Characterization of Inhibition Kinetics of (S)-Warfarin Hydroxylation by Noscaphine: Implications in Warfarin Therapy

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ABSTRACT

Noscaphine is an antitussive and potential anticancer drug. Clinically significant interactions between warfarin and noscaphine have been previously reported. In this study, to provide a basis for warfarin dosage adjustment, the inhibition kinetics of noscaphine against warfarin metabolism was characterized. Our enzyme kinetics data obtained from human liver microsomes and recombinant CYP2C9 proteins indicate that noscaphine is a competitive inhibitor of the (S)-warfarin 7-hydroxylation reaction by CYP2C9. Interestingly, noscaphine also inhibited (S)-warfarin metabolism in a NADPH- and time-dependent manner, and removal of unbound noscaphine and its metabolites by ultrafiltration did not reverse inhibition of (S)-warfarin metabolism by noscaphine, suggesting mechanism-based inhibition of CYP2C9 by noscaphine. Spectral scanning of the

reaction between CYP2C9 and noscaphine revealed the formation of an absorption spectrum at 458 nm, indicating the formation of a metabolite-intermediate complex. Surprisingly, noscaphine is a 2- to 3-fold more efficient inactivator of CYP2C9.2 and CYP2C9.3 variants than it is of the wild type, by unknown mechanisms. Based on the inhibitory kinetic data, (S)-warfarin exposure is predicted to increase up to 7-fold (depending on CYP2C9 genotypes) upon noscaphine coadministration, mainly due to mechanism-based inactivation of CYP2C9 by noscaphine. Together, these results indicate that mechanism-based inhibition of CYP2C9 by noscaphine may dramatically alter pharmacokinetics of warfarin and provide a basis for warfarin dosage adjustment when noscaphine is coadministered.

Introduction

Noscaphine (Fig. 1), a nonaddictive phthalideisoquinoline alkaloid obtained from opium poppy latex, has been broadly recognized as a safe and promising oral antitussive agent (Karlsson et al., 1990). Recently, noscaphine has shown antiproliferative activity against a wide variety of tumor cell types (Mahmoudian and Rahimi-Moghaddam, 2009). Pretreatment with oral noscaphine was also proven to limit lymphatic metastasis of human prostate cancer PC3 cells in nude mouse models (Barken et al., 2010). Noscaphine is currently undergoing phase I/II trials for non-Hodgkin's lymphoma or hematological malignancy treatment (Aneja et al., 2007), and combination chemotherapy regimens with noscaphine are being investigated for non-small-cell lung cancer and triple negative breast cancer (Chougule et al., 2011a,b). As

an anticancer agent, noscaphine is used at 6- to 15-fold higher doses than as a cough suppressant (MedInsight Research Institute, <http://www.pcref.org/MedInsight%20-%20PCREF%20Noscaphine%20Review.pdf>). In humans, the pharmacokinetic behavior of noscaphine shows a relatively high interindividual variation, as well as extensive "first pass" metabolism due to carbon-carbon bond cleavage, *O*-demethylation, monohydroxylation, and demethylenation reactions (Tsunoda and Yoshimura, 1981). Several cytochrome P450 enzymes (CYP1A2, CYP2C9, CYP2C19, and CYP3A4/5) and flavin-containing mono-oxygenase 1 are responsible for these reactions (Fang et al., 2012).

Venous thromboembolism (VTE) is a common complication of cancer and cancer therapy (Streiff, 2009). The prevalence of VTE in cancer patients is 2- to 6-fold higher than in the general population (Rahme et al., 2013). The anticoagulant warfarin is frequently prescribed for the initial phase treatment of VTE, and is used for long-term treatment to prevent recurrent thrombosis (Lee, 2009). Of note, warfarin is a narrow therapeutic index drug that can cause life-threatening bleeding; thus, better understanding of factors that can influence the pharmacokinetics of warfarin is essential to achieve

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ABBREVIATIONS: ABT, 1-aminobenzotriazole; amu, atomic mass unit; AUC, area under the plasma drug concentration versus time curve; AUC_i/AUC, predicted ratio of in vivo exposure of (S)-warfarin with versus without coadministration of noscaphine; C_{max,u}, unbound maximal plasma concentration; DDI, drug-drug interaction; EM, extensive metabolizer; f_{m(CYP2C9)}, extent of CYP2C9-mediated metabolism in overall (S)-warfarin elimination; f_u, free fraction of noscaphine in pooled human serum; f_{u,m}, free fraction of noscaphine in the primary microsomal incubation; HLM, human liver microsome; *I*, concentration of inhibitor; [I]_{in vivo}, inhibitor concentration at the enzyme active site; INR, international normalized ratio; K_i, inactivator concentration required for half the maximal inactivation rate; K_i, enzyme-inhibitor constant; k_{inact}, maximal inactivation rate; K_{i,u}, unbound K_i adjusted by the free fraction of noscaphine in pooled HLMs; K_{i,u}, reversible inhibition constant adjusted by the free fraction of noscaphine in pooled HLMs; K_m, substrate concentration that yields a half-maximal velocity; k_{obs}, observed rate of inactivation; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MBI, mechanism-based inactivation; MI, metabolite intermediate; NADP⁺, nicotinamide adenine dinucleotide phosphate; PBS, phosphate-buffered saline; PM, poor metabolizer; S, concentration of substrate; v, velocity of the enzymatic reaction; VTE, venous thromboembolism.

Fig. 1. Chemical structures of noscaphine and (S)-warfarin. Arrows denote the proposed oxidation sites by CYP2C9.

optimal warfarin therapy. Clinical evidence indicates that noscaphine causes significant drug-drug interactions (DDIs) with warfarin when administered as an antitussive agent (Ohlsson et al., 2008; Scordo et al., 2008; Myhr, 2009). Considering the higher doses of noscaphine used for cancer treatment (Madan et al., 2011), clinically significant interactions between noscaphine and warfarin are expected. However, the magnitude and underlying mechanisms of noscaphine-warfarin interaction remain unknown.

Warfarin is administered as a racemic mixture and undergoes stereoselective metabolic clearance in the human liver. (S)-Warfarin (Fig. 1) has a 5-fold higher anticoagulant activity than the (R)-isomer (Hirsh et al., 2001), and is primarily metabolized by CYP2C9 (Rettie et al., 1992). A previous study demonstrated that noscaphine inhibited CYP2C9 turnover in a noncompetitive and time-dependent manner in human liver microsomes (HLMs) when diclofenac was used as the probe substrate (Fang et al., 2010). Of note, CYP2C9 inhibitors have shown substrate-dependent differences in inhibitory potencies, with warfarin generally being more susceptible to CYP2C9 inhibition. For example, the reversible inhibition by CYP2C9 inhibitors including benzbromarone, sulfamethizole, and progesterone was greater with (S)-warfarin than phenytoin, tolbutamide, and diclofenac were as probe substrates (Kumar et al., 2006). Also, (S)-warfarin was a more sensitive probe substrate of CYP2C9 to mechanism-based inactivation (MBI) in comparison with (S)-flurbiprofen and diclofenac (Hutzler et al., 2009). In addition to exhibiting substrate-dependent inhibition kinetics, CYP2C9 also shows genotype-dependent inhibition patterns. For example, CYP2C9.3 (I359L), the enzyme product of a predominant CYP2C9 variant in Caucasians, was found to be less susceptible than the wild-type CYP2C9.1 to a reversible inhibitor fluconazole as compared with the wild-type CYP2C9.1 (Kumar et al., 2006) when flurbiprofen was used as a probe substrate. Whether CYP2C9 variants exhibit different inhibition kinetics for warfarin metabolism remains unknown. Together, the inhibition kinetics of noscaphine remains to be characterized specifically for warfarin considering its narrow therapeutic index.

In the present study, we examined the inhibitory mechanisms of noscaphine against (S)-warfarin 7-hydroxylation at noscaphine concentrations required for cancer treatment, and characterized the in vitro inhibition kinetics using pooled HLMs and cDNA-expressed CYP2C9. Furthermore, we examined the effects of the CYP2C9 genotype on the inhibitory potency of noscaphine to guide warfarin dosage adjustment. The extent of the in vivo noscaphine-warfarin interaction was then predicted from the obtained kinetic data.

Materials and Methods

Chemicals and Reagents. (S, R)-Noscaphine, (S)-warfarin, 7-hydroxywarfarin, mebendazole, phenytoin, phosphate-buffered saline (PBS), isocitric acid, magnesium chloride, isocitric acid dehydrogenase, 1-aminobenzotriazole (ABT),

sulfaphenazole, nicotinamide adenine dinucleotide phosphate (NADP⁺), and NADPH were purchased from Sigma-Aldrich (St. Louis, MO). Pooled HLMs (*n* = 50 donors) were from Invitrogen (Carlsbad, CA). cDNA-expressed CYP2C9.1 (wild type), CYP2C9.2 (R144C), and CYP2C9.3 (I359L) coexpressing human NADPH-cytochrome P450 reductase and human cytochrome b5 were obtained from BD Biosciences (Woburn, MA). Formic acid (American Chemical Society grade), acetonitrile, and methanol (Optima grade) were purchased from Fisher Scientific (Pittsburgh, PA). All other reagents were of high-performance liquid chromatography grade or the highest grade commercially available.

Determination of *K_m*. Pooled HLMs (0.1 mg/ml) were incubated with (S)-warfarin (0–20 μM) in a NADPH-regenerating system (5 mM isocitric acid, 0.2 units/ml isocitric acid dehydrogenase, and 5 mM magnesium chloride in 100 mM Tris-HCl buffer, pH 7.4; 100 μl total volume). After preincubation at 37°C for 5 minutes, the reactions were started by addition of NADP⁺ (1 mM) and further incubated for another 20 minutes. The reactions were then terminated by adding 100 μl of ice-cold acetonitrile containing mebendazole (1 μM) as the internal standard and kept on ice for 30 minutes, followed by centrifugation at 16,100*g* for 15 minutes at 4°C to obtain the supernatant. The concentrations of 7-hydroxywarfarin in the supernatants were measured using liquid chromatography–tandem mass spectrometry (LC-MS/MS). 7-Hydroxywarfarin formation rates were determined, and the *K_m* values were estimated using GraphPad Prism 5 software (GraphPad, La Jolla, CA). The incubations were performed in duplicate, and the experiments were repeated at least two times. The final concentrations of organic solvent in the incubation media were kept below 1% (v/v) for all microsomal reactions.

Determination of *IC*₅₀. Pooled HLMs (0.1 mg/ml) or recombinant enzymes (40 pmol/ml) were incubated with (S)-warfarin (2.5 μM) in the presence of noscaphine (0–100 μM) in a NADPH-regenerating system for 20 minutes. The warfarin concentration was selected based on the apparent *K_m* values in pooled HLMs or cDNA-expressed CYP2C9 (2–9 μM) (Liu et al., 2012) and the clinically relevant concentration range (*C*_{max}, ~5 μM) (Maddison et al., 2013). 7-Hydroxywarfarin formation rates were determined, and the half-maximal inhibitory concentration (*IC*₅₀) values were estimated.

Determination of *K_i*. (S)-Warfarin (1.0, 2.5, 5.0, and 10 μM) was incubated with pooled HLMs or recombinant CYP2C9.1 in the presence of noscaphine at different concentrations (0–25 μM), and the concentration of 7-hydroxywarfarin was measured. The *K_i* values (enzyme-inhibitor constant) of noscaphine were calculated via nonlinear regression of the data to the equations for competitive inhibition (eq. 1), noncompetitive inhibition (eq. 2), or mixed inhibition (eq. 3), using GraphPad Prism 5 software:

$$v = (V_{\max}S)/(K_m(1 + I/K_i) + S) \quad (1)$$

$$v = (V_{\max}S)/((K_m + S)(1 + I/K_i)) \quad (2)$$

$$v = (V_{\max}S)/(K_m(1 + I/K_i) + S(1 + I/\alpha K_i)) \quad (3)$$

where *v* represents the velocity of the reaction; *S* and *I* are the concentrations of substrate and inhibitor, respectively; and *K_m* is the substrate concentration that yields a half-maximal velocity (*V*_{max}). The inhibition type was determined from the Lineweaver-Burk plot and the Dixon plot, and fits to the enzyme inhibition kinetic models. Comparisons of goodness of the fits were determined by examination of the residues, *R*² values, the parameter standard error estimates, and 95% confidence intervals. Kinetic constants were reported as the mean ± S.E. of the parameter estimate. *K_i* values were further adjusted for nonspecific binding to 0.1 mg/ml microsomes to get unbound *K_i* (*K_{i,u}*).

Time- and NADPH-Dependent Inhibition. Noscaphine (15 μM) was preincubated with CYP2C9.1, CYP2C9.2, or CYP2C9.3 (400 pmol/ml) for 30 minutes at 37°C with a regeneration system in the presence or absence of NADP⁺. The concentration of noscaphine was selected to be 10-fold higher than the concentration exhibiting 25% of the maximum reversible inhibition according to a previous study (Obach et al., 2007). An aliquot of the inactivation mixture (10 μl) was then added to an incubation mixture containing (S)-warfarin (2.5 μM), NADP⁺, and the regeneration system for the measurement of residual CYP2C9 activity.

Determination of *K_i* and *k_{inact}*. Noscaphine (0–50 μM) was preincubated with pooled HLMs (1 mg/ml) or recombinant CYP2C9 enzymes (400 pmol/ml) in a NADPH-regenerating system. At different time points (0–30 minutes), an

aliquot (10 μ l) was transferred into a secondary incubation system containing (S)-warfarin (25 μ M), and 7-hydroxywarfarin concentrations were measured by LC-MS/MS. The concentration of warfarin in the secondary incubation system was determined to be >4 times higher than the apparent K_m values obtained from different enzyme sources (Hutzler et al., 2009; Liu et al., 2012). The observed rates of inactivation (k_{obs}) were determined from the slopes of residual CYP2C9 activity versus time plot. Inactivation kinetic parameters [theoretical maximal inactivation rate (k_{inact}) and inactivator concentration required for half the maximal inactivation rate (K_I)] were calculated using nonlinear regression of the data according to eq. 4:

$$k_{obs} = k_{obs}[I]=0 + \frac{k_{inact} \cdot [I]}{K_I + [I]} \quad (4)$$

where $[I]$ represents the initial concentrations of nescapine in the inactivation preincubations; $k_{obs}[I]=0$ is the apparent inactivation rate constant measure in the absence of nescapine; and unbound K_I ($K_{I,u}$) is K_I multiplied by $f_{u,m}$, where $f_{u,m}$ is the free fraction of nescapine in the primary microsomal incubation (1 mg/ml).

Effects of Ultrafiltration on CYP2C9 Inactivation. HLMs (1 mg/ml) were preincubated with ABT (1 mM), nescapine (50 μ M), sulfaphenazole (10 μ M), or vehicle (control) in a NADPH-regenerating system for 20 minutes at 37°C. The preincubation mixtures (100 μ l) were chilled on ice, 5-fold diluted, and then filtered through Amicon Ultra-0.5 centrifugal filter devices (Millipore, Billerica, MA; cutoff of 30 kDa) according to the manufacturer's instructions. Samples were washed with 450 μ l of 100 mM Tris-HCl buffer (pH 7.4), centrifuged at 14,000g for 10 minutes, collected by a reverse spin at 1000g for 2 minutes, and then resuspended with buffer to the original volume. Aliquots (10 μ l) were removed and diluted 10-fold to determine the residual CYP2C9 activities by incubating with warfarin (2.5 μ M).

Determination of $f_{u,m}$. The Single-Use Rapid Equilibrium Dialysis plate from Thermo Scientific-Pierce Biotechnology (Rockford, IL) with a cutoff molecular mass of 8000 Da was used. HLM (0.1 or 1 mg/ml) solutions (100 μ l) containing nescapine (0.3–50 μ M) were placed into the sample chamber, and 300 μ l of PBS dialysis buffer was added into the adjacent chamber in triplicate. The content of organic solvent in the final microsomal solutions was kept below 1%. The plates were then covered with sealing tape (MicroAmp; Applied Biosystems, Foster City, CA) and incubated at 37°C for 4 hours on an orbital shaker at 100 rpm. Aliquots (50 μ l) were removed from each side of the insert and placed in separate tubes. The same volume (50 μ l) of blank microsomes was added to the buffer samples, and an equal volume of PBS was added to the collected microsome samples for matrix matching. To each sample, 200 μ l of acetonitrile containing phenytoin (1 μ M; internal standard) was added. These samples were vortexed for 30 seconds and chilled on ice for 30 minutes, then centrifuged at 16,100g for 15 minutes at 4°C. Nescapine concentrations in the supernatant were determined by LC-MS/MS.

Measurement of 7-Hydroxywarfarin and Nescapine. 7-Hydroxywarfarin concentrations in microsomal samples were determined by an LC-MS/MS method as previously described, with slight modifications (Liu et al., 2012). An Agilent 1200 high-performance liquid chromatography (Agilent Technologies, Santa Clara, CA) interfaced with an Applied Biosystems Qtrap 3200 equipped with an electrospray ion source was used. Chromatographic separation was carried out with a Waters XTerra MS C18 column (2.1 $\times$ 50 mm, 3.5 μ m; Waters Corporation, Milford, MA). The mobile phase was delivered at 250 μ l/min, and the gradient was initiated at 90% A–10% B (A, 5 mM ammonium acetate buffer, pH 4.6; B, acetonitrile). The proportion of mobile phase B was increased to 60% over 1 minute, held constant for 1 minute, and then restored to the initial composition. 7-Hydroxywarfarin was detected by MS/MS [323/176 atomic mass unit (amu)], and mebendazole was used as the internal standard (294/262 amu) in negative ion mode. The mobile phase for nescapine consisted of 0.1% formic acid in water (A) and acetonitrile (B). The column was first equilibrated at 25% mobile phase B for 1.5 minutes at a 200 μ l/min flow rate. The elution was then ramped linearly to 90% mobile phase B over 1.5 minutes, maintained for 5 minutes, and followed by a return to initial conditions. Multiple reaction monitoring for nescapine (MS/MS 414/220 amu) was performed in positive ionization mode, and phenytoin (MS/MS 253/182 amu) was used as the internal standard.

Spectral Analysis of Metabolite-Intermediate Formation. Recombinant CYP2C9.1 was diluted into buffer (0.1 M potassium phosphate buffer, pH 7.4)

to yield a final concentration of 0.3 μ M, and a stock solution of reduced NADPH was added to yield a final concentration of 1 mM NADPH. The solution was split into sample and reference cuvettes and spectra recorded on an Olis-modernized Aminco DW-2 spectrophotometer (Olis, Bogart, GA). After 3 minutes of preincubation at 37°C, the experiment was initiated by adding vehicle to the reference cuvette (2 μ l methanol) and substrate to the sample cuvette (2 μ l of nescapine stock solution) to yield a final concentration of 50 μ M nescapine. The final volume in both cuvettes was 0.40 ml and contained 0.5% methanol. The spectrophotometer was operated in split-beam mode, and repetitive scans were taken from 400- to 500-nm wavelength light (readings at 1-nm intervals) at 1, 2, 4, 8, and 16 minutes. Once metabolite-intermediate (MI) complex formation had ceased, the incubation was quenched by adding a few crystals of dithionite to each cuvette. Following a 3-minute incubation period, a new baseline scan was taken and the spectrum blanked. CO gas was then bubbled gently into the sample cuvette only, and scans were taken to determine the amount of residual cytochrome P450 capable of binding CO. The amount of CO-bound cytochrome P450 was quantified by applying a molar extinction coefficient of 91 $\text{cm}^{-1} \times \text{mM}^{-1}$ to the 450–490-nm absorbance difference (Omura and Sato, 1964). Control runs were performed in the absence of NADPH. The kinetic profile of MI complex formation was captured by measuring the absorbance at 458 and 490 nm from 0 to 20 minutes and plotting the increase in difference in absorbance over time. The concentration of MI complexes was approximately calculated by dividing the absorbance difference at 458 and 490 nm by the molar extinction coefficient of 65 $\text{cm}^{-1} \text{mM}^{-1}$ (Manno et al., 1988). The initial rates of MI complex formation (k) and maximal concentration of MI complex (MIC_{max}) were calculated by fitting the data to the standard monoexponential function $\text{MIC}_t = \text{MIC}_{\text{max}} (1 - e^{-kt})$, where MIC_t is the concentration of MI complex at each time point.

Quantitative Predictions of In Vivo Drug Interactions. The relative changes in area under the curve (AUC) of (S)-warfarin upon coadministration of nescapine were predicted based on reversible inhibition (eq. 5) or time-dependent inhibition mechanism (eq. 6) (Brown et al., 2005; Grimm et al., 2009):

$$\frac{\text{AUC}_i}{\text{AUC}} = \frac{1}{1 - f_{m(\text{CYP2C9})} + \frac{f_{m(\text{CYP2C9})}}{1 + \frac{[I]_{\text{in vivo}}}{K_{I,u}}}} \quad (5)$$

$$\frac{\text{AUC}_i}{\text{AUC}} = \frac{1}{\frac{f_{m(\text{CYP2C9})}}{1 + \frac{k_{inact} \times [I]_{\text{in vivo}}}{K_{I,u} \times k_{deg(\text{CYP2C9})}}} + (1 - f_{m(\text{CYP2C9})})} \quad (6)$$

where AUC_i/AUC is the predicted ratio of in vivo exposure of (S)-warfarin with versus without coadministration of nescapine; $k_{deg(\text{CYP2C9})}$ is the first-order rate constant of in vivo degradation of CYP2C9 [0.00026 minute^{-1} (Obach et al., 2007)]; $K_{I,u}$ is K_I adjusted by the free fraction of nescapine in 0.1 mg/ml pooled HLMs ($= K_I \times f_u$); and $K_{I,u}$ is the unbound inhibitor concentration at 1 mg/ml microsomes yielding 0.5 times k_{inact} . $[I]_{\text{in vivo}}$ represents the inhibitor concentration at the enzyme active site. The unbound C_{max} at steady state (defined as $f_u \times C_{\text{max}}$, $C_{\text{max},u}$) was used for $[I]_{\text{in vivo}}$ because $C_{\text{max},u}$ yields the most accurate predictions of drug-drug interactions (Obach et al., 2007), and the use of total C_{max} typically leads to overprediction of DDI risk (Fujioka et al., 2012). The free fraction of nescapine (f_u) in pooled human serum is about 7% (Karlsson and Dahlstrom, 1990). In previous clinical pharmacokinetic studies of nescapine, single doses of 100-, 200-, and 300-mg nescapine tablets led to the C_{max} values of 0.31, 0.95, and 2.1 μ M, respectively (Karlsson et al., 1990). A recent clinical study with a dosage of 50 mg demonstrated that the nescapine concentration at 4 hours after dosing ranged from 0.02 to 0.19 μ M (Rosenborg et al., 2010). Based on all these previous data, C_{max} values ranging from 0.02 to 2.1 μ M were selected to estimate the AUC_i/AUC ratio.

The extent of CYP2C9-mediated metabolism in overall (S)-warfarin elimination [$f_{m(\text{CYP2C9})}$] was estimated by using the following equations (Ito et al., 2005):

$$CL^{EM} = f_{m(\text{CYP2C9})}^{EM} CL^{EM} + (1 - f_{m(\text{CYP2C9})}^{EM}) CL^{EM} \quad (7)$$

$$CL^{XM} = f_{m(\text{CYP2C9})}^{XM} CL^{XM} + (1 - f_{m(\text{CYP2C9})}^{XM}) CL^{EM} \quad (8)$$

where XM refers to poor (PM; patients with the *2/*2, *2/*3, or *3/*3 genotype), intermediate (patients with the *1/*2 or *1/*3 genotype), or extensive (EM; patients with the *1/*1 genotype) metabolizer phenotype predicted based on CYP2C9 genotype information. Combining eqs. 7 and 8, the following equation describes the $f_{m(CYP2C9)}^{XM}$ decrease by the CL^{EM}/CL^{XM} ratio:

$$f_{m(CYP2C9)}^{XM} = 1 - \frac{(1 - f_{m(CYP2C9)}^{EM})CL^{EM}}{CL^{XM}} \quad (9)$$

The $f_{m(CYP2C9)}^{EM}$ value was set as 0.82–0.92 based on the urinary recovery of metabolites, biliary excretion, and the recovery of unchanged drug (Kunze and Trager, 1996). Then, the $f_{m(CYP2C9)}^{XM}$ of (S)-warfarin ranged from 0 to 0.92 for different CYP2C9 phenotypes, based on the previous in vivo (S)-warfarin clearance data obtained in subjects with different CYP2C9 genotypes (Scordo et al., 2002). AUC_i/AUC values were subsequently calculated and plotted against $[I]_{in vivo}$ and $f_{m(CYP2C9)}$ using MATLAB R2009b (MathWorks, Natick, MA). The fraction of warfarin dose required to obtain the same level of systemic exposure as in the wild-type carrier was predicted by using eq. 10 for the carriers of CYP2C9 variants (Castellan et al., 2013):

$$Dose\ Adjustment\ Factor_{Genotype} = \frac{AUC^{EM}}{AUC^{XM}} = 1 - f_{m(CYP2C9)}^{EM}(1 - FA) \quad (10)$$

in which FA represents the fraction of remaining CYP2C9 activity of CYP2C9 variants. The combined effects of CYP2C9 genotypes and inhibition by noscaphine on warfarin dose could be estimated using eq. 11:

$$Dose\ Adjustment\ Factor = Dose\ Adjustment\ Factor_{Genotype} \times \frac{AUC}{AUC_i} \quad (11)$$

Results

Inhibition of (S)-Warfarin 7-Hydroxylation by Noscaphine. To determine the (S)-warfarin concentration used in inhibition assays, the enzyme kinetic studies were first performed by using pooled HLMs. The apparent kinetic parameters K_m and V_{max} of 7-hydroxywarfarin formation were estimated to be $3.3 \pm 0.3 \mu M$ and 6.9 ± 0.2 pmol/min/mg protein, respectively (Fig. 2A). In the subsequent experiments using $2.5 \mu M$ (S)-warfarin, noscaphine inhibited CYP2C9-mediated (S)-warfarin 7-hydroxylation with an IC_{50} of $6.5 \pm 0.7 \mu M$ in pooled HLMs (Fig. 2B). In cDNA-expressed enzyme preparations of CYP2C9.1, the IC_{50} value was $2.6 \pm 0.1 \mu M$. Relatively small decreases in inhibition potency were noted in CYP2C9.2 and CYP2C9.3 enzymes (IC_{50} : 3.6 ± 0.2 and $4.9 \pm 0.7 \mu M$, respectively).

As shown in Fig. 3, results from the inhibition kinetic assays suggest that noscaphine inhibited CYP2C9 activity in a competitive manner, with K_i values of $4.6 \pm 0.4 \mu M$ (in pooled HLMs) and $1.0 \pm 0.1 \mu M$ (in recombinant CYP2C9.1). These results indicate that noscaphine is a competitive inhibitor of (S)-warfarin metabolism by CYP2C9.

Time- and NADPH-Dependent Inhibition of CYP2C9. Previously, noscaphine was shown to inhibit diclofenac 4'-hydroxylation via a mechanism of time-dependent inhibition (Fang et al., 2010). To examine whether noscaphine inhibits warfarin metabolism by a similar mechanism, noscaphine at different concentrations was incubated with pooled HLMs for different time periods, and the residual CYP2C9 activity was determined using (S)-warfarin as the probe drug. The resulting inactivation kinetics was best fit to the standard hyperbolic equation (Fig. 4), with k_{inact} of $0.041 \text{ minute}^{-1}$ and K_I of $6.8 \mu M$. The estimated k_{inact}/K_I was $6.0 \text{ ml/min}/\mu M$, indicating high inactivation efficiency. To evaluate the effects of CYP2C9 genotypes on the time-dependent inhibition potential of noscaphine, single point inactivation experiments were performed using recombinant wild-type CYP2C9 and its variants. To this end, a recombinant CYP2C9 protein was preincubated with noscaphine (or vehicle) in the presence (or absence) of NADPH for 30 minutes, and then the residual CYP2C9 activity was determined by incubation with (S)-warfarin. A greater than 15% loss in CYP2C9 activity by preincubation with noscaphine (when compared with the activity in the absence of NADPH) was used as a cutoff value for significant time-dependent inhibition (Obach et al., 2007). The percentage of CYP2C9.1 activity loss in the presence of NADPH was calculated to be $42\% \pm 5\%$ (Fig. 5A), indicating significant inactivation of the enzyme by noscaphine. Interestingly, the CYP2C9 variants associated with the PM phenotype were found to be more susceptible to inactivation by noscaphine than the wild type; the decrease in activity was $71\% \pm 7\%$ (CYP2C9.2, $P < 0.01$) and $59\% \pm 5\%$ (CYP2C9.3, $P < 0.05$) (Fig. 5A). Similar results were also observed when inactivation kinetics was compared among different CYP2C9 variants (Fig. 5, B–D). CYP2C9.1 was inactivated by noscaphine with a K_I of $4.4 \mu M$ and k_{inact} of $0.013 \text{ minute}^{-1}$. Although K_I values for CYP2C9.2 ($5.7 \mu M$) and CYP2C9.3 ($2.6 \mu M$) were similar to that of CYP2C9.1, their k_{inact} values increased noticeably ($0.033 \text{ minute}^{-1}$ for CYP2C9.2 and $0.022 \text{ minute}^{-1}$ for CYP2C9.3), resulting in moderately higher inactivation efficiencies in comparison with that of the wild-type enzyme (i.e., 2-fold and 3-fold for CYP2C9.2 and CYP2C9.3, respectively). Taken together, noscaphine is

Fig. 2. Enzyme kinetics of (S)-warfarin 7-hydroxylation in pooled HLMs (A) and the inhibition of (S)-warfarin 7-hydroxylation by noscaphine in HLMs and recombinant CYP2C9 enzymes (B).

Fig. 3. Representative Lineweaver-Burk plots and Dixon plots showing the effects of nescapine on (S)-warfarin 7-hydroxylation in pooled HLMs (A and B) and recombinant CYP2C9.1 (C and D). Ctrl, control.

both a competitive and time-dependent inhibitor of CYP2C9, and its inhibitory potency differs among CYP2C9 variants.

Effect of Ultrafiltration on CYP2C9 Inactivation by Nescapine.

To determine the reversibility of the inactivation of CYP2C9 by nescapine, NADPH, unbound nescapine, and its metabolites were removed from the preincubation samples by ultrafiltration, and the remaining CYP2C9 activity was determined by measuring (S)-warfarin 7-hydroxylation rates. ABT (a mechanism-based inactivator of cytochrome P450s) and sulfaphenazole (a reversible inhibitor of CYP2C9) were used as controls in parallel. The filtered samples of microsomes preincubated with nescapine and ABT showed less than 10% recovery of (S)-warfarin 7-hydroxylase activity after ultrafiltration, whereas > 30% recovery was detected with sulfaphenazole (Fig. 6). Together, these results indicate that nescapine-mediated CYP2C9 inhibition is irreversible, and that, similarly to ABT, nescapine inhibits CYP2C9 by MBI.

Spectral Analysis of MI Complex Formation. To examine MI complex formation, absorption spectra of the incubation mixtures of recombinant CYP2C9.1 with nescapine were monitored. A time-dependent increase in the Soret peak at 458 nm, characteristic of MI complex formation, was observed upon the addition of NADPH

(Fig. 7A). When the difference between the absorbance at 458 nm and 490 nm (dAbs) was plotted over time, a kinetic profile for MI complex formation was determined (Fig. 7B). Based on the data for the first 30 seconds of reaction, the initial rate of MI complex formation was calculated to be 0.0133 dAbs/ μ M cytochrome P450/min. Approximately 41% of the total enzyme was converted to an MI complex. Remaining uncomplexed, CYP2C9 was determined by a subsequent CO-binding study. The results showed that a significant fraction (40%–60%) was able to form a ferrous CO complex after MI complex formation had ceased (data not shown). In summary, all these spectra data combined suggested that MBI of (S)-warfarin 7-hydroxylation by nescapine was mainly due to the formation of MI complex with CYP2C9.

Prediction of In Vivo (S)-Warfarin-Nescapine Interactions. The clinical impact of CYP2C9 inhibition by nescapine was evaluated by estimating the ratio of (S)-warfarin AUC in the presence and absence of the inhibitor, based on the inhibitory mechanisms (i.e., competitive inhibition and MBI). The K_i and K_I values were first adjusted for nonspecific binding to 0.1 mg/ml ($f_{u,m}$: 97% $\pm$ 3%) and 1.0 mg/ml ($f_{u,m}$: 22% $\pm$ 1%) HLM proteins, respectively, yielding the adjusted values ($K_{i,u}$ of 4.5 μ M and $K_{I,u}$ of 1.5 μ M). AUC changes upon nescapine

Fig. 4. Time- and concentration-dependent inhibition of (S)-warfarin 7-hydroxylation by noscapine (0–50 μM) in pooled HLM incubations. Aliquots were removed from the preincubation mixtures at the indicated time points and diluted 10-fold for measurement of residual CYP2C9 activity using (S)-warfarin as the probe substrate. The rate of inactivation of CYP2C9 activity at different inhibitor concentrations (k_{obs}) was determined by linear regression analysis of the natural logarithm of the percentage of activity remaining versus preincubation time data (A). K_i and k_{inact} were calculated by nonlinear regression analysis of the k_{obs} versus noscapine concentration data according to the equation described in *Materials and Methods* (B).

coadministration were predicted for varying $f_{\text{m(CYP2C9)}}$ and noscapine concentrations. Values of $f_{\text{m(CYP2C9)}}$ ranging from 0 to 0.92 reflect the differential CYP2C9 enzyme activity of CYP2C9 variants (Table 1;

see *Materials and Methods* for details), whereas noscapine concentrations ranging from 0.02 to 2.1 μM reflect C_{max} obtained from a typical oral dose of noscapine for antitussive or anticancer effects.

Fig. 5. NADPH-dependent inactivation of (S)-warfarin 7-hydroxylation by noscapine (15 μM) in recombinant CYP2C9 (A). Kinetic plots demonstrating observed rates of inactivation (k_{obs}) by noscapine (0–50 μM) in recombinant CYP2C9.1 (B), CYP2C9.2 (C), and CYP2C9.3 (D).

Fig. 6. Effects of ultrafiltration on the time- and NADPH-dependent inhibitory potential of noscaphine toward CYP2C9-mediated (*S*)-warfarin 7-hydroxylation.

Figure 8 shows three-dimensional (and contour) plots for the estimated AUC_i/AUC ratios when noscaphine inhibits CYP2C9 activity only by MBI (Fig. 8A) or competitive inhibition mechanism (Fig. 8B). The results indicate that, in carriers of wild-type CYP2C9, a typical dose of noscaphine is predicted to cause a >7 -fold increase in the exposure of (*S*)-warfarin by MBI (Fig. 8A) but a minimal increase ($\sim 3\%$) by the competitive inhibition mechanism (Fig. 8B). The dose adjustment factor was then estimated for warfarin based on the combined effects of noscaphine-mediated inhibition and CYP2C9 genotypes on warfarin exposure (Fig. 8C; Table 1). The coadministration of noscaphine was estimated to require up to a 7-fold decrease in warfarin dose in CYP2C9*1/*1 carriers, whereas the need for dose change was smaller in the carriers of CYP2C9 variants. Together, our results indicate that MBI of CYP2C9 by noscaphine may significantly increase the systemic exposure of (*S*)-warfarin, and the required warfarin dose adjustments show genotype dependency.

Discussion

Altered CYP2C9-mediated (*S*)-warfarin metabolism is one of the major causes of high interindividual variability in warfarin response (Eriksson and Wadelius, 2012). Noscaphine is both a substrate and an

inhibitor for CYP2C9 (Rosenborg et al., 2010; Fang et al., 2012), and is known to cause significant increases in international normalized ratio (INR) when coadministered with warfarin (Ohlsson et al., 2008; Scordo et al., 2008; Myhr, 2009). However, direct in vitro evidence of the noscaphine-warfarin interaction or a detailed analysis of the inhibition kinetics to predict the magnitude of in vivo DDI is lacking. In addition, the effect of genetic polymorphism in CYP2C9 on noscaphine-mediated inhibition remains unknown. The objective of this study was to investigate the inhibitory mechanisms of (*S*)-warfarin metabolism by noscaphine and examine the effects of common genetic polymorphisms of CYP2C9 on the extent of noscaphine-mediated CYP2C9 inhibition. Combined with genotype-dependent inhibition kinetic data, the magnitude of pharmacokinetic interactions and the required warfarin dosage adjustments were estimated for subjects of different genotypes.

In this study, noscaphine was found to be a competitive inhibitor of CYP2C9-mediated (*S*)-warfarin 7-hydroxylation in both HLMs and recombinant enzyme systems ($K_i < 10 \mu\text{M}$) (Fig. 3). Interestingly, when diclofenac 4'-hydroxylation was used as the probe reaction, noscaphine inhibited CYP2C9 activity in a noncompetitive manner in pooled HLMs (Fang et al., 2010). Similar substrate-dependent differences in inhibitory mechanism were previously reported for CYP3A4 inhibitors. For example, fluconazole exhibits competitive inhibition of CYP3A4-mediated (*R*)-warfarin hydroxylation (Kunze et al., 1996) but noncompetitive inhibition of midazolam 1'-hydroxylation. This was attributed to the multiple binding regions for substrates or inhibitors in the voluminous active site (Gibbs et al., 1999). CYP2C9 also exhibits a capacity for multiple ligands binding (Williams et al., 2003; Wester et al., 2004). Possibly, noscaphine prevents (*S*)-warfarin from accessing the substrate-binding site in the CYP2C9 active pocket, but not prevent diclofenac's access, resulting in the competitive inhibition of (*S*)-warfarin metabolism. This hypothesis remains to be verified through molecular modeling of CYP2C9. In the competitive inhibition of CYP2C9 activity by noscaphine, the IC_{50} value for CYP2C9.3 was 1.9-fold higher than that for CYP2C9.1 (Fig. 2). This result is in good agreement with a previous study in which the fluconazole K_i value for CYP2C9.3 was found to be 2-fold higher than that for CYP2C9.1 when flurbiprofen was used as a probe substrate (Kumar et al., 2008). This genotype-dependent CYP2C9 inhibitory potency of noscaphine is likely due to differences in the conformation of the active site and the orientation of inhibitor binding (Kumar et al., 2006). Taken together, noscaphine inhibits CYP2C9-mediated (*S*)-warfarin metabolism via a competitive

Fig. 7. Spectral evidence for the formation of an MI complex with CYP2C9.1 and noscaphine ($50 \mu\text{M}$) (A) and the time course for complex formation (B).

TABLE 1

Estimation of (S)-warfarin clearance fraction [$f_{m(CYP2C9)}$], ratio change of in vivo exposure to (S)-warfarin (AUC_i/AUC), and suggested dose adjustment factor for patients with different CYP2C9 genotypes

All simulations were performed at noscaphine C_{max} values ranging from 0.02 to 2.1 μM , and the fraction of wild-type CYP2C9 activity (FA) for each mutant was obtained directly from the literature (Castellan et al., 2013).

Genotype	Fraction of Dose Expected to be Cleared by CYP2C9 [$f_{m(CYP2C9)}$] ^a	Magnitude of the Potential (S)-Warfarin-Noscaphine Interactions (AUC_i/AUC)	Dose Adjustment Factor ^b	
			Based Solely on Genotypes	Based on Both Genotypes and Inhibitory Effects of Noscaphine
CYP2C9*1/*1	0.82–0.92	1.12–7.36	1.00	0.14–0.88
CYP2C9*1/*2	0.69–0.86	1.09–5.20	0.83	0.16–0.74
CYP2C9*1/*3	0.66–0.85	1.09–4.96	0.60	0.12–0.53
CYP2C9*2/*2	0.44–0.75	1.06–3.38	0.72	0.21–0.65
CYP2C9*2/*3	0.23–0.66	1.03–2.63	0.44	0.17–0.40
CYP2C9*3/*3	0–0.13	1.00–1.14	0.20	0.18–0.19

^aValues were estimated from mass balance data as described previously (Kunze and Trager, 1996).

^bDose adjustment ratios were predicted at the upper most value of estimated $f_{m(CYP2C9)}$.

inhibition mechanism, and the inhibitory profiles exhibit substrate- and genotype-dependent variation.

Our study revealed that noscaphine is also a mechanism-based inhibitor of CYP2C9, exhibiting time- and NADPH-dependent inhibition (Fig. 4). Removal of free noscaphine and its metabolites from the HLM incubation using ultrafiltration did not reverse noscaphine-mediated inhibition of (S)-warfarin metabolism, indicating irreversibility of the inhibition (Fig. 6). Our spectral analysis further demonstrated a time-dependent increase in the maximum absorbance at 458 nm (Fig. 7A), suggesting the formation of an MI complex by noscaphine. This is reasonable because noscaphine has a methylenedioxyphenyl group incorporated into its structure (Fig. 1A). The oxidation of this group often leads to the formation of carbene complexes with ferrous heme iron of cytochrome P450 (Franklin, 1971). Since the formation of an MI complex is dependent upon catalytic rate, the

susceptibility to MBI is expected to be dependent upon the relative activities of cytochrome P450 variants (Polasek and Miners, 2007; Orr et al., 2012). To verify that formation of a reactive metabolite(s) of noscaphine by CYP2C9 is responsible for the MBI by noscaphine, the kinetics of CYP2C9 inhibition were characterized using CYP2C9.2 and CYP2C9.3 variants that are known to exhibit decreased enzyme activity (Liu et al., 2012). Surprisingly, our results showed that the inactivation of CYP2C9 by noscaphine was >2-fold more efficient (i.e., k_{inact}/K_I) for CYP2C9.2 and CYP2C9.3 than for CYP2C9.1. This suggests that formation of a reactive metabolite(s) of noscaphine may be greater from CYP2C9.2 and CYP2C9.3. In fact, it was previously shown that a metabolite of noscaphine is produced 1.4-fold more rapidly by CYP2C9.2 than CYP2C9.1 (Fang et al., 2012), suggesting that the CYP2C9 variants may show increased (rather than decreased) catalytic activity for certain reactions. This may be due to the structural

Fig. 8. Three-dimensional and contour plots for the calculated AUC_i/AUC ratios with $f_{m(CYP2C9)}$ and C_{max} of noscaphine in plasma resulting from MBI (A) or competitive inhibition (B). Suggested dose adjustment factors based on the range of noscaphine plasma C_{max} values and patients of different genotypes (C).

features of CYP2C9 [i.e., a big active pocket allowing simultaneous binding of multiple ligands (Williams et al., 2003)] that potentially lead to highly substrate-dependent functionality of CYP2C9 variants (Maekawa et al., 2009). It will be of interest to examine whether the partition ratios, metabolite formation rates, and nescapine depletion rates are significantly different among these enzymes.

Nescapine is a mechanism-based inhibitor of CYP2C9-mediated (S)-warfarin 7-hydroxylation and, as expected of a P450 substrate, also acts as a competitive inhibitor. However, the clinical impact of competitive inhibition on (S)-warfarin elimination was predicted to be minimal (i.e., at most 3%) at typical doses of nescapine, whereas that of MBI could be significant (Fig. 8). Our study predicted that coadministration of nescapine and warfarin could result in up to 7-fold increases in AUC of (S)-warfarin by MBI mechanism. Overall, the extent of the increase in (S)-warfarin AUC appears consistent with the result from a clinical study in which a >2-fold increase in the INR was seen in patients coadministered with nescapine (as an antitussive drug) and warfarin (Ohlsson et al., 2008). The typical dose of nescapine as a cough suppressant (50 mg) produces a C_{max} of $\sim 0.5 \mu M$ (Haikala et al., 1986; Olsson et al., 1986). This concentration would require up to a 70% decrease in warfarin dose in both extensive and intermediate metabolizer patients but little change in PM patients, especially in carriers of CYP2C9*3/*3 (Fig. 8; Table 1).

Our estimation is based on the previously reported plasma C_{max} after a single oral dose of nescapine ranging from 50 to 300 mg, but a higher C_{max} may be achieved clinically. In fact, the dosage of off-label use of nescapine as an anticancer agent ranges from 1000 to 2250 mg per day divided into three doses (MedInsight Research Institute, <http://www.pcref.org/MedInsight%20-%20PCREF%20Nescapine%20Review.pdf>). Furthermore, nescapine is known to exhibit a nonlinear pharmacokinetic behavior in humans (Karlsson et al., 1990); a 3-fold increase in dose (from 100 to 300 mg) leads to a 9-fold increase in AUC. Thus, the extent of pharmacokinetic interactions between warfarin and nescapine may be greater than what was estimated in this study, indicating that careful monitoring of the INR will be required when nescapine is combined with warfarin.

In conclusion, we have shown that nescapine is both a competitive inhibitor and a mechanism-based inactivator of (S)-warfarin 7-hydroxylation by CYP2C9. Our in-depth kinetic analysis of the inhibition by nescapine provided a basis to predict genotype-dependent changes in warfarin exposure upon various concentrations of nescapine. Significant changes in warfarin dosage are required due to the MBI of CYP2C9 by nescapine. These findings should shed light on a better understanding and prediction of drug-drug interaction involving nescapine and CYP2C9.

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Authorship Contributions

Participated in research design: Y.-Y. Zhang, Kunze, Jeong.

Conducted experiments: N. Zhang, Seguin.

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