Analysis of *R*- and *S*-Hydroxywarfarin Glucuronidation Catalyzed by Human Liver Microsomes and Recombinant UDP-Glucuronosyltransferases

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ABSTRACT

Coumadin (*R*-, *S*-warfarin) is a challenging drug to accurately dose, both initially and for maintenance, because of its narrow therapeutic range and wide interpatient variability and is typically administered as a racemic (Rac) mixture, which complicates the biotransformation pathways. The goal of the current work was to identify the human UDP-glucuronosyltransferases (UGTs) involved in the glucuronidation of the separated *R*- and *S*-enantiomers of 6-, 7-, and 8-hydroxywarfarin and the possible interactions between these enantiomers. The kinetic and inhibition constants for human recombinant 1A family UGTs toward these separated enantiomers have been assessed using high-performance liquid chromatography (HPLC)-UV-visible analysis, and product confirmations have been made using HPLC-mass spectrometry/mass spectrometry. We found that separated *R*- and *S*-enantiomers of 6-, 7-, and 8-hydroxywarfarin demonstrate significantly different glucuronidation

kinetics and can be mutually inhibitory. In some cases significant substrate inhibition was observed, as shown by K_m , V_{max} , and K_i comparisons. In particular, UGT1A1 and extrahepatic UGT1A10 have significantly higher capacities than other isoforms for *S*-7-hydroxywarfarin and *R*-7-hydroxywarfarin glucuronidation, respectively. Activity data generated using a set of well characterized human liver microsomes supported the recombinant enzyme data, suggesting an important (although not exclusive) role for UGT1A1 in glucuronidation of the main warfarin metabolites, including Rac-6- and 7-hydroxywarfarin and their *R*- and *S*-enantiomers in the liver. This is the first demonstration that the *R*- and *S*-enantiomers of hydroxywarfarins are glucuronidated, with significantly different enzymatic affinity and capacity, and supports the importance of UGT1A1 as the major hepatic isoform involved.

Introduction

Warfarin is a coumarin anticoagulant drug that is used worldwide to manage thromboembolic disease. Despite difficulties in effective patient management, warfarin remains one of the most commonly prescribed cardiovascular medica-

tions. Warfarin is primarily administered as an oral medication consisting of a racemic (Rac) mixture of enantiomers. In humans, the *S*-enantiomer exhibits approximately two to five times more anticoagulant activity than the *R*-enantiomer (Breckenridge, 1977). Variations in hepatic metabolism are thought to be a major determinant of warfarin-response variations, in part because warfarin is readily oxidized via hepatic cytochromes P450 (P450) to produce *R*- and *S*-enantiomers of 4'-, 6-, 7-, 8-, and 10-hydroxywarfarin. The *R*- and *S*-enantiomers are oxidized by multiple hepatic cytochromes P450 at five different positions to form 10 different hydroxylated metabolites, which can be conjugated by UGTs to form 10 different glucuronides. Recently, we have shown that ~30

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ABBREVIATIONS: Rac, racemic; UDP-GlcUA, UDP-glucuronic acid; UGT, UDP-glucuronosyltransferase; P450, cytochrome P450; HPLC, high-performance liquid chromatography; HLM, human liver microsomes; MS, mass spectrometry; LC-MS/MS, liquid chromatography-tandem mass spectrometry; K_i s, substrate inhibition kinetics; MRM, multiple reaction monitoring.

metabolites (including sulfates) of warfarin are generated in vivo and that glucuronidation of several Rac-hydroxywarfarins is catalyzed by several UGT isoforms localized in both the liver and intestine.

Glucuronidation activity of human liver microsomes (HLM) and human intestinal microsomes and eight human recombinant UGTs toward *R*- and *S*-warfarin, Rac-warfarin, and the major P450 metabolites of warfarin (4'-, 6-, 7-, 8-, and 10-hydroxywarfarin) has been assessed (Zielinska et al., 2008). This was the first study identifying and characterizing the specific human UGT isozymes that glucuronidate the major P450 metabolites of warfarin with metabolic rates similar to those of the parent compound. This was also the first demonstration of the significant role of the exclusively extrahepatic intestinal UGTs, UGT1A10 and UGT1A8, in the clearance of warfarin metabolites. However, glucuronidation of specific enantiomers has not been studied, and understanding these specific metabolic pathways will provide valuable insight into further characterization of warfarin detoxification and excretion pathways.

The primary goal of this study was to identify the human UGTs involved in the metabolism of *R*- and *S*-hydroxywarfarins and characterize their kinetic parameters. First, we investigated all available recombinant UGT1A isoforms for their ability to glucuronidate each hydroxywarfarin enantiomer and determined the variability in glucuronidation within a well characterized human liver bank in relation to UGT probe activities, isoform protein expression, genetic polymorphisms, and donor demographics. We also determined whether individual *R*- and *S*-enantiomers of hydroxywarfarins inhibit the glucuronidation of other hydroxywarfarins. These experiments have been compared with data already generated for Rac-hydroxywarfarin mixtures and are being used to provide insight into the mechanism of warfarin biotransformation. Second, we analyzed correlations between altered hydroxywarfarin metabolism and known UGT1A1 polymorphisms as well as demographic and environmental factors, including age, sex, and histories of smoking and alcohol consumption using a set of 53 human liver microsomes from donors with no liver disease. These data support recombinant enzyme data and identify factors that might be important in the glucuronidation of the main warfarin metabolites, including Rac-6- and 7-hydroxywarfarin and their *R*- and *S*-enantiomers. This knowledge could potentially constitute a basis for the development of safer approaches to warfarin treatment that could include the administration of enantiomerically pure drugs.

Materials and Methods

Materials. All chemicals used for this study were of at least reagent grade. 4'-Hydroxywarfarin, 6-hydroxywarfarin, 7-hydroxywarfarin, 8-hydroxywarfarin, UDP-glucuronic acid (UDP-GlcUA), and all other chemicals and reagents, unless otherwise specified, were purchased from Sigma-Aldrich (St. Louis, MO). Acetonitrile and formic acid were from Thermo Fisher Scientific (Waltham, MA).

Separation and Purification of *R*- and *S*-Hydroxywarfarin Enantiomers. Although the *R*- and *S*-enantiomers of warfarin are available commercially, the hydroxywarfarins are only available as Rac mixtures. Because the experiments presented here required enantiomerically pure compounds, a semipreparative high-performance liquid chromatography (HPLC)-based method for the separation and purification of enantiomers of warfarin and hy-

droxywarfarins was developed using a semipreparative Chirobiotic V chiral liquid chromatography column (Sigma-Aldrich). Two milligrams of each Rac mixture were dissolved in ethanol and applied to the column, and compounds were eluted using a 70:30 ratio of 0.1% acetic acid to methanol. Each separated enantiomer was collected, extracted from the mobile phase using ethyl acetate, dried under nitrogen, and dissolved in ethanol. Milligram quantities of pure enantiomers of each compound were purified for use as reagents and analytical standards using this method. Purity of each compound was assessed using previously described methods (Miller et al., 2009) to ensure that substrates were enantiomerically pure and free of other hydroxywarfarins.

Screening of Human Liver Microsomes and Recombinant UGT Isoforms. HLM from a single donor (50 μ g) or recombinant UGT membrane protein (5 μ g) were assayed for activity toward *R*- and *S*-hydroxywarfarins. The cloning and expression of UGT1A1, UGT1A3, UGT1A4, and UGT1A6 through UGT1A10 in baculovirus-infected Sf9 insect cells as His-tagged proteins and the preparation of enriched membrane fractions have been reported previously (Kurkela et al., 2003; Kuuranne et al., 2003). These preparations have been shown to contain similar amounts of protein by Western blot analysis using an anti-His antibody directed at the His tag on each of these recombinant isoforms. Each enzyme tested in this study is known to be active toward substrates specific for that isoform.

Protein was incubated in 100 μ M Tris-HCl, pH 7.4/5 mM MgCl₂/5 mM saccharolactone with 750 μ M substrate in a total volume of 30 μ l. Substrates were added in dimethyl sulfoxide (final concentration: 2%), and controls omitting substrate/cosubstrate were run with each assay. All incubations were performed in duplicate, and no additional detergent or other activators were used. Reactions were started by the addition of UDP-GlcUA (4 mM) and incubated for 90 min at 37°C. The rates of glucuronidation of these enzymes have been shown to be linear with time (up to 3 h) and protein concentration (data not shown). The reactions were stopped by the addition of 30 μ l of ethanol followed by centrifugation at 14,000 rpm for 8 min to pellet the protein. HPLC analyses of the supernatants were performed using an HP1050 HPLC system using the Agilent ChemStation software package (Agilent Technologies, Santa Clara, CA). Samples were separated using either a Supelcosil LC-18 (25 cm $\times$ 4.6 mm, 5 μ m) or a Supelco Astec Chirobiotic V (25 cm $\times$ 4.6 mm, 5 μ m) column at 37°C. The solvent system used with the LC-18 column consisted of 0.1% acetic acid in water (A) and methanol (B) at a flow rate 1 ml/min, with the following elution gradient: 100% A (5 min), linear gradient from 100% A to 100% B (5–25 min), 100% B (25–30 min). The column was re-equilibrated at initial conditions for 10 min between runs. For the chiral column, the solvent system was isocratic 0.1% acetic acid in water (80%) and acetonitrile (20%) at 0.8 ml/min. The elution of each warfarin metabolite was monitored with a UV-visible diode array detector at 313 nm. Primary standards for the glucuronidated monohydroxylated warfarin metabolites are not available; therefore, product concentrations were calculated using the external standard response for each hydroxywarfarin substrate. It has been shown previously that the addition of the glucuronic acid moiety does not alter the extinction coefficient from that of the unreacted substrate (Doerge et al., 2000).

Steady-State Enzyme Kinetics Assays. Kinetic parameters were determined by incubating recombinant UGT membrane protein (5 μ g) in the presence of varying concentrations of substrate (Rac mixture, or purified *R*- and *S*-enantiomers; 50–3000 μ M) at a fixed concentration of UDP-GlcUA (4 mM) for 90 min. All other conditions were identical to those of the screening experiments.

Curve-fitting and statistical analyses were conducted using GraphPad Prism version 4.0b (GraphPad Software, Inc., San Diego, CA). Kinetic constants were obtained by fitting experimental data to

the following kinetic models using the nonlinear regression (Curve Fit) function.

Michaelis-Menten equation for one-enzyme model is shown in eq. 1:

$$v = \frac{V_{\max} \times [S]}{K_m + [S]} \quad (1)$$

Uncompetitive substrate inhibition model, where K_i is the inhibition constant describing the reduction in rate, is shown in eq. 2:

$$v = \frac{V_{\max}}{1 + \frac{K_m}{[S]} + \frac{[S]}{K_i}} \quad (2)$$

Goodness of fit for each model to glucuronidation kinetic data was assessed from the standard error, 95% confidence intervals, and R^2 values. Kinetic curves were also analyzed as Eadie-Hofstee plots to support kinetic models. Kinetic constants were reported as the mean $\pm$ S.E. of the parameter estimated.

Steady-State Enzyme Inhibition Assays. Assays for determination of substrate inhibition kinetics (K_i s) were carried out by incubating a fixed concentration of UDP-GlcUA (4 mM) and increasing concentrations of "substrate" (S-7-hydroxywarfarin, S-7-hydroxywarfarin, or R-8-hydroxywarfarin; 50–1500 μ M) with five fixed concentrations of "inhibitor" (R-7-hydroxywarfarin, R-7-hydroxywarfarin, or S-8-hydroxywarfarin; 0, 100, 500, 1000, and 1500 μ M) and 5 μ g of UGT1A10 for 90 min at 37°C. Activities (velocity (v), nanomoles per milligram of protein per minute) were determined and K_i values were calculated from the resulting curves using Dynafit (BioKin, Ltd., Watertown, MA) (Kuzmic, 1996, 2006). The best fit of the data to traditional models of reversible inhibition mechanisms (competitive, uncompetitive, noncompetitive, or mixed) was determined. The inhibition parameters were used as the variables K_i and K_i' and the kinetic parameters $V_{\max}$ and K_m were constants based on values obtained from uninhibited reactions. The most probable mechanism was identified using the Akaike Information Criterion from model discrimination (Burnham and Anderson, 2002).

Liquid Chromatography-Tandem Mass Spectrometry Instrumentation and Conditions. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analyses for product confirmation were performed using an API-4000 Q TRAP tandem mass spectrometer (Applied Biosystems, Foster, CA) interfaced with an Agilent 1200 Series quaternary liquid chromatography system (Agilent Technologies). Analyst software (version 1.5; Applied Biosystems) was used to control the overall operation of the HPLC system and the mass spectrometer. Samples were loaded and resolved at a flow rate of 1 ml/min on a Agilent ZORBAX Eclipse 5 μ XDB-C18 (15 cm $\times$ 4.6 mm) column maintained at 40°C. Mobile phases were 0.1% acetic acid in water (A) and 0.1% acetic acid in methanol (B). Compounds of interest were eluted using the following gradient: 50% B (0 min), linear gradient from 50% B to 60% B (0–2 min), linear gradient from 60% B to 90% B (2–3 min), linear gradient from 90% B to 100% B (3–4.1 min), 100% B (4.1–7.9 min), linear gradient from 100% B to 50% B (7.9–8 min), and 50% B (8.0 min and after). Total run time, including a 2-min column pre-equilibration period, was 12 min. Injection volume was 5 μ l. All MS/MS analyses were performed in positive ion mode by electrospray ionization using a TurboIonSpray source. Curtain, ion 1, and ion 2 gases were 40, 50, and 65 psi gauge, respectively. Collisionally activated dissociation gas was

set to high. Turbo heater temperature was 510°C, and ion spray voltage was 5500 V. Specific MS/MS experimental conditions are noted in Table 1.

Correlation Studies Using Human Liver Microsomes. Liver samples from donors with no known liver disease were provided by either the Liver Tissue Procurement and Distribution System (University of Minnesota, Minneapolis, MN) or the National Disease Research Interchange (Philadelphia, PA) with the approval of the Tufts University Institutional Review Board. All liver samples were either intended for transplantation but had failed to tissue match, were normal tissue adjacent to surgical biopsies, or were autopsy specimens. Donors were primarily white ($n = 44$) but also included four African-Americans and two Hispanics. Additional demographic details about the donors, as well as the preparation of the HLM, have been described previously (Court, 2010).

Glucuronidation activities for Rac-6- and 7-hydroxywarfarin and their R- and S-enantiomers were measured in duplicate using the entire set of HLMs. Incubations (100- μ l total volume) were carried out at 37°C for 3 h in 50 mM phosphate buffer, pH 7.5, with 5 mM MgCl₂, 5 mM UDP-glucuronic acid, 100 μ M hydroxywarfarin, 5 μ g of alamethicin, and 25 μ g of microsomal protein. Incubations were terminated by the addition of 50 μ l of acetonitrile containing 10% acetic acid and 37.5 μ M warfarin (internal standard), vortexed, and placed on ice. Samples were centrifuged, and the supernatants were analyzed by an HPLC-MS system consisting of a Surveyor HPLC with Deca XP Plus ion trap detector and electrospray source operating in the positive ion mode (Thermo Fisher Scientific). Chromatographic separation was achieved using a 150 $\times$ 2 mm Synergi Fusion column (Phenomenex, Torrance, CA) with the mobile phase at 0.3 ml/min over an 11-min total run time. The mobile phases were 0.1% formic acid in water (A) with acetonitrile (B). Separation was achieved using the following gradient: 30% B (1 min), linear gradient from 30% B to 100% B (1–7 min), and linear gradient from 100% B to 30% B (7–8 min). The column was then re-equilibrated to initial conditions for 3 min between runs. A full ion scan from m/z of 100 to 1000 mass units was recorded, with monitoring of the parent ion (m/z) of 309 units for warfarin, 325 units for hydroxywarfarin, and 501 units for hydroxywarfarin glucuronide.

Data and Statistical Analysis. Statistical analyses of the correlation data were conducted using SigmaPlot (version 10; SPSS, Inc., Chicago, IL) with a p value of less than 0.05 considered statistically significant. Activity values for isoform-specific glucuronidation reactions and immunoblot-quantified UGT protein levels that had been previously determined for a range of UGT1A and UGT2B isoforms in the liver bank samples (Court, 2010) were correlated against the glucuronidation activities determined for each of the hydroxywarfarin compounds using the Spearman rank-order correlation test. Liver bank hydroxywarfarin glucuronidation activity data were also evaluated for effects of liver donor demographics and UGT1A1 genotype UGT1A1*28 (Girard et al., 2005). Effects of donor sex and histories of smoking and alcohol ingestion were evaluated using an unpaired t test, whereas one-way analysis of variance was used to test the influence of race and UGT genotype. For both the t test and one-way analysis of variance, it was necessary to log-transform data to ensure the normality of distribution.

TABLE 1
MS/MS experimental conditions for product ion and Multiple Reaction Monitoring (MRM) scans

Analyte		Q1	Q3	CE	CES	EP	DP	CXP
		m/z		V				
Product ion MRM	Hydroxywarfarin glucuronide	501	50–550	25	10	10	52	5–20 (2-s cycle)
	6-, 7-, and 8-Hydroxywarfarin	325	179	20	0	10	52	10
	6-, 7-, and 8-Hydroxywarfarin glucuronide	501	179	45	0	10	52	12

CE, collision energy; CES, collision energy spread; EP, entrance potential; DP, declustering potential; CXP, collision exit potential; Q1, quadrupole 1; Q3, quadrupole 3.

TABLE 2

Kinetic constants for selected recombinant UGT isoforms compared with substrates in Rac mixtures and as purified enantiomers

Glucuronidation activities of recombinant proteins were measured by incubating membrane fractions containing recombinant UGT1A1 and UGT1A10 (5 μ g) with increasing concentrations (shown in Fig. 2) of substrate at a constant concentration of UDP-GlcUA (4 mM). Curve fits and kinetic constants were determined using GraphPad Prism 4 software. Data were fit to the Michaelis-Menten model except where indicate with ^a. These data were fit to the uncompetitive substrate inhibition model. K_m and K_s values are presented in micromolars, and V_{max} values are presented in nanomoles per milligram per minute.

Enzyme	Substrate	Purified			In Racemic Mixture		
		K_m	V_{max}	K_s			K_s
UGT1A1	S-7-Hydroxywarfarin	230 $\pm$ 36	1.7 $\pm$ 0.09		150 $\pm$ 38	1.4 $\pm$ 0.1	
	R-7-Hydroxywarfarin	130 $\pm$ 30	1.9 $\pm$ 0.1		54 $\pm$ 13	1.4 $\pm$ 0.07	
UGT1A10	S-6-Hydroxywarfarin	360 $\pm$ 39	1.2 $\pm$ 0.04		250 $\pm$ 23	0.60 $\pm$ 0.02	
	R-6-Hydroxywarfarin	210 $\pm$ 38	0.67 $\pm$ 0.03		140 $\pm$ 25	0.65 $\pm$ 0.03	
	S-7-Hydroxywarfarin	160 $\pm$ 29	1.0 $\pm$ 0.05		150 $\pm$ 17	0.55 $\pm$ 0.02	
	R-7-Hydroxywarfarin	44 $\pm$ 8.4	0.04 $\pm$ 0.04		83 $\pm$ 9.8	0.042 $\pm$ 0.001	
	S-8-Hydroxywarfarin ^a	139 $\pm$ 27	28 $\pm$ 2.5	3300 $\pm$ 1200	317 $\pm$ 73	38 $\pm$ 5	1900 $\pm$ 810
	R-8-Hydroxywarfarin ^a	105 $\pm$ 33	7.2 $\pm$ 1.0	2600 $\pm$ 1500	85 $\pm$ 19	3.5 $\pm$ 0.4	930 $\pm$ 250

Results

Screening of Human Liver Microsomes and Recombinant UGT Isoforms for Activity toward Enantiomerically Pure Hydroxywarfarins

HLM and human recombinant UGT1A isoforms were screened using purified *R*- and *S*-enantiomers of 4', 6-, 7-, and 8-hydroxywarfarin as substrates. During these preliminary assessments, the screening studies were purposely designed at a high substrate concentration (750 μ M) to maximize the formation and identification of potential glucuronide conjugates (Table 2; Fig. 1). UGT1A1 and UGT1A10 showed activity toward all of the hydroxywarfarins assayed, with *R*- and *S*-7-hydroxywarfarin as the best substrates for UGT1A1 and *R*- and *S*-8-hydroxywarfarin as the best substrates for UGT1A10. Moderate activity was seen toward *S*-8-hydroxywarfarin for both UGT1A8 and UGT1A9. There was no noticeable reaction for any of the hydroxywarfarins with UGT1A4 or UGT1A6.

Human Liver Bank Analysis

A well characterized human liver bank was used to support the recombinant enzyme data and identify factors that might be important in glucuronidation of the main warfarin metabolites, including Rac-6- and 7-hydroxywarfarin as well as their *R*- and *S*-enantiomers. Correlation with specific UGT activity probes and corresponding im-

munoquantified protein levels (Tables 3 and 4) showed highest correlations with bilirubin glucuronidation (UGT1A1) and UGT1A1 protein content (Spearman correlation *R* values 0.6–0.7) for Rac-6-hydroxywarfarin and Rac-7-hydroxywarfarin as well. It is noteworthy that the *R*- and *S*-7-hydroxywarfarin glucuronidation activities did not show distinct correlations with any one UGT1A probe activity or protein content. All of the UGT2B activities showed very low correlation values with all of the hydroxywarfarins evaluated. Evaluation of effects of liver demographics (Tables 5 and 6) showed no effect of sex or smoking history. However, significant alcohol use (>14 drinks per week) was associated with significantly higher (50–100%) mean activities for both 6- and 7-hydroxywarfarin (Rac and enantiomeric) glucuronidation. Given the observed correlations with UGT1A1 activities, the effect of UGT1A1 polymorphisms was evaluated. As shown in Tables 5 and 6, UGT1A1*28 was not associated with significantly lower 6- or 7-hydroxywarfarin glucuronidation when considering the entire liver bank (*p* > 0.05). However, by excluding livers that had significant alcohol exposure, which may have obscured any genotype effect, we observed significantly lower (by 50–60%) mean Rac-7-hydroxywarfarin (*p* = 0.038) and *R*-7-hydroxywarfarin (*p* = 0.028) glucuronidation activities in the homozygous UGT1A1*28 livers versus livers with other genotypes. Although more than 50% lower mean Rac-6-hydroxywarfarin glucuroni-

Fig. 1. Sites of hydroxywarfarin glucuronidation catalyzed by human UGTs. Top, the combined structures of 8-, 7-, 6-, and 4'-hydroxywarfarin (OHWarfarin). Bottom, specific glucuronide products formed by the indicated UGT isoforms. Glucuronidation activities of UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, and UGT1A10 were measured using 750 μ M substrate, and membrane fractions of recombinant UGTs expressed as His-tagged proteins in baculovirus-infected Sf9 insect cells (5 μ g) were incubated together at 37°C for 90 min as described under *Materials and Methods*. Activities are expressed as follows: +, <0.5 nmol/mg protein/min; ++, 0.5 to 2.0 nmol/mg protein/min; +++, 2.0 to 10 nmol/mg protein/min; and +++++, >10 nmol/mg protein/min. No product formation was seen for UGTs 1A4 or 1A6, and human liver microsomes showed >0.5 nmol/mg protein/min for each of the substrates analyzed.

TABLE 3

Correlation of Rac-6-, *R*-6-, and *S*-6-hydroxywarfarin glucuronidation rates with UGT probe activities and immunoquantified protein measured in a bank of 50 human liver microsomes

UGT Isoform	Probe	Rac-6-hydroxywarfarin Glucuronidation		<i>R</i> -6-Hydroxywarfarin Glucuronidation		<i>S</i> -6-Hydroxywarfarin Glucuronidation	
		Spearman <i>R</i> Value	<i>p</i> Value	Spearman <i>R</i> Value	<i>p</i> Value	Spearman <i>R</i> Value	<i>p</i> Value
UGT1A1	Bilirubin	0.722	0.0000002	0.69	0.0000002	0.685	0.0000002
UGT1A4	Trifluoperazine	0.491	0.000324	0.56	0.0000269	0.475	0.000546
UGT1A6	Serotonin	0.592	0.00000842	0.597	0.00000668	0.508	0.000217
UGT1A9	Propofol	0.643	0.00000151	0.617	0.00000653	0.543	0.000132
UGT2B7	Zidovudine	−0.0926	0.521	−0.135	0.349	−0.0661	0.647
UGT2B15	<i>S</i> -Oxazepam	0.447	0.00121	0.364	0.00952	0.451	0.00108
UGT1A1	Protein	0.674	1.53×10^{-8}	0.668	1.34×10^{-7}	0.664	0.00000141
UGT1A6	Protein	0.422	0.00328	0.437	0.00224	0.325	0.0262
UGT1A9	Protein	0.353	0.0176	0.34	0.0224	0.232	0.124
UGT2B7	Protein	−0.275	0.0535	−0.282	0.0471	−0.196	0.171

TABLE 4

Correlation of Rac-7-, *R*-7-, and *S*-7-hydroxywarfarin glucuronidation rates with UGT probe activities and immunoquantified protein measured in a bank of 50 human liver microsomes

UGT Isoform	Probe	Rac-7-Hydroxywarfarin Glucuronidation		<i>R</i> -7-Hydroxywarfarin Glucuronidation		<i>S</i> -7-Hydroxywarfarin Glucuronidation	
		Spearman <i>R</i> Value	<i>p</i> Value	Spearman <i>R</i> Value	<i>p</i> Value	Spearman <i>R</i> Value	<i>p</i> Value
UGT1A1	Bilirubin	0.742	0.0000002	0.493	0.000304	0.488	0.000364
UGT1A4	Trifluoperazine	0.554	0.0000343	0.498	0.000265	0.429	0.00201
UGT1A6	Serotonin	0.611	0.00000332	0.435	0.0019	0.505	0.00024
UGT1A9	Propofol	0.607	0.0000104	0.472	0.00114	0.522	0.000262
UGT2B7	Zidovudine	−0.232	0.105	−0.175	0.223	−0.266	0.0617
UGT2B15	<i>S</i> -Oxazepam	0.231	0.107	0.112	0.437	0.175	0.223
UGT1A1	Protein	0.681	0.0000002	0.496	0.000577	0.46	0.00159
UGT1A6	Protein	0.405	0.00491	0.492	0.000496	0.338	0.0204
UGT1A9	Protein	0.353	0.0176	0.404	0.00612	0.228	0.131
UGT2B7	Protein	−0.43	0.00192	−0.28	0.0492	−0.412	0.00312

TABLE 5

Effects of liver donor demographics and UGT1A1 polymorphisms on Rac-6-, *R*-6-, and *S*-6-hydroxywarfarin glucuronidation activities measured in a bank of 50 human liver microsomes

		<i>n</i>	Rac-6-Hydroxywarfarin- Glucuronidation			<i>R</i> -6-Hydroxywarfarin- Glucuronidation			<i>S</i> -6-Hydroxywarfarin- Glucuronidation		
			Mean	S.D.	<i>p</i> Value	Mean	S.D.	<i>p</i> Value	Mean	S.D.	<i>p</i> Value
<i>pmol equivalents* · min^{−1} · mg protein^{−1}</i>											
Race/ethnicity	White	44	27.0	19.8	0.123	11.0	8.9	0.28	25.5	13.7	0.125
	Black	4	23.3	11.3		7.9	2.8		25.2	6.9	
	Hispanic	2	7.9	5.9		3.2	1.4		10.5	3.0	
Sex	Male	35	26.6	20.0	0.527	10.8	8.9	0.596	25.9	14.1	0.39
	Female	15	24.3	17.8		9.5	7.7		22.5	11.4	
Smoking	Yes	18	27.7	17.3	0.439	11.2	8.0	0.42	26.1	11.4	0.448
	No	27	26.2	21.9		10.8	9.4		24.9	15.3	
Alcohol (>14 per week)	Yes	13	38.3	18.4	0.003	16.9	9.6	0.001	33.5	12.8	0.004
	No	31	21.7	19.0		8.4	7.3		21.7	12.9	
UGT1A1*28	6/6	23	26.3	16.4	0.335	10.6	8.0	0.551	26.0	12.4	0.555
	6/7	16	29.4	24.6		11.8	10.5		26.2	16.4	
	7/7	6	18.7	20.4		7.4	7.6		20.1	13.0	
UGT1A1*28 (no alcohol)	6/6 or 6/7	28	23.8	19.4	0.085	8.9	7.4	0.807	22.9	12.7	0.986
	7/7	5	10.5	5.4		10.0	9.6		21.3	11.5	

* Using substrate standard curve.

dation activity was also observed in homozygous UGT1A1*28 livers without alcohol exposure, this difference did not reach statistical significance ($p = 0.085$). Other activities evaluated in the alcohol-free livers, including *R*-6-, *S*-6-, and *S*-7-hydroxywarfarin glucuronidation activities did not appear to vary by UGT1A1*28 genotype ($p > 0.05$).

Kinetic Analysis

Purified Enantiomers. Steady-state kinetic experiments were done with purified *R*- and *S*-enantiomers of 6-, 7-, and 8-hydroxywarfarin and UGT1A1 and UGT1A10 (Fig. 2A).

UGT1A10 exhibited higher K_m and V_{max} values for the *S*-enantiomers of 6- and 7-hydroxywarfarin compared with those of the corresponding *R*-enantiomers. Although the *S*-enantiomer of 8-hydroxywarfarin also had a higher V_{max} with UGT1A10 than that for *R*-8-hydroxywarfarin, the affinity of this enzyme for the two enantiomers was approximately equal. UGT1A1 showed similar V_{max} values for *R*- and *S*-7-hydroxywarfarin, with a slightly higher K_m value for the *S*-enantiomer.

Racemic Mixtures. Comparison of the kinetic values obtained from separated hydroxywarfarin enantiomers to those from analyses of 6-, 7-, and 8-hydroxywarfarin Rac mixtures

TABLE 6

Effects of liver donor demographics and UGT1A1 polymorphisms on Rac-7-, R-7-, and S-7-hydroxywarfarin glucuronidation activities measured in a bank of 50 human liver microsomes

		<i>n</i>	Rac-7-Hydroxywarfarin Glucuronidation			R-7-Hydroxywarfarin Glucuronidation			S-7-Hydroxywarfarin Glucuronidation		
			Mean	S.D.	<i>p</i> Value	Mean	S.D.	<i>p</i> Value	Mean	S.D.	<i>p</i> Value
<i>pmolequivalents* · min⁻¹ · mgprotein⁻¹</i>											
Race/ethnicity	White	44	78.6	67.9	0.205	12.0	10.8	0.653	8.8	7.4	0.16
	Black	4	49.4	37.6		6.0	2.625		5.7	2.7	
	Hispanic	2	22.4	21.8		15.1	13.9		2.0	1.2	
Sex	Male	35	75.6	69.5	0.738	11.4	10.6	0.708	8.6	7.5	0.569
	Female	15	70.3	57.9		12.0	10.5		7.5	6.6	
Smoking	Yes	18	80.2	60.5	0.453	11.3	10.7	0.719	8.6	6.2	0.605
	No	27	78.0	72.8		12.3	11.3		9.1	8.1	
Alcohol (>14 per week)	Yes	13	126.9	73.7	0.002	18.5	11.1	0.003	13.4	7.5	0.009
	No	31	59.0	55.5		9.5	9.9		7.2	6.6	
UGT1A1*28	6/6	23	79.4	63.0	0.165	11.4	10.4	0.91	7.1	6.3	0.236
	6/7	16	81.8	80.1		12.6	11.8		10.9	9.1	
	7/7	6	44.9	59.2		12.2	12.5		5.6	5.2	
UGT1A1*28 (no alcohol)	6/6 or 6/7	28	62.4	57.1	0.038	11.687	9.6	0.028	6.101	5.1	0.852
	7/7	5	21.6	17.9		6.054	8.6		7.318	9.4	

* Using substrate standard curve.

with UGT1A1 and UGT1A10 was also done (Fig. 2B). Racemic mixtures contain equal concentrations of each enantiomer; therefore, at each point, there is assumed to be an equal amount of the two enantiomers present in the reaction. Any decrease in the amount of glucuronide product relative to that of the purified compounds represents enzyme inhibition. For UGT1A10, the glucuronidation of S-6- and S-7-hydroxywarfarin was clearly inhibited (decrease in $V_{\max}$ of ~50%) by the corresponding R-enantiomer in the Rac mixture. However, no significant inhibition of R-enantiomer glucuronidation was seen. The activity of UGT1A10 toward S-8-hydroxywarfarin was not significantly affected by the presence of R-8-hydroxywarfarin in the Rac mixture, but R-8-hydroxywarfarin was significantly inhibited by the presence of S-8-hydroxywarfarin (decrease in $V_{\max}$ of ~65%). Glucuronidation of 7-hydroxywarfarin by UGT1A1 showed only minimal inhibition of glucuronidation in each case. The S-7-hydroxywarfarin was observed to inhibit the glucuronidation of R-7-hydroxywarfarin by UGT1A1 only at concentrations above 500 μ M and to decrease the observed $V_{\max}$ value by ~25%. A similar effect was seen for the inhibition of UGT1A1 glucuronidation of S-7-hydroxywarfarin by R-7-hydroxywarfarin, with a decrease in $V_{\max}$ of ~15%.

Inhibition Studies

K_i Values. Assays to establish K_i values were done to determine the specific effects of the inhibitor enantiomers on the glucuronidation of their corresponding substrate enantiomers. Three sets of experiments showed measurable inhibition of S-6-hydroxywarfarin by R-6-hydroxywarfarin, S-7-hydroxywarfarin by R-7-hydroxywarfarin, and R-8-hydroxywarfarin by S-8-hydroxywarfarin, which corresponds to those reactions where we observed a $\geq 50\%$ decrease in $V_{\max}$ values for products produced when the Rac mixtures of hydroxywarfarins were used (Fig. 2B). The three experiments each resulted in data that best fit the noncompetitive inhibition model, with K_i values of 400 ± 28 , 333 ± 25 , and 344 ± 35 μ M for the 6-hydroxywarfarins, 7-hydroxywarfarins, and 8-hydroxywarfarins, respectively (Table 7).

Product Confirmation and MS Spectral Interpretation. Products from assays of glucuronidation of the R- and S-enantiomers of 6- and 7-hydroxywarfarin by UGT1A1 and

UGT1A10 were analyzed by LC-MS/MS to confirm product formation (Fig. 3) and structure (Fig. 4). The presence of m/z 325 in product ion analyses suggests the loss of glucuronic acid, and identification of other fragment ions has been proposed.

The MS/MS spectra for R- and S-6-hydroxywarfarin glucuronides (Fig. 4, C and D) and S-7-hydroxywarfarin glucuronides generated in assays with UGT1A1 and UGT1A10 (Fig. 4, B and F) are virtually identical with regard to the masses of the fragment ions, although there are slight differences in relative abundances of the fragments. The primary fragment ion in the MS/MS spectrum appears at m/z 355, which corresponds to the loss of the side chain from the glucuronidated hydroxycoumarin skeleton. However, the MS/MS spectra of R-7-hydroxywarfarin glucuronide products of incubation with UGT1A1 and UGT1A10 (Fig. 4, A and E) were different from each other. The spectrum for the R-7-hydroxywarfarin glucuronide produced by UGT1A1 resembled those of R- and S-6-hydroxywarfarin and S-7-hydroxywarfarin glucuronides. The primary fragment ion of the R-7-hydroxywarfarin glucuronide produced by UGT1A10 was m/z 325 rather than 355 (Fig. 4E).

Discussion

This work represents the first study identifying and characterizing the specific human UGT isozymes responsible for the glucuronidation of the individual R- and S-enantiomers of the major hydroxylated P450 metabolites of warfarin. These are preliminary studies and are essential for understanding the fate of warfarin in the human body and determining how warfarin is fully detoxified and excreted.

Studies with recombinant UGT1A enzymes suggest that UGT1A1 may be an important UGT1A isoform mediating glucuronidation of a number of the hydroxywarfarin metabolites in human liver. This is supported by human liver bank data for the 6- and 7-hydroxywarfarin metabolites that showed highest correlation with UGT1A1 probe activity (bilirubin glucuronidation) and UGT1A1 protein content. It is noteworthy that UGT1A1 correlations for S- and R-7-hydroxywarfarin glucuronidation were lower than they were for the Rac mixture. The reason for this is unclear; however, the

A Comparison of Separated Enantiomers

UGT1A1

UGT1A10

B Comparison of Separated Enantiomers to Enantiomers in Racemic Mixtures

UGT1A1

UGT1A10

■ S-OHWar-Gluc from reactions with Purified Enantiomer ◆ R-OHWar-Gluc from reactions with Purified Enantiomer
 ■ S-OHWar-Gluc from reactions with Racemic Mixtures ◆ R-OHWar-Gluc from reactions with Racemic Mixtures

Fig. 2. A and B, kinetic constants for hydroxywarfarin glucuronide (OHWar-Gluc) formation by selected recombinant UGT isoforms and comparison of those from purified R- and S-enantiomers to those from Rac mixtures. Glucuronidation activities of recombinant proteins were measured by incubating membrane fractions containing recombinant protein (5 μ g) with increasing concentrations (shown in the figure) of substrate at a constant concentration of UDP-GlcUA (4 mM). Kinetic parameters were compared between reactions with purified enantiomers of each selected hydroxywarfarin. These curves were then compared with those from reactions with Rac mixtures. Curve fits and kinetic constants were determined using GraphPad Prism 4 software. The graphical fits of the data from each of the analyses with each substrate are shown. Red and blue curves in a single row represent the same data.

TABLE 7

Inhibition experiments

Assays to determine K_i values were done for those experiments in which $\geq 50\%$ decreases in $V_{\max}$ values were seen for products produced in Rac mixtures (Fig. 2). K_i curve fits and constants were determined using Dynafit.

Enzyme	Substrate	Inhibitor	K_i μM	Inhibition Type
UGT1A1	<i>S</i> -7-Hydroxywarfarin	<i>R</i> -7-Hydroxywarfarin	—	No measurable inhibition
	<i>R</i> -7-Hydroxywarfarin	<i>S</i> -7-Hydroxywarfarin	—	No measurable inhibition
UGT1A10	<i>S</i> -6-Hydroxywarfarin	<i>R</i> -8-Hydroxywarfarin	401 ± 28	Noncompetitive
	<i>R</i> -8-Hydroxywarfarin	<i>S</i> -6-Hydroxywarfarin	—	No measurable inhibition
	<i>S</i> -7-Hydroxywarfarin	<i>R</i> -7-Hydroxywarfarin	333 ± 25	Noncompetitive
	<i>R</i> -7-Hydroxywarfarin	<i>S</i> -7-Hydroxywarfarin	—	No measurable inhibition
	<i>S</i> -8-Hydroxywarfarin	<i>R</i> -8-Hydroxywarfarin	—	No measurable inhibition
	<i>R</i> -8-Hydroxywarfarin	<i>S</i> -8-Hydroxywarfarin	344 ± 35	Noncompetitive

—, not calculated.

Fig. 3. Positive ion mode LC-MS/MS chromatograms. MRM experiments for (*R/S*)-7-hydroxywarfarin glucuronidation by UGT1A1 (A and B), (*R/S*)-6-hydroxywarfarin glucuronidation by UGT1A10 (C and D), and (*R/S*)-7-hydroxywarfarin glucuronidation by UGT1A10 (E and F) scanned for both 7-hydroxywarfarin (m/z 325) and its resulting glucuronide (m/z 501).

results might suggest an interaction between the enantiomers that enhances the contribution of UGT1A1 to metabolism when both are present. With regard to demographic effects, higher 6- and 7-hydroxywarfarin glucuronidation activities were consistently associated with a liver donor history of significant alcohol consumption. This has been observed in our liver bank samples previously for other UGT1A substrates but not for UGT2B substrates (Court, 2010), supporting the role of UGT1A (over UGT2B) isoforms in 6- and

7-hydroxywarfarin glucuronidation. This potential interaction between alcohol consumption and warfarin metabolism could have clinical implications, predicting higher dose requirements in chronic alcohol users. Although few studies have evaluated this possibility, a recent study (Takeuchi et al., 2010) showed that Japanese subjects that consumed alcoholic beverages required 4 mg of higher doses per week compared with nonconsumers. However, it is not known whether this represents a pharmacokinetic or pharmacody-

Fig. 4. MS2 spectra for (*R/S*)-7-hydroxywarfarin glucuronidation by UGT1A1 (A and B), (*R/S*)-6-hydroxywarfarin glucuronidation by UGT1A10 (C and D), and (*R/S*)-7-hydroxywarfarin glucuronidation by UGT1A10 (E and F).

namic interaction and thus deserves further study. Given the strong effect of alcohol on the 6- and 7-hydroxywarfarin glucuronidation, it was necessary to stratify livers based on exposure history to examine the effect of the well known UGT1A1*28 TA repeat polymorphism. Although we observed a clear association between this polymorphism and glucuronidation activities for Rac-7- and *R*-7-hydroxywarfarin, only a weak association or no association was observed for other substrates, suggesting that UGT1A1 is not the sole isoform glucuronidating those warfarin metabolites.

This study also provides important information on where in the body this final step in warfarin biotransformation takes place. Although hepatic UGT1A1 does show activity toward each of the enantiomers, the exclusively extrahepatic

isoform, UGT1A10, also shows high levels of activity toward *R*- and *S*-hydroxywarfarins tested in these experiments; therefore, extrahepatic glucuronidation of hydroxywarfarins, particularly in the intestine, must be taken into account in examining the fate of this drug in humans.

Another unexpected finding showed that *R*-7-hydroxywarfarin glucuronidation by UGT1A1 and UGT1A10 produced two different glucuronide products. Although these two products had similar UV spectra, there were significant differences in their retention times during HPLC analysis (UGT1A1 product: 13.9 min; UGT1A10 product: 11.2 min; data not shown), and in their MS2 spectra (Fig. 4). We postulate that, in these two compounds, the glucuronide moiety is added to different hydroxyl groups located at C4 and C7

positions in R-7-hydroxywarfarin. Further analysis of these products is needed to determine the exact cause of these differences.

UGT1A10 is highly expressed in numerous target tissues, including the gastrointestinal tract (Mojarrabi et al., 1996; Strassburg et al., 1997, 1998), and the upper aerodigestive tract (Zheng et al., 2002). The broad tissue distribution and substrate specificity of UGT1A10 indicate that it may contribute to the extrahepatic detoxification of endogenous and xenobiotic compounds, especially orally administered drugs such as warfarin, and our past and current studies show that it is also involved in the glucuronidation of hydroxywarfarins (Miller et al., 2008; Zielinska et al., 2008). Although there are many studies focused on the substrate specificity of UGT1A10, studies attempting to identify catalytically important amino acids and the characteristics of the binding site are less prevalent (Martineau et al., 2004; Basu et al., 2005; Xiong et al., 2006, 2008; Starlard-Davenport et al., 2007; Miller et al., 2008).

Our studies presented here have allowed us to systematically investigate the kinetics of intestinal UGT1A10 with Rac-S/R-hydroxywarfarin mixtures as well as the structurally similar, enantiomerically pure hydroxywarfarins. For glucuronidation of 8-hydroxywarfarin compounds, K_i experiments produced data that fit the noncompetitive inhibition model. At this point, we do not have convincing evidence for the existence of multiple binding sites in UGT1A10. However, we can speculate that the inhibitor enantiomers (R-6-, R-7-, and S-8-hydroxywarfarin) could potentially bind to a second inhibitory site within UGT1A10 before the corresponding substrate enantiomers (S-6-, S-7-, or R-8-hydroxywarfarin) can be bound. The binding of the inhibitor could alter the shape of the enzyme, thereby preventing the substrate from binding to its active site. More experiments and the use of different kinetic models are needed for unambiguous proof of this phenomenon. We would like to emphasize that the presence of multiple binding sites was suggested previously in our studies with UGT1A10 binding site mutants (Xiong et al., 2006; Starlard-Davenport et al., 2007; Miller et al., 2008). These experiments open the door for more in-depth analysis of the presence of multiple binding sites in this isoform as well as interactions between these enantiomers in vivo.

In conclusion, these studies bring us a step closer in understanding the complex metabolic process responsible for hydroxywarfarin glucuronidation. It is anticipated that further elucidation of warfarin metabolic pathways leading to detoxification and excretion will provide additional insight into potential drug-drug interactions and the specific molecular mechanisms responsible for the adverse reactions associated with warfarin therapy.

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Authorship Contributions

Participated in research design: Bratton, Court, Moran, and Radominska-Pandya.

Conducted experiments: Bratton, Khallouki, and Mosher.

Contributed new reagents or analytic tools: Finel.

Performed data analysis: Bratton, Moran, and Radominska-Pandya.

Wrote or contributed to the writing of the manuscript: Bratton, Court, Moran, and Radominska-Pandya.

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