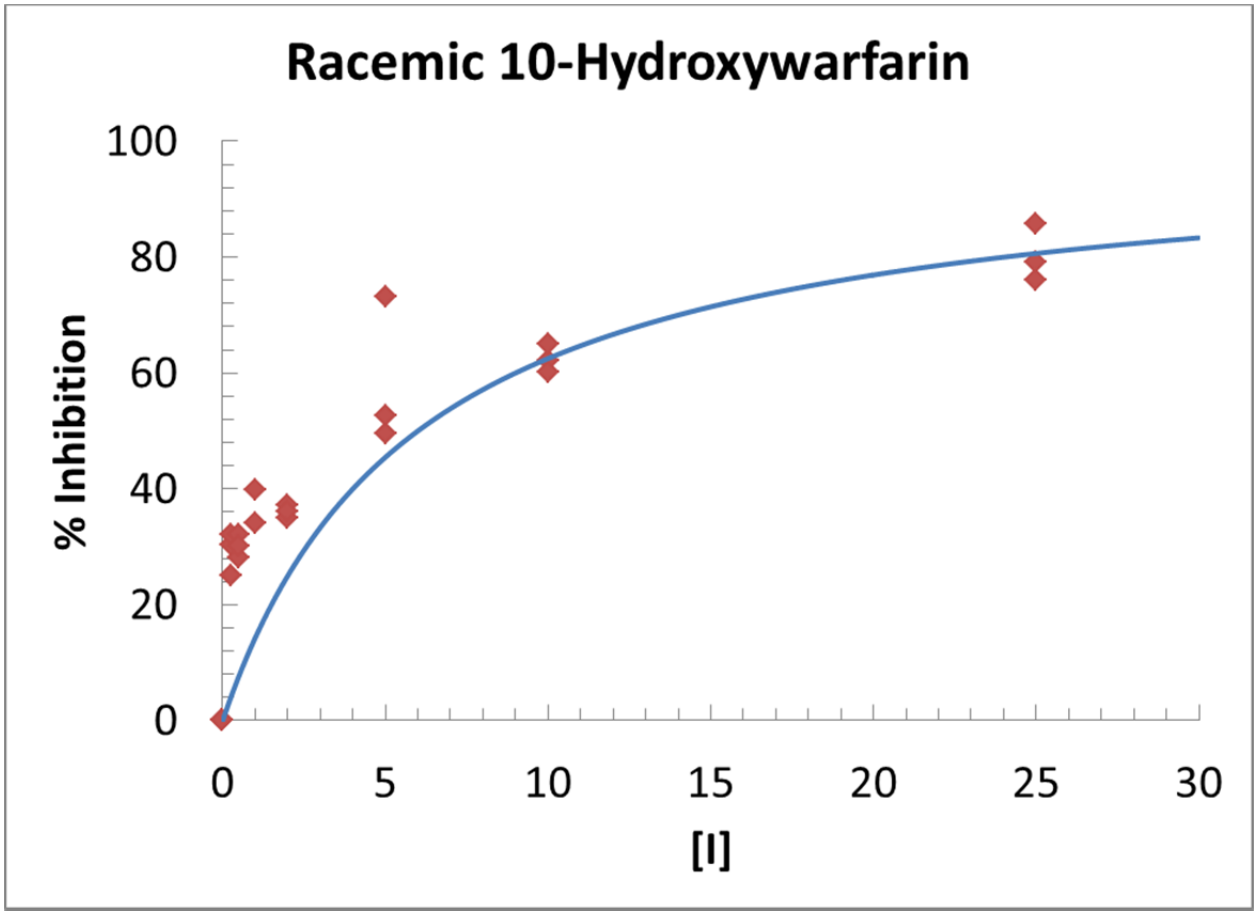
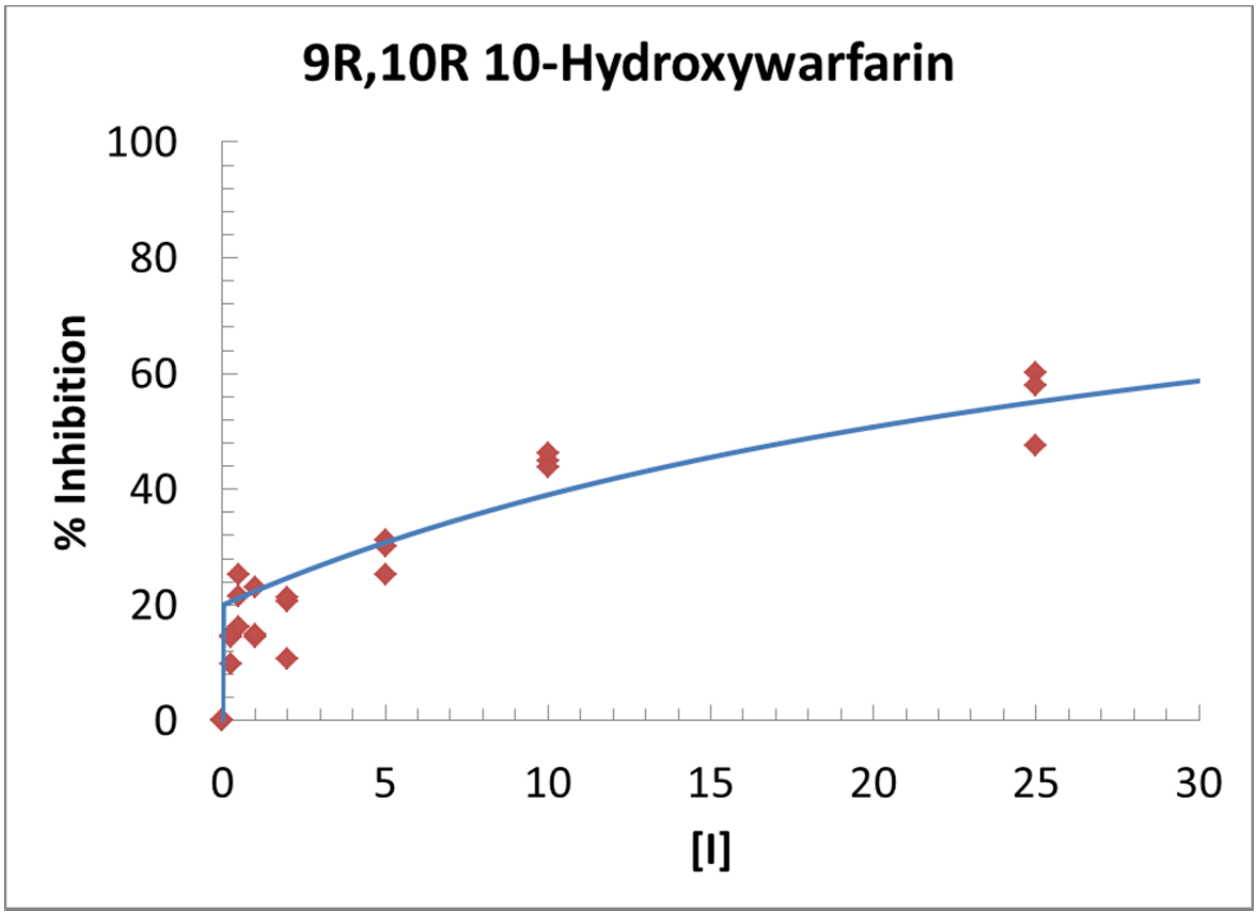


Figure 2.3. IC50 inhibition curves of the percent inhibition of human liver microsomal S-warfarin 7-hydroxylase activity by 10-hydroxywarfarin (Panel A: racemic 10-hydroxywarfarin, Panel B: 9R,10R-hydroxywarfarin, Panel C: 9R,10S-hydroxywarfarin, Panel D: 9S,10R-hydroxywarfarin, Panel E: 9S,10S-hydroxywarfarin). Metabolic incubations contained 0.45 mg protein/mL. Shown are the results of triplicate incubations at each inhibitory concentration with predicted inhibitory curve superimposed on the data.

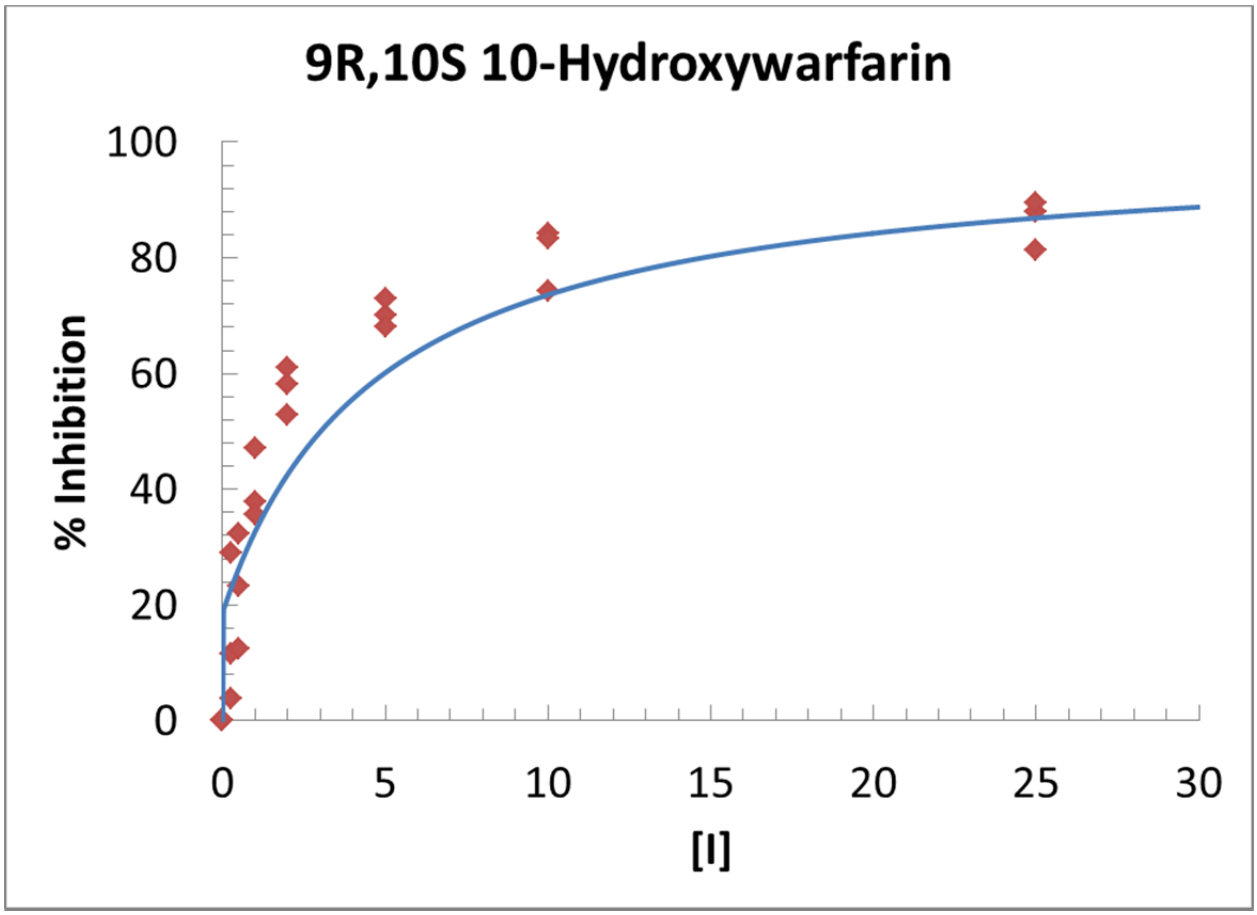
A



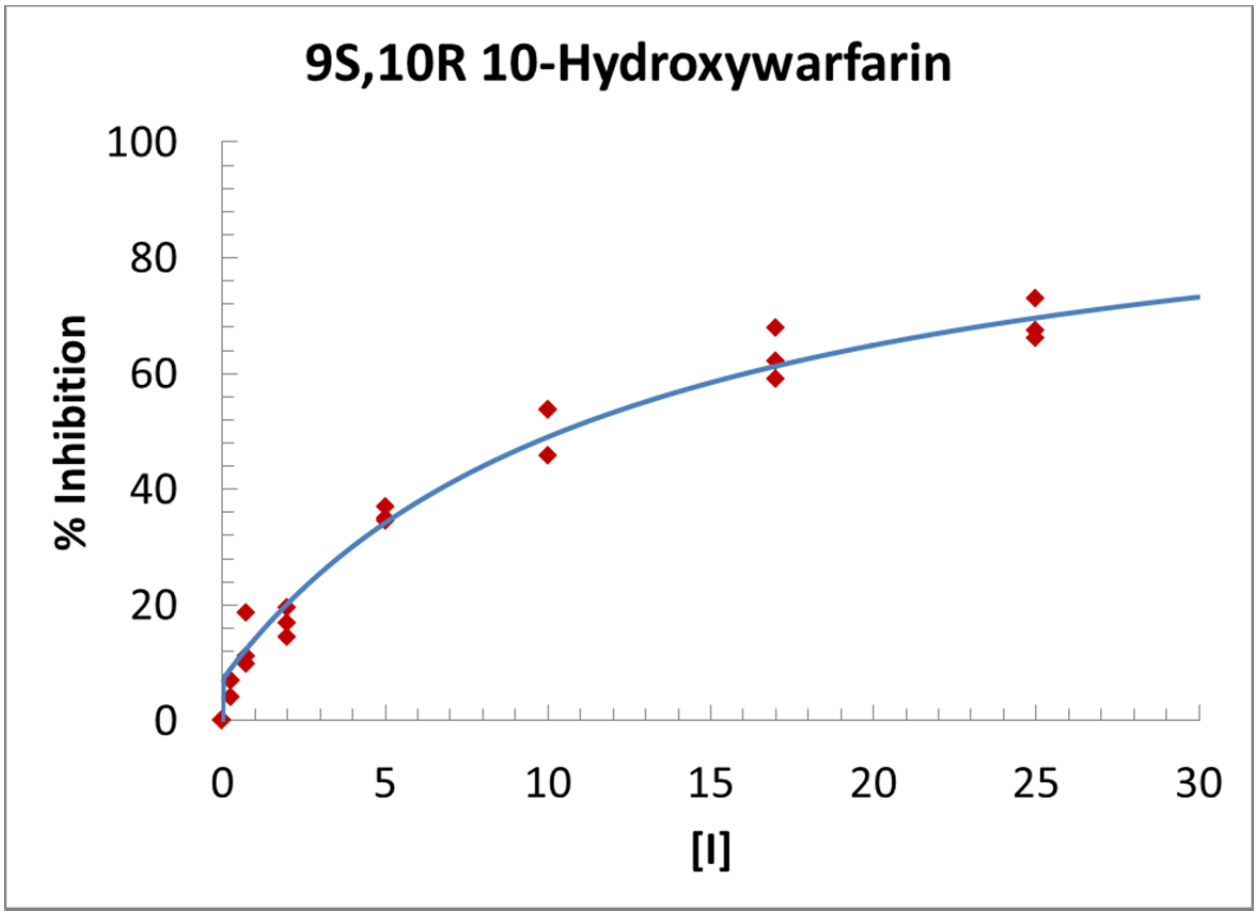
B


9R,10R 10-Hydroxywarfarin
% Inhibition
100
80
60
40
20
0
0
5
10
15
20
25
30
[I]

C


9R,10S 10-Hydroxywarfarin
% Inhibition
100
80
60
40
20
0
0
5
10
15
20
25
30
[I]

D


9S,10R 10-Hydroxywarfarin
% Inhibition
100
80
60
40
20
0
0
5
10
15
20
25
30
[I]

E

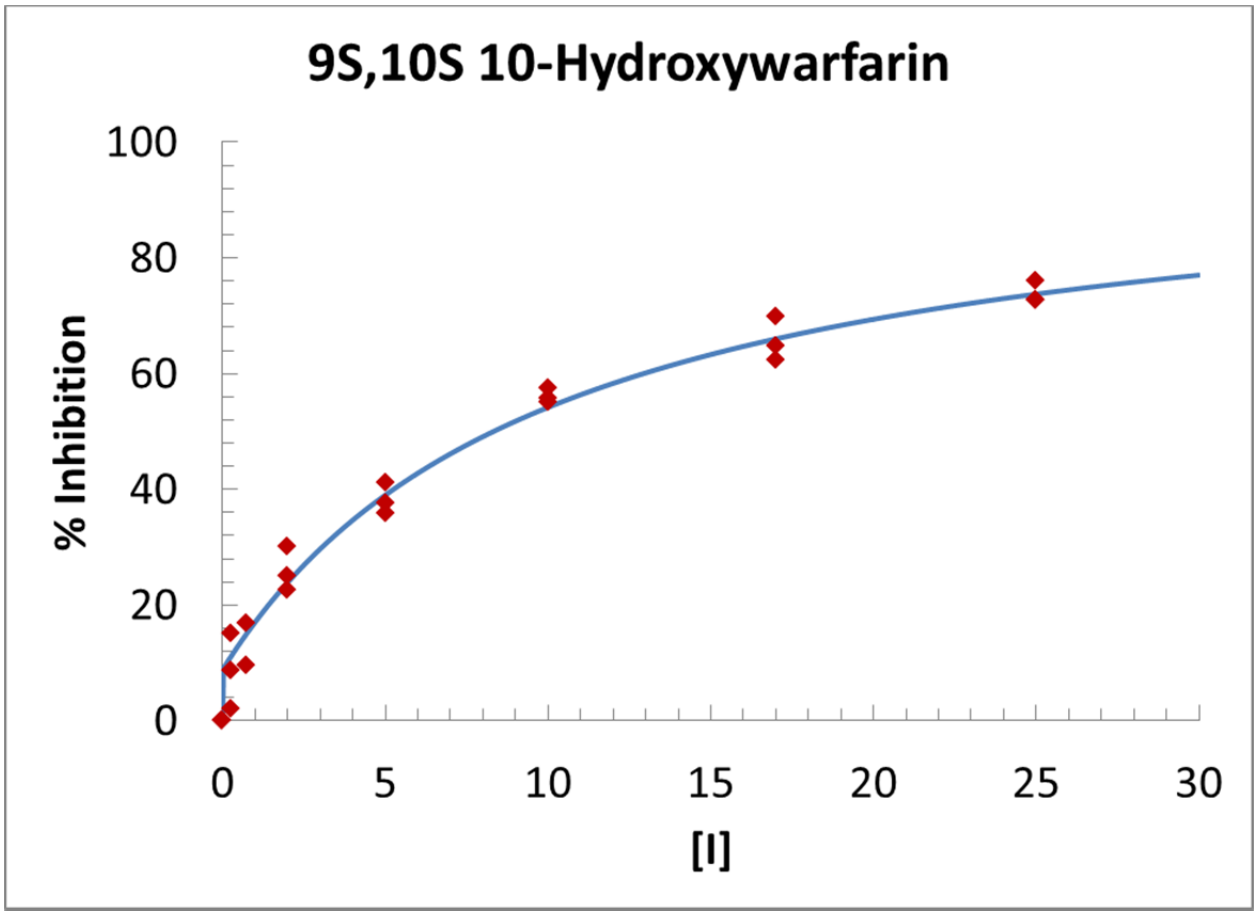
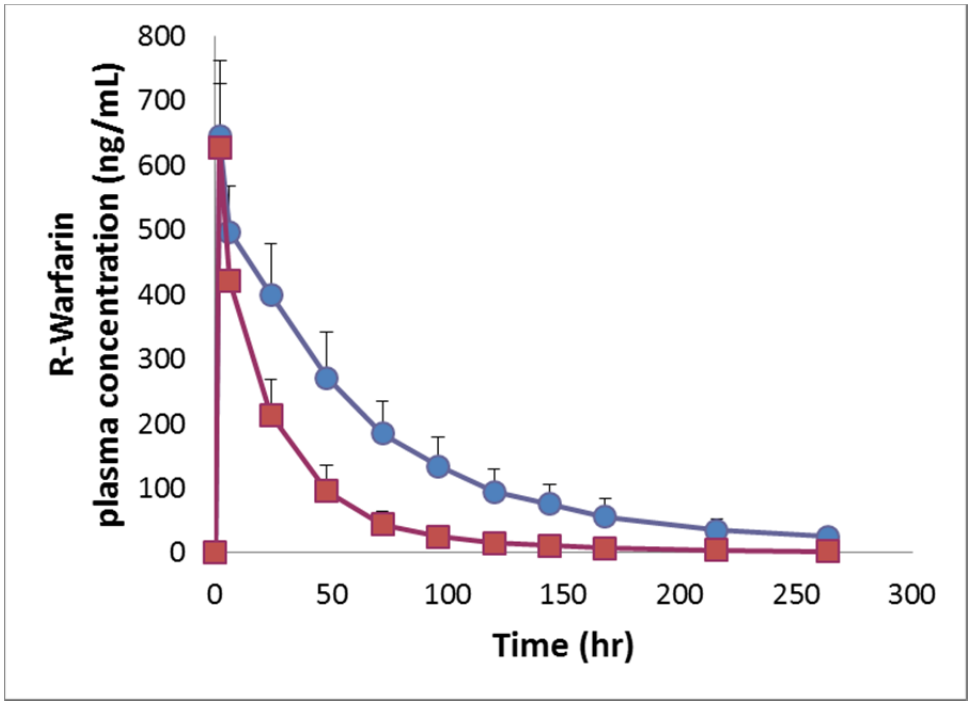
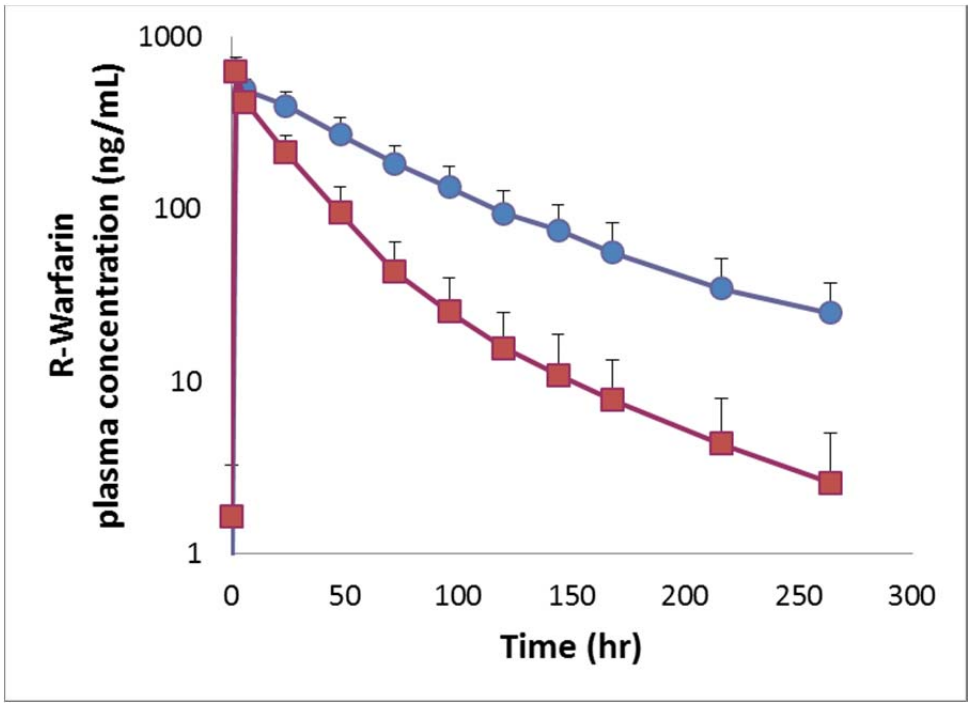

9S,10S 10-Hydroxywarfarin
% Inhibition
100
80
60
40
20
0
0
5
10
15
20
25
30
[I]

Figure 2.4. Plasma concentration curves (mean ± sd, n=13) of R-warfarin (Panel A & B), S-warfarin (Panel C & D), R-10-hydroxywarfarin (Panel E & F), and S-7-hydroxywarfarin (Panel G & H) after a single dose of 10 mg warfarin in 13 healthy volunteers prior to and following daily 300 mg rifampin treatment (1 week prior to warfarin dosing; total duration 3 weeks). Warfarin alone and in the presence of rifampin is shown in blue and red, respectively.

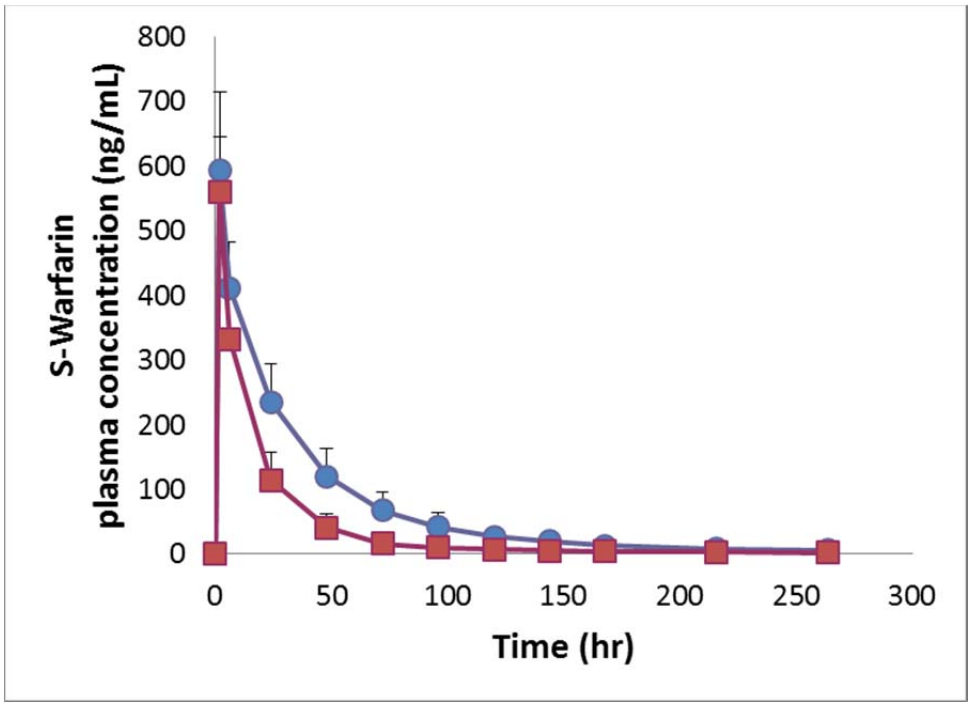
A



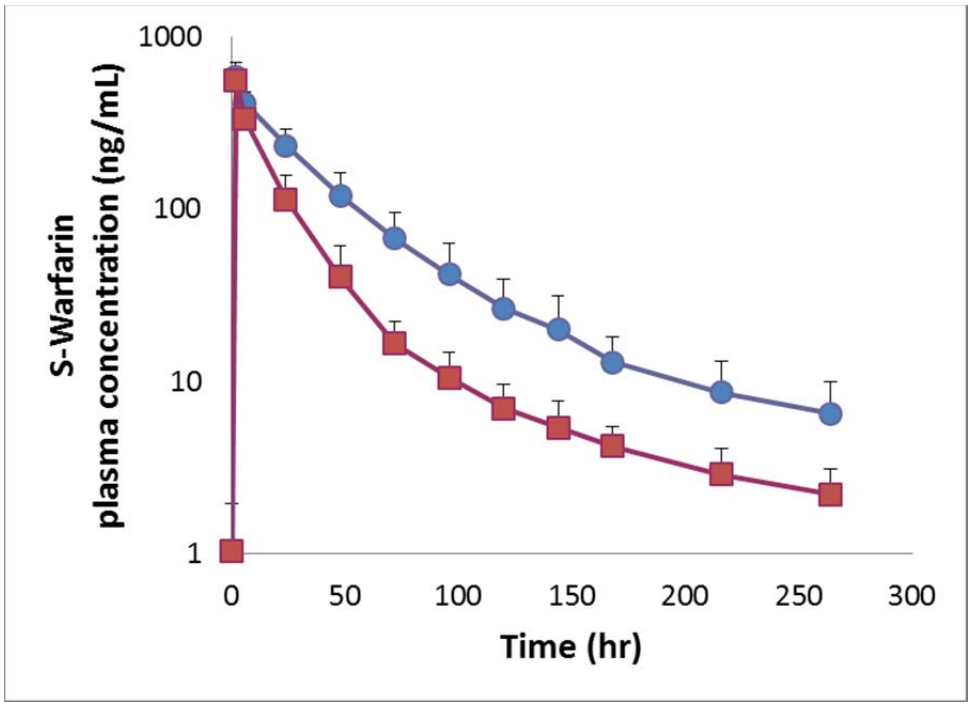
B


1000
100
10
1
R-Warfarin
plasma concentration (ng/mL)
0
50
100
150
200
250
300
Time (hr)

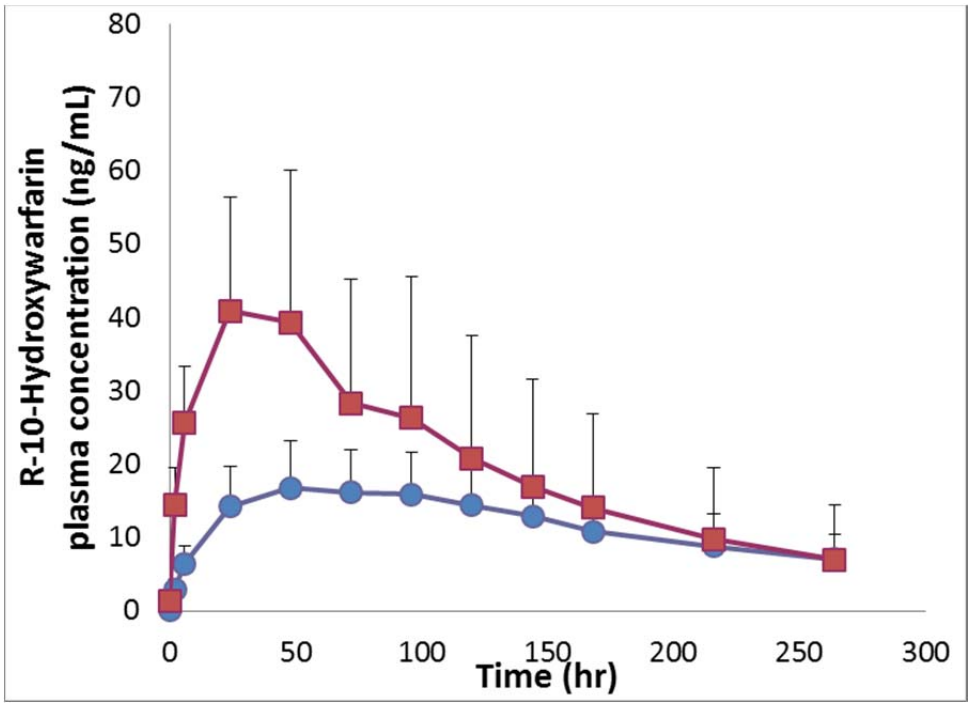
C


S-Warfarin plasma concentration (ng/mL)
800
700
600
500
400
300
200
100
0
0
50
100
150
200
250
300
Time (hr)

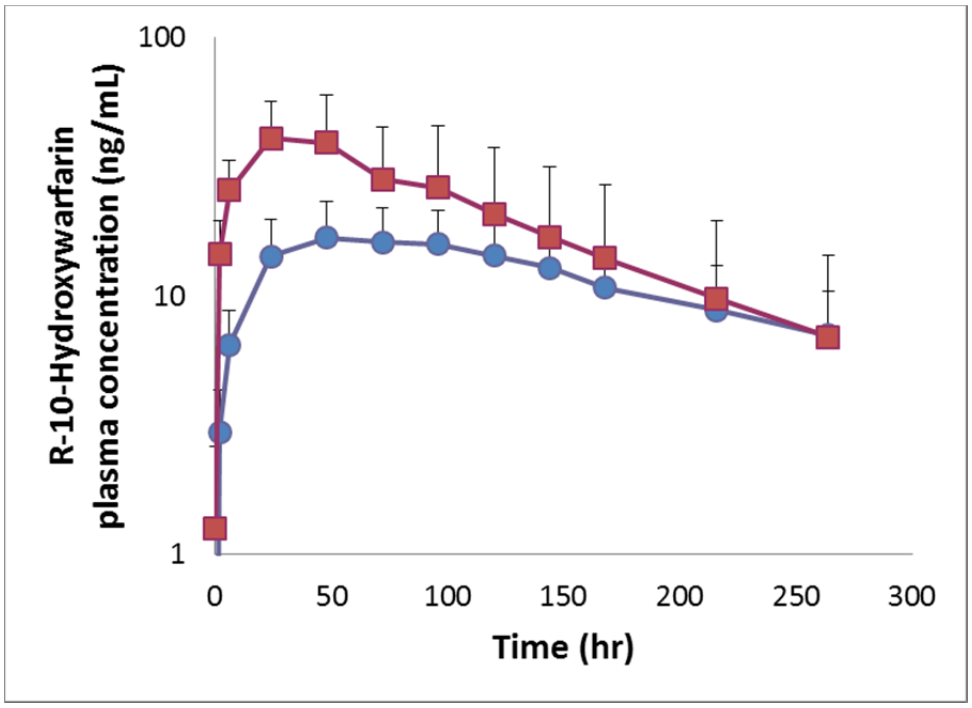
D


1000
100
10
1
S-Warfarin
plasma concentration (ng/mL)
0
50
100
150
200
250
300
Time (hr)

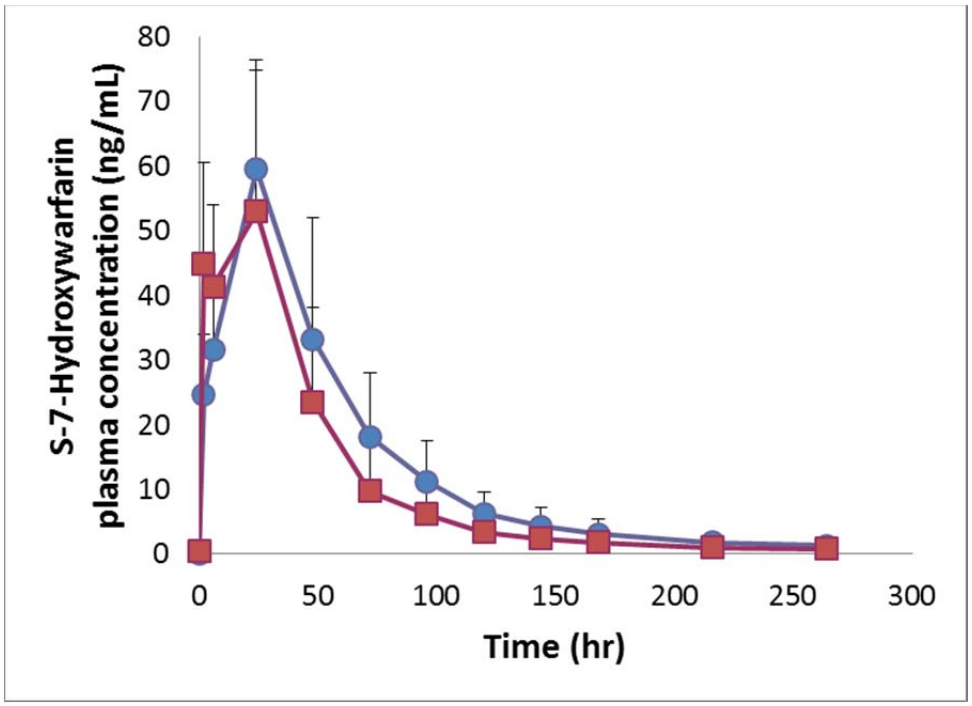
E


R-10-Hydroxywarfarin plasma concentration (ng/mL)
80
70
60
50
40
30
20
10
0
0
50
100
150
200
250
300
Time (hr)

F


R-10-Hydroxywarfarin plasma concentration (ng/mL)
100
10
1
0
50
100
150
200
250
300
Time (hr)

G


S-7-Hydroxywarfarin plasma concentration (ng/mL)
80
70
60
50
40
30
20
10
0
0
50
100
150
200
250
300
Time (hr)

H

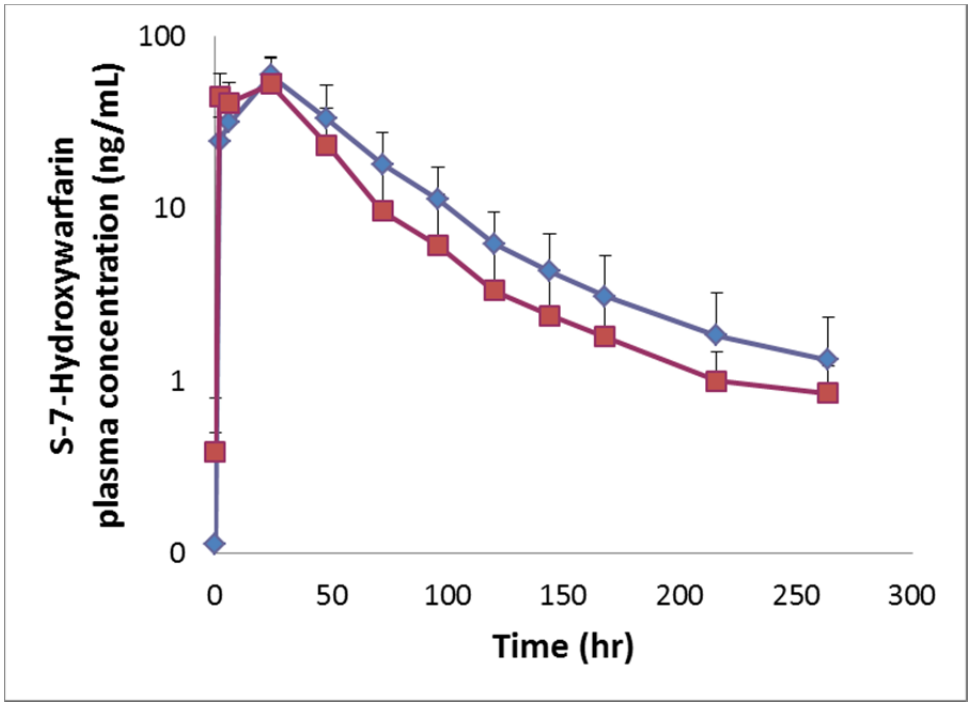

S-7-Hydroxywarfarin plasma concentration (ng/mL)
100
10
1
0
0
50
100
150
200
250
300
Time (hr)

Table 2.1. Determination of the free fraction of 10-hydroxywarfarin in human plasma. Experiments were performed in triplicate or quadruplicate on three separate occasions. Error measurement represents standard deviation of the means.

	EXP #1	EXP #2	EXP #3	
	Free Drug Concentration (nM)			
A	65.0	91.0	56.0	
B	43.2	78.9	63.4	
C	58.1	100.4	89.4	
D	44.8	-	-	
Mean	**52.8**	**90.1**	**69.6**	
SD	*10.5*	*10.8*	*17.5*	
	Total Drug Concentration (μM)			
A	5.51	7.31	8.71	
B	6.27	7.40	8.54	
C	5.15	7.28	8.87	
D	6.18	-	-	
Mean	**5.78**	**7.33**	**8.71**	
SD	0.54	0.06	0.16	**Mean ± SD**
Fraction Unbound (%)	**0.91**	**1.23**	**0.80**	**0.98 ± 0.22**

Table 2.2. Comparison of inhibitory constants (IC50 and Ki) for the inhibition of human liver microsomal S-warfarin 7-hydroxylation by various warfarin metabolites

CYP2C9 inhibitors	IC50 (μM)	Ki (μM)	Ref
10OHWAR	6.0 ± 1.2	5.0 ± 1.0	this work
9R,10R-OH	30.2 ± 4.34	25.3 ± 3.6	this work
9R,10S-OH	4.4 ± 0.57	3.7 ± 0.5	this work
9S,10R-OH	12.1 ± 0.85	10.2 ± 0.71	this work
9S,10S-OH	10.1 ± 0.61	8.5 ± 0.51	this work
4'-OH WAR		17 (13 - 24) [a]	6
10-OH WAR		2.2 (1.7 – 13.0)	6
6-OH WAR		167 (117 - 257)	6
7-OH WAR[b]		64 (55 – 74)	6
8-OH WAR		13 (11 – 16)	6

[a] 95% confidence intervals are shown in parentheses
[b] Results are based on S-6 hydroxylation formation

Table 2.3. Demographic characteristics of subjects

Subjects Demographics	
Total number of subjects	13
Age (yr)[a]	26 [19-54]
Weight (kg)	65.7 [43.1-87.0]
Sex	54
Female	6 (46%)
Male	7 (54%)
Race	62
American Indian or Alaska Native	1 (8%)
Asian	4 (31%)
White	8 (62%)
Ethnicity	
Hispanic or Latino	1 (8%)
Not Hispanic or Latino	12 (92%)
Genotypes	
*CYP2C9*1/*1* (n =)	5
*CYP2C9*1/*1B* (n =)	3
*CYP2C9*1B/*1B* (n =)	5

[a] Mean and [range]

Table 2.4. Pharmacokinetic parameters of warfarin enantiomers, S-7-hydroxywarfarin and R-10-hydroxywarfarin following a single 10 mg dose of warfarin in 13 healthy volunteers prior to and following treatment with 300 mg rifampin. Values are presented as mean ± sd. Tmax data are given as median and range. SD, standard deviation; AUC, area under the plasma concentration time curve; Cmax, peak concentration; Tmax, time to Cmax; t1/2, elimination half-life; CL/F, apparent oral clearance.

	Control	**Rifampin**	**p-value**
R-warfarin			
Cmax (ng/mL)	646 ± 116	628 ± 100	n.s.
Tmax (hr)	2 ± 1	2 ± 0	n.s.
t1/2 (hr)	71 ± 10	52 ± 9.2	<0.001
$AUC_{(0\text{-}264)}$ (μg*hr/mL)	38.4 ± 9.5	16.3 ± 4.5	<0.001
$AUC_{(0\text{-}\infty)}$ (μg*hr/mL)	41.0 ± 10.7	16.5 ± 4.6	<0.001
CL/F (mL/hr)	132 ± 44	329 ± 102	<0.001
S-warfarin			
Cmax (ng/mL)	594 ± 121	560 ± 85	n.s.
Tmax (hr)	2 ± 0	2 ± 0	--
t1/2 (hr)	69 ± 8.5	89 ± 16	0.001
$AUC_{(0\text{-}264)}$ (μg*hr/mL)	19.0 ± 5.4	10.1 ± 2.8	<0.001
$AUC_{(0\text{-}\infty)}$ (μg*hr/mL)	19.6 ± 5.6	10.4 ± 2.8	<0.001
CL/F (mL/hr)	271 ± 64	506 ± 102	<0.001
R-10-hydroxywarfarin			
Cmax (ng/mL)	18 ± 6.2	43 ± 18	<0.001
Tmax (hr)	72 ± 28	33 ± 12	<0.001
t1/2 (hr)	136 ± 32	77 ± 28	<0.001
$AUC_{(0\text{-}264)}$ (μg*hr/mL)	3.2 ± 1.2	5.6 ± 3.6	0.013
$AUC_{(0\text{-}\infty)}$ (μg*hr/mL)	4.7 ± 2.0	6.6 ± 4.8	n.s.
S-7-hydroxywarfarin			
Cmax (ng/mL)	60 ± 17	62 ± 19	n.s.
Tmax (hr)	26 ± 6.7	16 ± 14	0.03
t1/2 (hr)	45 ± 15	29 ± 9	0.002
$AUC_{(0\text{-}264)}$ (μg*hr/mL)	3.6 ± 1.4	2.8 ± 1.1	0.008
$AUC_{(0\text{-}\infty)}$ (μg*hr/mL)	3.7 ± 1.4	2.9 ± 1.1	0.006

Table 2.5. Comparisons of [I]/KI values for the inhibition of S-warfarin 7-hydroxylation by R-10-hydroxywarfarin diastereomers prior to and following rifampin treatment. Ki values were predicted based on IC50 estimates determined from HLM.

Compound	fu	[I] (μM)	Ki (μM)	1 + [I]/Ki
Warfarin alone				
9R,10R-hydroxywarfarin	0.010	0.61 ± 0.27	25.3 ± 3.6	1.02
9R,10S-hydroxywarfarin			3.7 ± 0.5	1.16
In the presence of rifampin				
9R,10R-hydroxywarfarin	0.010	0.85 ± 0.62	25.3 ± 3.6	1.03
9R,10S-hydroxywarfarin			3.7 ± 0.5	1.23