Stereospecific Metabolism of *R*- and *S*-Warfarin by Human Hepatic Cytosolic Reductases

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Received March 12, 2017; accepted June 6, 2017

ABSTRACT

Coumadin (rac-warfarin) is the most commonly used anticoagulant in the world; however, its clinical use is often challenging because of its narrow therapeutic range and interindividual variations in response. A critical contributor to the uncertainty is variability in warfarin metabolism, which includes mostly oxidative but also reductive pathways. Reduction of each warfarin enantiomer yields two warfarin alcohol isomers, and the corresponding four alcohols retain varying levels of anticoagulant activity. Studies on the kinetics of warfarin reduction have often lacked resolution of parent-drug enantiomers and have suffered from coelution of pairs of alcohol metabolites; thus, those studies have not established the importance of individual stereospecific reductive pathways. We report the first steady-state analysis of *R*- and *S*-warfarin reduction in vitro by pooled human liver cytosol. As determined by authentic standards,

the major metabolites were 9*R*,11*S*-warfarin alcohol for *R*-warfarin and 9*S*,11*S*-warfarin alcohol for *S*-warfarin. *R*-warfarin ($V_{\max}$ 150 pmol/mg per minute, K_m 0.67 mM) was reduced more efficiently than *S*-warfarin ($V_{\max}$ 27 pmol/mg per minute, K_m 1.7 mM). Based on inhibitor phenotyping, carbonyl reductase-1 dominated *R*- and *S*-warfarin reduction, followed by aldo-keto reductase-1C3 and then other members of that family. Overall, the carbonyl at position 11 undergoes stereospecific reduction by multiple enzymes to form the *S* alcohol for both drug enantiomers, yet *R*-warfarin undergoes reduction preferentially. This knowledge will aid in assessing the relative importance of reductive pathways for *R*- and *S*-warfarin and factors influencing levels of pharmacologically active parent drugs and metabolites, thus impacting patient dose responses.

Introduction

Coumadin is a commonly used blood thinner worldwide that consists of a 1:1 mixture of *R*- and *S*-warfarin (Fig. 1) (Ansell et al., 2008). The anticoagulant effect of warfarin reflects inhibition of *VKORC*, which plays a critical role in vitamin K cycling and the maturation of coagulation factors. Although quite efficacious, treating patients optimally with warfarin can be problematic because of warfarin's narrow

therapeutic range and the interindividual variations in response. Doses must be individually established for each patient based on how that patient responds to the medication. Failure to achieve and maintain an optimal dose for patients increases risks for potentially life-threatening adverse events caused by underdosing (strokes) and overdosing (bleeding) (Wysowski et al., 2007). Knowledge of the causes of this variability in dose responses can then lead to better management strategies.

Interindividual variations in patient dose responses to warfarin often reflect the importance of drug metabolism (Jones and Miller, 2011). Cytochromes P450 oxidize *R*- and *S*-warfarin into hydroxywarfarins, and as major routes for drug clearance, those metabolic pathways have dominated the focus of most studies. Through minor, less well characterized pathways, both drugs also undergo reduction at the carbonyl at position 11 to yield warfarin alcohols. Warfarin reduction introduces a second chiral center, making two warfarin alcohol diastereomers for each warfarin enantiomer. For *R*-warfarin, the two metabolites are 9*R*,11*R*-, and 9*R*,11*S*-warfarin alcohol (*RR*- and *RS*-warfarin alcohol respectively), and for *S*-warfarin, they are 9*S*,11*S*- and 9*S*,11*R*- warfarin alcohol (*SS*- and *SR*-warfarin alcohol respectively). Warfarin alcohols retain pharmacologic activity that is 6-fold lower than that for the parent drugs (Gebauer, 2007); however, clearance of *RS*-warfarin alcohol is 2.5-fold lower than the other alcohols, indicating selective accumulation of the metabolite in plasma based on

Financial support for undergraduate research was provided in part by the Summer Undergraduate Research Fellowship program sponsored by the Biochemistry and Molecular Biology Department at the University of Arkansas for Medical Sciences (UAMS) (to B.P.J. and D.L.P.), and the UAMS Summer Undergraduate Research Program to Increase Diversity in Research, National Institutes of Health (NIH) National Heart, Lung and Blood Institute award [Grant R25HL108825] to D.L.P. In addition, financial support was provided to GPM through a grant-in-aid from the SouthWest Affiliate of the American Heart Association [Grant 13GRNT16960043] and a pilot study grant provided by the NIH [Grant UL1 TR000039] awarded to the UAMS along with grants from the NIH National Library of Medicine [Grants R01LM012222 and R01LM012482]. The content of this manuscript is solely the responsibility of the authors and does not necessarily represent the official views of the UAMS, the American Heart Association, or NIH. Funding was provided by the U.S. National Science Foundation for the nuclear magnetic resonance spectrometer [Grant 1040470].

<https://doi.org/10.1124/dmd.117.075929>.

ABBREVIATIONS: AKR, aldo-keto reductase; AKRIC, aldo-keto reductase; CBR1, carbonyl reductase 1; DMSO, dimethylsulfoxide; HLC, human liver cytosol; HPLC, high-performance liquid chromatography; INDO, indomethacin; NMR, nuclear magnetic resonance; QUE, quercetin; TLC, thin-layer chromatography.

Fig. 1. Reduction of *R*- and *S*- warfarin introduces a second chiral center to the molecule. The molecular structure of warfarin contains a single chiral center (letter designation in blue italics). The reduction of *R*- and *S*- warfarin introduces a second chiral center to yield a warfarin alcohol so that there are four possible metabolites.

stereochemistry (Lewis et al., 1973), which increases their relative potency during maintenance dosing (Haque et al., 2014). According to a previous report (Haque et al., 2014), warfarin alcohols are the most potent of the warfarin metabolites, contributing 15% of the anticoagulant effect of *S*-warfarin for the average patient at a maintenance dosing. Consequently, knowledge of the impact of stereochemistry on *R*- and *S*-warfarin reduction is critical for adequately assessing the clinical relevance of those pathways.

Recent studies using subcellular fractions from rats (Alshogran et al., 2015a) and humans (Malátková et al., 2016) have provided limited insights on reductive pathways for warfarin because of their reliance on rac-warfarin and not the actual drug enantiomers as a substrate and the subsequent lack of chiral resolution of metabolites from the reaction. First, the drug is administered as a 1:1 mixture of enantiomers, but those conditions do not reflect patient serum levels and mask actual clearance of individual warfarin enantiomers. Reported *R*-warfarin levels in patients are on average 2-fold higher than *S*-warfarin (Breckenridge et al., 1974; Jones et al., 2010; Haque et al., 2014). Knowledge of the metabolic kinetics for individual drug enantiomers would provide perspective for assessing their relative significance in drug clearance.

Moreover, the use of a mixture of substrates in those studies masks possible drug-drug interactions between the enantiomers, which were implicated in one of the studies (Malátková et al., 2016). Second, it is not possible to extrapolate the metabolic kinetics for *R*- and *S*-warfarin from those for rac-warfarin so that knowledge of the impact of stereochemistry on metabolic clearance of the drugs remains unknown. Third, current studies on warfarin reduction failed to resolve all four diastereomers generated during rac-warfarin reduction. Instead, reported kinetic parameters reflect the formation of mixtures of metabolites, i.e., alcohol 1 (*RR*- and *SS*-warfarin alcohols) and alcohol 2 (*RS*- and *SR*-warfarin alcohols), generated during rac-warfarin metabolism and hence provide no information on the kinetics for individual pathways leading to specific metabolites. Taken together, the current findings provide only a qualitative perspective on warfarin reduction and lack specific quantitative details on the individual pathways that would determine their potential clinical relevance.

Herein, we report the impact of stereochemistry on the efficiency of rac-, *R*-, and *S*- warfarin reduction by pooled human liver cytosolic (HLC150) cell fractions. Because of the lack of commercially available standards, we synthesized and purified all four diastereomeric warfarin

Fig. 2. Control experiments to assess optimal conditions for steady-state studies. (A) Impact of buffer on activity. Total warfarin alcohol formation rates over 30 minutes measured for 500 μM warfarin, 1 mM NADPH, and 0.5 mg/ml protein reactions in 50 mM potassium phosphate, pH 7.4, with or without 5 mM MgCl₂ or 50 mM Tris hydrochloride pH 7.4 (Tris-HCl), with or without 5 mM MgCl₂. (B) Impact of cosolvent on activity. Total warfarin alcohol formation rates measured for reactions with 50 μM warfarin, 1 mM NADPH, and 0.5 mg/ml protein at 0.5%, 1.0%, 2.0%, or 5.0% methanol or dimethylsulfoxide (DMSO) cosolvent in 50 mM potassium phosphate, pH 7.4. (C) Impact of protein levels on activity. After 40-minute reaction, total warfarin alcohol levels measured for 50 μM warfarin and 1 mM NADPH reactions with 0.25, 0.5, and 1.0 mg protein/ml pooled human liver cytosol in 50 mM potassium phosphate, pH 7.4. (D) Impact of time on activity. Total warfarin alcohol levels measured for reactions of 50 μM warfarin at 10, 20, 40, and 60 minutes after initiation with NADPH in 50 mM potassium phosphate, pH 7.4.

alcohols using green chemistry techniques. We then optimized reaction conditions to achieve steady-state conditions and carried out kinetic studies with *R*- and *S*-warfarin along with rac-warfarin for comparison with findings from other studies. We assessed potential interactions between the drug enantiomers by simulating rac-warfarin metabolism using parameters for the individual drugs and compared them with those measured for rac-warfarin in our study and kinetic values reported in the literature that used a limited pool of cytosol fraction from five cadavers (Malátková et al., 2016). Lastly, we used inhibitor phenotyping to identify possible reductases responsible for warfarin metabolism in cytosolic fractions.

Materials and Methods

Materials. High-performance liquid chromatography (HPLC)-grade methanol, acetic acid, dimethylsulfoxide (DMSO), and NADPH were purchased from Thermo Fisher (Pittsburgh, PA), and pooled human liver cytosol (HLC150) was from BD Biosciences (Franklin Lakes, NJ). Diethyl ether, hydrochloric acid, ethyl acetate, acetone, and acetic acid were obtained from PHARMCO-AAPER (Brookfield, CT). Celite, sodium borohydride, and DMSO- d_6 was purchased from Sigma-Aldrich (St. Louis, MO). Sorbtech Silica XHL TLC plates were purchased from Sorbtech (Norcross, GA). Rac-warfarin, warfarin-alcohol mixed isomers, and hydroxywarfarins (internal standards) were obtained from Toronto Research Chemicals (Toronto, Canada). Individual *R*- and *S*-warfarin stereoisomers were prepared in a previous study (Pugh et al., 2014); the metabolites *9R,11R*-, *9R,11S*-, *9S,11R*-, and *9S,11S*-warfarin alcohols were synthesized and purified to >98% purity.

Synthesis of Warfarin Alcohols through Green Chemistry. We prepared authentic isomers of all four diastereomeric warfarin alcohols by synthesizing *R*- and *S*-warfarin and then reducing them individually, followed by isolating the individual alcohols by thin-layer chromatography and cycles of recrystallization. First, *R*- and *S*-warfarin were prepared with >99% enantiomeric excess using a green chemistry procedure as we (Pugh et al., 2014) and others (Kim et al., 2006; Wong et al., 2010) have described. Second, reduction of *R*- and *S*-warfarin to warfarin alcohols followed a procedure from the literature (Trager et al., 1970; Chan et al., 1972). The *R*- or *S*-warfarin (50 mg, 1.62 mmol) was stirred in 1 ml of deionized water with sodium borohydride (7 mg, 1.85 mmol) at the ambient temperature for 2 hours. Excess sodium borohydride was neutralized by the addition of 1 ml of 0.5 M hydrochloric acid added dropwise with stirring. The mixture was filtered and washed with water. The resulting white solid was allowed to air-dry. The reduction step for each warfarin enantiomer introduced a new chiral center resulting in a pair of diastereomers (i.e., *RS* and *RR* warfarin alcohols for *R*-warfarin and the *SS*- and *SR*-warfarin alcohols for *S*-warfarin). The typical yield of each pair of diastereomers was >70%.

Isolation of individual alcohol diastereomers was achieved through silica gel thin-layer chromatography (TLC), followed by recrystallization. For example, a diastereomer mixture of two warfarin alcohols (either *RS* and *RR*, or *SR* and *SS*) was dissolved in the smallest amount of ethyl acetate and dropped onto a Sorbtech Silica XHL TLC plate W/UV254 (glass-backed, 250- μ m thickness, 20 $\times$ 20 cm dimensions). A solvent mix of 197:3 diethyl ether:acetic acid was used to effect a separation. The bottom bands of each separation were the *RR*- and *SS*-warfarin alcohols, while the top bands were the *RS*- and *SR*-warfarin alcohols. TLC identity of the aforementioned diastereomers was based on the previous research by others (Chan et al., 1972). Each band was carefully scraped from the glass TLC plate, and ethyl acetate was added to dissolve the alcohols. After stirring, the mixture was filtered over Celite, and ethyl acetate was removed by rotary evaporation. The white solid was dissolved in the smallest amount of warm acetone, and then drops of warm water were added slowly until the solution remained cloudy. At this point, the solution was heated again until clear and allowed to cool at the ambient temperature until crystals appeared, at which point the solution was cooled in ice. When crystallization ceased, the supernatant liquid was removed by pipet and the solid allowed to air-dry at ambient temperature.

Nuclear magnetic resonance (NMR) peak assignments were made using ^1H , ^{13}C , COSY, DEPT, HMQC, and HMBC techniques. *RR*-warfarin alcohol: ^1H NMR (DMSO- d_6 , 400 MHz): δ 1.21 (3H, d, J = 6.4 Hz, H-14), 2.40 (2H, m, H-12a, H-12b), 3.66 (1H, ddq, J = 6.0, 6.4, 6.4 Hz, H-13), 4.62 (1H, dd, J = 7.8, 8.2 Hz, H-11), 7.13 (1H, dd, J = 7.3, 7.3 Hz, H-4'), 7.24 (2H, dd, J = 7.3, 7.3 Hz, H-3', H-3''), 7.28 (1H, dd, J = 0.9, 8.1 Hz, H-8), 7.33 (1H, ddd, J = 0.9, 7.4,

7.8 Hz, H-6), 7.46 (2H, d, J = 7.3 Hz, H-2', H-2''), 7.56 (1H, ddd, J = 1.4, 7.4, 8.1 Hz, H-7), 7.93 (1H, dd, J = 1.4, 7.8 Hz, H-5); ^{13}C NMR (DMSO- d_6 , 400 MHz): δ 23.9 (C-14), 38.5 (C-11), 41.6 (C-12), 67.2 (C-13), 110.2 (C-3), 117.4 (C-8), 117.8 (C-10), 124.1 (C-5), 125.1 (C-6), 127.2 (C-4'), 129 (C-3'), 129.3 (C-2'), 132.9 (C-7), 144.2 (C-1'), 153.8 (C-9), 162.7 (C-4), 165.1 (C-2); *RS*-warfarin alcohol: ^1H NMR (DMSO- d_6 , 400 MHz): δ 1.22 (3H, d, J = 6.4 Hz, H-14), 2.09 (1H, ddd, J = 4.8, 9.4, 14.0 Hz, H-12b), 2.60 (1H, ddd, J = 3.2, 11.1, 14.0 Hz, H-12a), 3.66 (1H, m, H-13), 4.67 (1H, dd, J = 4.8, 11.1 Hz, H-11), 7.12 (1H, dd, J = 7.3 Hz, H-4'), 7.22 (2H, dd, J = 7.3, 7.8 Hz, H-3'), 7.27 (1H, d, J = 7.9 Hz, H-8), 7.32 (1H, dd, J = 7.9, 8.0 Hz, H-6), 7.39 (2H, d, J = 7.8 Hz, H-2'), 7.55 (1H, ddd, J = 1.4, 7.9, 7.9 Hz, H-7), 7.95 (1H, dd, J = 1.4, 8.0 Hz, H-5); ^{13}C NMR (DMSO- d_6 , 400 MHz): δ 24.1 (C-14), 38.3 (C-11), 41 (C-12), 67.1 (C-13), 108.7 (C-3), 117.3 (C-8), 118 (C-10), 124.4 (C-5), 125.1 (C-6), 127 (C-4'), 128.9 (C-3'), 129 (C-2'), 132.9 (C-7), 144.7 (C-1'), 154 (C-9), 164 (C-4), and 164.9 (C-2).

Identification of Initial Rate Conditions for Warfarin Reduction. Before steady-state studies, control experiments with HLC150 were carried out to determine optimal cosolvents, reaction buffers, reaction time, and protein concentration to ensure steady-state conditions and avoid confounding effects from experimental reagents. Warfarin stocks were prepared in methanol solutions to minimize cosolvent effects on reductase activity, as shown in our control experiments (see *Results*). Individual warfarin reactions were prepared with

Fig. 3. Kinetic profiles for steady-state reduction of *R*- and *S*-warfarin by HLC. Reduction of *R*- and *S*-warfarin as described in *Materials and Methods* yielded two alcohols for each set of reactions. (A) Kinetic profile for *R*-warfarin reduction showing the major (*RS*-warfarin alcohol, dark blue circle) and minor (*RR*-warfarin alcohol, light blue circle) metabolites. Both sets of data were fit to the Michaelis-Menten model and the resulting kinetic constants reported in Table 1. (B) Expanded view of kinetic profile for *R*-warfarin reduction to *RR*-warfarin alcohol and its corresponding fit of the data. (C) Kinetic profile for *S*-warfarin reduction showing the major metabolites (*SS*-warfarin alcohol, dark red circle). *SR*-warfarin alcohol was not quantifiable because of signal at the limit of detection. Data for *SS*-warfarin alcohol were fit to Michaelis-Menten model, and resulting kinetic constants are reported in Table 1.

TABLE 1
Steady-state kinetic constants for *R*-, *S*-, and rac-warfarin reduction by human liver cytosol

Substrate	Metabolite	Best-Fit Model ^a	$V_{\max}$ (pmol/min/mg protein)	K_m (μ M)	Efficiency ($V_{\max}/K_m$)
<i>R</i> -warfarin	<i>RS</i> -warfarin alcohol	Michaelis-Menten	155 $\pm$ 18	710 $\pm$ 160	0.22
	<i>RR</i> -warfarin alcohol	Michaelis-Menten	0.63 $\pm$ 0.18	560 $\pm$ 330	0.0011
<i>S</i> -warfarin	<i>SR</i> -warfarin alcohol	NA ^b	NA ^b	NA ^b	NA ^b
	<i>SS</i> -warfarin alcohol	Michaelis-Menten	41 $\pm$ 18	3500 ^c	0.012
Rac-warfarin	Alcohol 2 (<i>RS</i> / <i>SR</i> -warfarin alcohols)	Michaelis-Menten	78 $\pm$ 10	710 $\pm$ 170	0.11
	Alcohol 1 (<i>RR</i> / <i>SS</i> -warfarin alcohols)	Michaelis-Menten	4.8 $\pm$ 0.7	330 $\pm$ 130	0.015
Rac-warfarin (Malátková et al., 2016)	Alcohol 2 (<i>RS</i> / <i>SR</i> -warfarin alcohols)	Michaelis-Menten	76.4 $\pm$ 6.0	746 $\pm$ 93	0.10
	Alcohol 1 (<i>RR</i> / <i>SS</i> -warfarin alcohols)	Michaelis-Menten	2.0 $\pm$ 0.2	248 $\pm$ 51	0.0080

NA, not applicable.

^aBest fit of data was determined between hyperbolic (Michaelis-Menten) and nonhyperbolic (Hill equation) models using extra sum-of-squares *F* test in GraphPad Prism.

^b*SR*-warfarin alcohol was consistently quantifiable only at *S*-warfarin concentrations of 600 μ M or greater, yet data could not be reliably fit to any kinetic model.

^cThe predicted K_m was greater than the highest substrate concentration used in this study (1000 μ M) owing to solubility limits; thus, there is more error in the kinetic constants than predicted by a fit, hence the exclusion of S.E. from the table.

0.25 mg/ml pooled HLC150 from 150 donors and initiated on the addition of 1 mM NADPH (final). Reactions were incubated at 37°C with 350 rpm rotation. After 30 minutes, reactions were quenched with an equal volume of acetonitrile containing 1 μ M rac-8-hydroxywarfarin (final), an internal standard, which did not coelute with warfarin or the warfarin alcohols. Quenched reactions were centrifuged at 5°C at 2500 rpm for 15 minutes, and the supernatant was removed for analysis by HPLC. Unless otherwise stated, all control reactions were carried out using rac-warfarin and experimental effects monitored by measuring total product formation by combining areas of alcohols 1 and 2 to take all four alcohol diastereomers into account. All control reactions were carried out in duplicate.

Analysis of Warfarin Reduction by HPLC. The resolution and quantitation of individual drugs and metabolites were achieved through HPLC. Samples were injected onto a Waters HPLC Breeze system equipped with a 4.6 $\times$ 150-mm Zorbax Eclipse 3.5 μ M XDB-C18 column (Agilent, Santa Clara, CA) heated to 45°C, and analytes were resolved over 13 minutes using a gradient method with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)/NaOH solution, pH 6.80 and 25:75 acetonitrile:methanol and monitored by fluorescence (excitation: 325 nm; emission: 393 nm). Peak areas were normalized to the internal standard and then quantitated relative to known standards. Reaction rates were reported as total product formed per reaction time per amount of protein (pmol/min per milligram of protein).

Steady-State Kinetics for *R*-, and *S*-, and Rac-Warfarin Reduction. After optimizing reaction conditions, we determined reaction kinetics for *R*-, and *S*-, and rac-warfarin. Substrate concentrations were 1000, 500, 250, 200, 125, 100, 50, and 25 μ M initially in 50 mM potassium phosphate, pH 7.4. *S*-warfarin reactions contained an additional reaction with 750 μ M substrate. Reactions were performed in at least triplicate for each substrate concentration. Reaction products were separated and analyzed by HPLC and fluorescence detection as described previously. Alcohol peaks 1 and 2 or individual alcohol diastereomers were quantitated and assessed individually. Warfarin alcohol formation rates were plotted as a function of substrate concentration and analyzed by comparing the fit of the data to the Michaelis-Menten equation (hyperbolic curve) and Hill equation (nonhyperbolic curve) using in GraphPad Prism (San Diego, CA). The best fit was determined using extra sum-of-squares *F* test.

Inhibitor Phenotyping of *R*- and *S*-Warfarin Reduction. HLC150 phenotyping reactions were carried out using the steady-state conditions established in the control experiments (see *Results*). We prepared all inhibitor stocks in methanol and assessed their effects against a control reaction containing just methanol at the same final percent (5% methanol). *R*- or *S*-warfarin concentrations at 50 and 500 μ M were used in the presence of three different inhibitors to selectively inhibit individual and classes of cytosolic reductases (as reviewed in Malátková and Wsól, 2014). Moreover, with broadly specific inhibitors, we used concentrations about 5-fold above the reported IC_{50} to ensure target inhibition while minimizing off-target effects. We used 2 μ M flufenamic acid, which inhibits all aldo-keto reductase family (AKR) 1C (AKR1C) members. This concentration has also been shown to inhibit AKR1B10 activity (Zhang et al., 2013); however, levels are typically too low to be relevant for our studies, and induction was observed only in liver cancer (Laffin and Petrash, 2012). For indomethacin (INDO), a high concentration (180 μ M) would similarly inhibit all

AKR1C members and carbonyl reductase 1 (CBR1); a lower concentration (5 μ M) has a more selective effect on AKR1C3 with only limited CBR1 inhibition expected. We targeted CBR1 activity for inhibition using 60 μ M quercetin (QUE). In addition, we combined inhibitors to assess the collective contributions of AKR1C and CBR1 enzymes. AKR1B1 is also inhibited by INDO and QUE (Byrns and Penning, 2009), but this enzyme is expressed only at minimal levels in the liver, except in cases of alcoholic liver disease (O'Connor et al., 1999). Thus, we did not expect it to contribute significantly to metabolism in our study.

Results

HLC150 Steady-State Kinetics for *R*-, *S*-, and Rac-Warfarin Reduction. Initially, we carried out control experiments to identify optimal conditions for steady-state studies, as shown in Fig. 2. Warfarin

Fig. 4. Kinetic profile for steady-state reduction of rac-warfarin by human liver cytosol. Reduction of rac-warfarin, as described in *Materials and Methods*, yielded unresolved mixtures of metabolites. (A) Kinetic profile for alcohol 1 (*RR*- and *SS*-warfarin alcohol, light purple circle) and alcohol 2 (*RS*- and *SR*-warfarin alcohols, dark purple circle). (B) Expanded view of kinetic profile for rac-warfarin reduction to alcohol 1 and its corresponding fit of the data. Both sets of data were fit to the Michaelis-Menten model, and the resulting kinetic constants are reported in Table 1.

Fig. 5. Inhibitor phenotyping of steady-state reduction of *R*- and *S*-warfarin by HLC150. Reactions were carried out with two substrate concentrations, 50 μ M (A and B) and 500 μ M (C and D), 0.25 mg/ml protein, and 1 mM NADPH with inhibitors for specific cytosol reductases at 37°C and pH 7.4. (A and C) depict selective inhibition of *R*-warfarin to *RS*-warfarin alcohol. (B and D) show selective inhibition of *S*-warfarin to *SS*-warfarin alcohol. Reaction activities were normalized to the average control rate measured for reactions in the absence of inhibition. Rates were reported as percentages of the control rate. The reported activities are averaged from a minimum of five individual experiments $\pm$ S.E. Asterisks indicate statistical significance of inhibition compared with control as calculated using Student's *t* test: **P* < 0.05; ***P* < 0.005; ****P* < 0.0005).

reduction was more efficient in potassium phosphate compared with Tris-HCl buffer, and MgCl₂ had no impact on reaction rates (Fig. 2A). Variations in methanol cosolvent levels had no effect on activity, whereas higher DMSO levels inhibited the reaction (Fig. 2B). Observed warfarin alcohol levels increased essentially linearly with total protein concentration (Fig. 2C). Over the course of 60 minutes, product formation levels were essentially linear (Fig. 2D). Based on these studies, the standard conditions for a 30-minute reaction with 0.25 mg/ml protein was 50 mM potassium phosphate 7.4, no MgCl₂, and 1% methanol as a cosolvent.

Stereospecificity played a significant role in the steady-state reduction of warfarin enantiomers and corresponding products from cytosolic reactions (Fig. 3; Table 1). The *R*-warfarin reactions yielded *RS*-warfarin alcohol as the major metabolite and *RR*-warfarin alcohol as the minor one. Both reactions were best fit to a simple Michaelis-Menten kinetic model as compared with a nonhyperbolic curve, described by the Hill equation. The metabolic efficiency for formation of the *RS*-warfarin alcohol was nearly 200-fold compared with the *RR*-warfarin alcohol, almost exclusively owing to the difference in the rate of the reaction. Similarly, the reduction of *S*-warfarin yielded the analogous metabolites (i.e., *SS*-warfarin alcohol and *SR*-warfarin alcohol). The kinetic profile for *SS*-warfarin alcohol was fit to the Michaelis-Menten model, yet the predicted *K_m* was greater than the highest substrate concentration used in

this study (1000 μ M) because of solubility limits. Thus, there is more error in the kinetic constants than predicted by a fit, and hence the exclusion of standard error from Table 1. The *SR*-warfarin alcohol was consistently measurable but remained at the limit of quantitation so that it was not possible to determine the kinetics for its formation. Taken together, these results show that the rate of *S*-warfarin conversion to *SS*-warfarin alcohol was about 9-fold lower than that for *R*-warfarin to *RS*-warfarin alcohol at 1000 μ M, indicating a strong preference for *R*-warfarin reduction and formation of *S* alcohols, regardless of substrate chirality.

In addition, we followed the experimental design reported by others (Alshogran et al., 2015a; Malátková et al., 2016) to carry out steady-state studies with rac-warfarin for comparative purposes. Unlike the previous experiments in this study, substrate concentration reflected an initial 1:1 mixture of *R*- and *S*-warfarin, and the corresponding diastereomeric metabolites were not resolved completely. We used the previously reported nomenclature (Alshogran et al., 2015a; Malátková et al., 2016) labeling the mixture of *RS*/*SR*-warfarin alcohols as warfarin alcohol 2 and the mixture of *RR*/*SS*-warfarin alcohols as warfarin alcohol 1. Both sets of data were fit to the Michaelis-Menten model indicating that the 8-fold difference in efficiency for the alcohol 2 reaction was due mainly to *V_{max}* and not *K_m* for this mixed metabolite assessment of activity (Fig. 4C; Table 1). The lack of resolution of the substrates or metabolites

Fig. 6. Assessment of potential interactions between *R*- and *S*-warfarin during metabolism. After experimentally determining rac-warfarin kinetics for alcohol 1 and 2 formation (light and dark purple circles, respectively), we predicted the apparent alcohol 1 (light purple circles) and 2 (dark purple circles) formation rates by combining our steady-state data for the reactions with the individual drug enantiomers (Fig. 3). The predicted versus experimentally measured alcohol 1 and 2 rates were plotted together for comparison. (A) Experimentally determined kinetic profile for rac-warfarin reaction (circles, solid lines), as well as that predicted by the metabolism of individual enantiomers (squares, dashed lines). (B) Expanded view of kinetic profile for rac-warfarin reduction to alcohol 1 and the predicted kinetic profile. Fit of the estimated data to Michaelis-Menten model is shown in dashed line for comparison with that for actual rac-warfarin reactions in solid line.

with this approach made it impossible to identify the actual major and minor metabolites for *R*- and *S*-warfarin without relying on our initial enantiospecific steady-state kinetic studies.

Inhibitor Phenotyping of Cytosolic Enzymes Responsible for *R*- and *S*-Warfarin Reduction. General and relatively specific reductase inhibitors indicated multiple enzymes likely contribute to overall reduction of *R*- and *S*-warfarin in the cytosol. The overall patterns of inhibition were similar in the formation of the corresponding major metabolites *RS*-warfarin alcohol and *SS*-warfarin alcohol (Fig. 5). The minor metabolites *RR*-warfarin alcohol and *SR*-warfarin alcohol were measured at levels too low for quantitative kinetics. Substrate concentrations had little effect on the patterns of inhibition for the compounds, with two exceptions. First, the effect of QUE inhibition was consistently significant but reduced for *S*-warfarin under low substrate conditions. Second, inhibition by low INDO concentration (5 μM) was reduced at lower concentrations for both warfarin enantiomers. By 180 μM INDO, all reduction rates were decreased >10-fold. These findings suggest a dominant role for AKR1C3 in the reactions and possible contributions from other AKR1C members and CBR1. QUE mainly inhibits CBR1, and, when present, reduction rates decreased by around 70%–85% for *RS*- and *SS*-warfarin alcohols. The addition of 5 μM INDO to the QUE reaction did not significantly impact the observed reduction rates; however, at 180 μM INDO, there was a further approximate 10% decrease in rates. Unlike INDO, flufenamic acid had little effect on reduction, despite both being previously shown to inhibit AKR1C family enzymes (Malátková and Wsól, 2014). This difference could be due to variability in AKR1C inhibition by flufenamic acid, depending on the substrate being metabolized, as shown in previous studies (Malátková and Wsól, 2014). Overall, these results suggest that AKR1C family enzymes and CBR1 participate in the reduction of *R*- and *S*-warfarin.

Discussion

Herein, we have demonstrated that stereospecificity played an important role in the efficiency of warfarin reduction and the

corresponding metabolites generated in the process. This metabolic pathway is low-affinity and high-capacity, although it is likely underutilized under in vivo conditions. These insights from the kinetics differ from previous reports relying on rac-warfarin and a failure to resolve parent drugs and their metabolites in the analyses. We replicated the findings of others and demonstrated how the experimental design confounds an accurate understanding of *R*- and *S*-warfarin reduction. Our subsequent phenotyping studies revealed the probable cytosolic reductases responsible for stereospecific metabolism of warfarin. Collectively, these findings provide a strong foundation for further studies, such as genotyping of reductases, to explore the clinical relevance of *R*- and *S*-warfarin in patients undergoing anticoagulant therapy.

Our data show that human cytosolic reductases more efficiently metabolized *R*-warfarin compared with *S*-warfarin and preferentially generate *S* alcohols in the process, which is consistent with previous studies involving both humans and animal models (Lewis et al., 1974; Hermans and Thijssen, 1992). According to our kinetics, warfarin reduction is a low-affinity but high-capacity metabolic pathway that is likely underutilized under in vivo conditions. Maintenance dosing to achieve a targeted therapeutic response results in low micromolar levels of *R*- and *S*-warfarin in plasma [1518–4500 nM as compiled in Haque et al. (2014)]. The amounts accessible for reduction are likely about 50-fold lower (mid-nanomolar range) as a result of high plasma protein binding (O'Reilly, 1986). A possible limitation of this estimation is the lack of consideration of warfarin accumulation in the liver. Studies with rats have shown liver warfarin levels are possibly 3-fold higher than that observed in plasma (Levy et al., 2003; Sun et al., 2015). The similarities in physiology between rats and humans suggest that warfarin accumulation in the liver possibly occurs in humans. Even taking this effect into consideration, in vivo levels of *R*- and *S*-warfarin would not approach saturation, so that their reductive elimination would present as essentially linear kinetics in patients.

Steady-state reduction of rac-warfarin by human liver cytosol yielded results similar to results reported by others (Moreland and Hewick,

1975; Alshogran et al., 2015b; Malátková et al., 2016), but we were able to provide a more in-depth analysis of the data given our kinetics for the individual drug enantiomers. Despite the use of different human liver cytosolic fractions, warfarin alcohol 2 was the major metabolite, and warfarin alcohol 1 was the minor metabolite. The $V_{\max}$ and K_m values for the alcohol 2 reactions were nearly identical in the studies, as shown in Table 1. The corresponding values for alcohol 1 were within 2-fold of one another; that difference could be due to error in the noise in data for the minor pathway. In our case, we can identify likely metabolites contributing to observed kinetic profiles for warfarin alcohols 1 and 2. The *RS*-warfarin alcohol is the primary contributor to the alcohol 2 and *SS*-warfarin alcohol to the alcohol 1. *SR*- and *RR*-warfarin alcohols would still contribute to observed mixtures of alcohols as metabolites of the reaction, such that the observed kinetics for rac-warfarin does not represent that for the individual warfarin enantiomers.

Based on the identification of the metabolites, we assessed potential interactions between the warfarin enantiomers that could occur when rac-warfarin served as a substrate. For comparative purposes, we predicted the apparent alcohol 1 and 2 formation rates for rac-warfarin if the *R*- and *S*-warfarin did not interact. Under those conditions, the observed rates would simply be the summation of the corresponding rates for metabolites contributing to alcohol 1 (*RR*- and *SS*-warfarin alcohols) and alcohol 2 (*RS*- and *SR*-warfarin alcohols) taken from our steady-state data for the reactions with the individual drug enantiomers. As shown in Fig. 6, the *predicted* formation rates for warfarin alcohols from rac-warfarin were calculated by summing individual warfarin alcohol rates from warfarin enantiomers, as described previously. The *predicted* kinetic profiles significantly differ from the *experimentally* measured values as shown by the dashed lines versus the solid lines. When both drug enantiomers are present, the apparent $V_{\max}$ and K_m are lower indicating *R*- and *S*-warfarin interact to impact their reduction to the corresponding alcohols, as has been suggested previously (Chan et al., 1972). Nevertheless, the quantitation of mixtures of the alcohol metabolites precludes the ability to identify the mechanism of interaction and thus understand its impact on major and minor pathways for *R*- and *S*-warfarin reduction. These findings further underscore the complications of relying on rac-warfarin metabolic kinetics to understand its relevance to human metabolism of the drug.

The inhibitor phenotyping indicated that AKR1C3 and CBR1 dominated reductive metabolism of *R*- and *S*-warfarin in the cytosol, whereas AKR1C1/2 may have made minor contributions. These major enzymes shared similar preference for *R*-warfarin over *S*-warfarin as a substrate and generated *S* alcohols regardless of substrate chirality. This stereospecificity for the cytosolic reductases results in the overall preference formation of the major *RS*-warfarin metabolite, followed by the minor *SS*-warfarin metabolite, with trace levels of the other warfarin alcohols. Carbonyl-reducing enzymes demonstrate stereospecificity that depends on substrate and the particular enzyme (Hoffmann and Maser, 2007). CBR1 substrates range in molecular mass from 207 g/mol for 4-methylnitrosamino-1-(3-pyridyl)-1-butanone (NNK) to 543 g/mol for doxorubicin (Malátková et al., 2010), which includes the mass of warfarin (308 g/mol). The enantiospecificity of these reactions has been reported only for NNK, in which there is a more than 90% preferential formation of the *S*-alcohol (Breyer-Pfaff et al., 2004), as observed for *R*- and *S*-warfarin reduction in this study. Nevertheless, stereospecificity does vary among individual members of the AKR1C family (Rižner and Penning, 2014).

As further evidence for their role in *R*- and *S*-warfarin metabolism, recombinant AKR1C3 and CBR1 readily catalyzed the reduction of rac-warfarin compared with AKR1B1, 1C1, 1C2, and 1C4 along with CBR3 (Malátková et al., 2016); however, the use of a mixture of *R*- and *S*-warfarin and lack of resolution of substrates and metabolites obscured

the stereospecificity of substrate recognition and metabolite formation by the reductases. When considered with respect to our observations, the importance and consequence of AKR1C3 and CBR1 in *R*- and *S*-warfarin become more clearly the cause for preferential metabolism of *R*-warfarin and formation of its *RS*-warfarin alcohol metabolite as major reductive pathways in humans.

Concluding Remarks. The kinetics for *R*- and *S*-warfarin metabolism by these reductases could impact clearance of the parent drugs and formation of pharmacologically active metabolites in patients undergoing anticoagulant therapy. For the average patient, *R*-warfarin will primarily undergo reduction compared with *S*-warfarin and thus impact its contributions to anticoagulation (Breckenridge et al., 1974), as well as the resulting less pharmacologically active warfarin alcohol metabolites (Lewis et al., 1973). The relevance of warfarin reduction on patient dose-response variability will depend on genetics, personal habits, and environmental factors that influence reductase activity (Kassner et al., 2008). Xenobiotics, including drugs and pollutants, interact with aryl hydrocarbon receptor to bind to xenobiotic response elements that regulate expression and activity of drug-metabolizing enzymes, including CBR1 (Lakhman et al., 2007). By contrast, AKR1C are likely regulated through antioxidant regulatory pathways (Burczynski et al., 1999) stimulated by habits such as smoking, which induces AKR1C3 levels 15-fold (Nagaraj et al., 2006). That effect could explain why smokers are resistant to warfarin anticoagulation by increasing drug metabolism. Similarly, genetic variants of AKR1C3 (Mindnich and Penning, 2009) and CBR1 (Malátková et al., 2010) would increase or decrease their roles in warfarin metabolism, and hence patient responses, so if shown to be relevant, the polymorphisms could be incorporated into current pharmacogenetic-based dosing algorithms (Shaw et al., 2015) to improve their predictability. Aside from those unexplored possibilities, the relative contribution of warfarin reduction to the more dominant oxidative pathways remains unknown, so that their collective effect on *R*- and *S*-warfarin metabolism requires further investigation. In sum, our knowledge of the stereospecificity and efficiency of warfarin reduction provides a critical advance in understanding the overall relevance of metabolism that could impact patient dose responses.

Acknowledgments

T.E.G. gratefully acknowledges funding from the Hendrix College Odyssey and Rwanda Presidential Scholars Programs, as well as from John and Laura Byrd.

Authorship Contributions

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