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

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ABSTRACT

Noscapine is an antitussive and potential anticancer drug. Clinically significant interactions between warfarin and noscapine have been previously reported. In this study, to provide a basis for warfarin dosage adjustment, the inhibition kinetics of noscapine against warfarin metabolism was characterized. Our enzyme kinetics data obtained from human liver microsomes and recombinant CYP2C9 proteins indicate that noscapine is a competitive inhibitor of the (S)-warfarin 7-hydroxylation reaction by CYP2C9. Interestingly, noscapine also inhibited (S)-warfarin metabolism in a NADPH- and time-dependent manner, and removal of unbound noscapine and its metabolites by ultrafiltration did not reverse inhibition of (S)-warfarin metabolism by noscapine, suggesting mechanism-based inhibition of CYP2C9 by noscapine. Spectral scanning of the reaction between CYP2C9 and noscapine revealed the formation of an absorption spectrum at 458 nm, indicating the formation of a metabolite-intermediate complex. Surprisingly, noscapine 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 noscapine coadministration, mainly due to mechanism-based inactivation of CYP2C9 by noscapine. Together, these results indicate that mechanism-based inhibition of CYP2C9 by noscapine may dramatically alter pharmacokinetics of warfarin and provide a basis for warfarin dosage adjustment when noscapine is coadministered.

Introduction

Noscapine (Fig. 1), a nonaddictive phthalideisouquinoline alkaloid obtained from opium poppy latex, has been broadly recognized as a safe and promising oral antitussive agent (Karlsson et al., 1990). Recently, noscapine has shown antiproliferative activity against a wide variety of tumor cell types (Mahmoudian and Rahimi-Moghaddam, 2009). Pretreatment with oral noscapine was also proven to limit lymphatic metastasis of human prostate cancer PC3 cells in nude mouse models (Barken et al., 2010). Noscapine 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 noscapine are being investigated for non–small-cell lung cancer and triple negative breast cancer (Chougule et al., 2011a,b). As an anticancer agent, noscapine is used at 6- to 15-fold higher doses than as a cough suppressant (MedInsight Research Institute, http://www.pcrf.org/MedInsight%20-%20PCREF%20Noscapine%20Review.pdf). In humans, the pharmacokinetic behavior of noscapine shows a relatively high interindividual variation, as well as extensive “first pass” metabolism due to carbon–carbon bond cleavage, O-demethylation, monohydroxylation, and demethylation 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 (Striff, 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...
optimal warfarin therapy. Clinical evidence indicates that noscapine 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 noscapine used for cancer treatment (Madan et al., 2011), clinically significant interactions between noscapine and warfarin are expected. However, the magnitude and underlying mechanisms of noscapine-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 noscapine 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 benz bromarone, sulfamethizole, and progesterone was greater with (S)-warfarin than phenytoin, tolbutamide, and diclofenac 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 the present study, we examined the inhibitory mechanisms of noscapine against (S)-warfarin 7-hydroxylation at noscapine 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 noscapine to guide warfarin dosage adjustment. The extent of the in vivo noscapine-warfarin interaction was then predicted from the obtained kinetic data.

Materials and Methods

Chemicals and Reagents. (S, R)-Noscapine, (S)-warfarin, 7-hydroxywarfarin, mebendazole, phenytoin, phosphate-buffered saline (PBS), isoxic acid, magnesium chloride, isoxic 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_{IC} Pooled HLMs (0.1 mg/ml) were incubated with (S)-warfarin (0–20 μM) in a NADPH-regenerating system (5 mM isoxic acid, 0.2 units/ml isoxic 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 16,100g 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-MSMS). 7-Hydroxywarfarin formation rates were determined, and the K_{IC} 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 noscapine (0–100 μM) in a NADPH-regenerating system for 20 minutes. The warfarin concentration was selected based on the apparent K_{IC} 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) (Madison et al., 2013). 7-Hydroxywarfarin formation rates were determined, and the half-maximal inhibitory concentration (IC_{50}) values were estimated.

Determination of K_{i} Pooled HLMs (1.0, 2.5, 5.0, and 10 μM) was incubated with pooled HLMs or recombinant CYP2C9.1 in the presence of noscapine at different concentrations (0–25 μM), and the concentration of 7-hydroxywarfarin was measured. The K_{i} values (enzyme-inhibitor constant) of noscapine 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:

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

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. Noscapine (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 noscapine 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 incubation 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_{m} and K_{m,u}. Noscapine (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 μl) was transferred into a secondary incubation system containing (S)-warfarin (25 μM), 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 Km values obtained from different enzyme sources (Hutzler et al., 2009; Liu et al., 2012). The observed rates of inactivation (kobs) were determined from the slopes of residual CYP2C9 activity versus time point. Inactivation kinetic parameters [theoretical maximal inactivation rate (kmax) and inactivator concentration required for half the maximal inactivation rate (Kf)] were calculated using nonlinear regression of the data according to eq. 4:

\[ k_{obs} = k_{inact},\text{eff} + \frac{k_{max}}{K_f} \left[ I \right] \]

where [I] represents the initial concentrations of noscapine in the inactivation preincubations; k_{inact,eff} is the apparent inactivation rate constant measured in the absence of noscapine; and unbound Kf (Kf\_u) is Kf multiplied by f_u, where f_u is the free fraction of noscapine in the primary microsomal incubation (1 mg/ml).

Effects of Ultrafiltration on CYP2C9 Inactivation. HLMs (1 mg/ml) were preincubated with ABT (1 mM), noscapine (50 μM), sulfaphenazole (10 μM), or vehicle (control) in a NADPH-regenerating system for 20 minutes at 37°C. The preincubation mixtures (100 μl) 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 μl 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 μl) were removed and diluted 10-fold to determine the residual CYP2C9 activities by incubating with warfarin (2.5 μM).

Determination of f_u. The Single-Use Rapid Equilibration 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 μl) containing noscapine (0.3–50 μM) were placed into the sample chamber, and 300 μl 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 μl) were removed from each side of the insert and placed in separate tubes. The same volume (50 μl) 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 μl of acetonitrile containing phenytoin (1 μM; 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. Noscapine concentrations in the supernatant were determined by LC-MS/MS.

Measurement of 7-Hydroxywarfarin and Noscapine. 7-Hydroxywarfarin concentrations in microsomal samples were determined by an LC-MS/MS method as previously described, with slight modifications (Li 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 X Terra MS C18 column (2.1 × 50 mm, 3.5 μm; Waters Corporation, Milford, MA). The mobile phase was delivered at 250 μl/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 [MS3/323/176 atomic mass unit (amu)], and mebendazole was used as the internal standard (294/262 amu) in negative ion mode. The mobile phase for noscapine 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 μl/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 noscapine (MS/MSMs 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 μM, 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 μl methanol) and substrate to the sample cuvette (2 μl of noscapine stock solution) to yield a final concentration of 50 μM noscapine. 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 cm⁻¹ × 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 cm⁻¹ × 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 monoeponential function MIC\_u = MIC\_max (1 - e⁻ctica), where MIC\_u 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 noscapine were predicted based on reversible inhibition (eq. 5) or time-dependent inhibition mechanism (eq. 6) (Brown et al., 2005; Grimm et al., 2009):

\[ \frac{AUC_i}{AUC} = 1 - \frac{f_u(CYP2C9)}{1 + \frac{K_{inact,u}}{f_u(CYP2C9)}} \]

(5)

\[ \frac{AUC_i}{AUC} = \frac{1}{1 + \frac{f_u(CYP2C9)}{K_{inact,u}} + (1 - f_u(CYP2C9))} \]

(6)

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

The extent of CYP2C9-mediated metabolism in overall (S)-warfarin elimination (f_u(CYP2C9)) was estimated by using the following equations (Ito et al., 2005):

\[ CL_{EM} = f_u(CYP2C9) CL_{EM} + (1 - f_u(CYP2C9)) CL_{EM} \]

(7)

\[ CL_{EM} = f_u(CYP2C9) CL_{EM} + (1 - f_u(CYP2C9)) CL_{EM} \]

(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 $f_{EM}^{CL_{CYP2C9}}$ decrease by the $CL_{EM}/CL_{CYP2C9}$ ratio:

$$f_{EM}^{CL_{CYP2C9}} = 1 - \left(1 - f_{EM}^{CL_{CYP2C9}}\right) \frac{CL_{EM}}{CL_{CYP2C9}}$$ (9)

The $f_{EM}^{CL_{CYP2C9}}$ 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 $f_{EM}^{CL_{CYP2C9}}$ 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/AUC values were subsequently calculated and plotted against $[I]_m$ using $f_{EM}^{CL_{CYP2C9}}$ 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):

$$Dose\; Adjustment\; Factor_{Genotype} = \frac{AUC_{EM}}{AUC_I} = 1 - f_{EM}^{CL_{CYP2C9}}(1 - FA)$$ (10)

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

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

**Results**

**Inhibition of (S)-Warfarin 7-Hydroxylation by Noscapine.** 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 ± 0.3 μM and 6.9 ± 0.2 pmol/min/mg protein, respectively (Fig. 2A). In the subsequent experiments using 2.5 μM (S)-warfarin, noscapine inhibited CYP2C9-mediated (S)-warfarin 7-hydroxylation with an $IC_{50}$ of 6.5 ± 0.7 μM in pooled HLMs (Fig. 2B). In cDNA-expressed enzyme preparations of CYP2C9.1, the $IC_{50}$ value was 2.6 ± 0.1 μM. Relatively small decreases in inhibition potency were noted in CYP2C9.2 and CYP2C9.3 enzymes ($IC_{50}$: 3.6 ± 0.2 and 4.9 ± 0.7 μM, respectively).

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

**Time- and NADPH-Dependent Inhibition of CYP2C9.** Previously, noscapine was shown to inhibit diclofenac 4'-hydroxylation via a mechanism of time-dependent inhibition (Fang et al., 2010). To examine whether noscapine inhibits warfarin metabolism by a similar mechanism, noscapine 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 minute$^{-1}$ and $K_i$ of 6.8 μM. The estimated $k_{inact}/K_i$ was 6.0 ml/min/μmol, indicating high inactivation efficiency. To evaluate the effects of CYP2C9 genotypes on the time-dependent inhibition potential of noscapine, single point inactivation experiments were performed using recombinant wild-type CYP2C9 and its variants. To this end, a recombinant CYP2C9 protein was preincubated with noscapine (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 noscapine (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% ± 5% (Fig. 5A), indicating significant inactivation of the enzyme by noscapine. Interestingly, the CYP2C9 variants associated with the PM phenotype were found to be more susceptible to inactivation by noscapine than the wild type; the decrease in activity was 71% ± 7% (CYP2C9.2, $P < 0.01$) and 59% ± 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 noscapine with a $K_i$ of 4.4 μM and $k_{inact}$ of 0.013 minute$^{-1}$. Although $K_i$ values for CYP2C9.2 (5.7 μM) and CYP2C9.3 (2.6 μM) were similar to that of CYP2C9.1, their $k_{inact}$ values increased noticeably (0.033 minute$^{-1}$ for CYP2C9.2 and 0.022 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, noscapine is
both a competitive and time-dependent inhibitor of CYP2C9, and its inhibitory potency differs among CYP2C9 variants.

**Effect of Ultrafiltration on CYP2C9 Inactivation by Noscapine.**

To determine the reversibility of the inactivation of CYP2C9 by noscapine, NADPH, unbound noscapine, and its metabolites were removed from the preincubation samples by ultrafiltration, and the remaining CYP2C9 activity was determined by measuring \((S)-\text{warfarin} \ 7\text{-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 noscapine and ABT showed less than 10\% recovery of } \,(S)-\text{warfarin} \ 7\text{-hydroxylase activity after ultrafiltration, whereas } >30\% \text{ recovery was detected with sulfaphenazole (Fig. 6). Together, these results indicate that noscapine-mediated CYP2C9 inhibition is irreversible, and that, similarly to ABT, noscapine 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 noscapine 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/μM 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)-\text{warfarin} \ 7\text{-hydroxylation by noscapine was mainly due to the formation of MI complex with CYP2C9.}

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

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**Fig. 3.** Representative Lineweaver-Burk plots and Dixon plots showing the effects of noscapine on \((S)-\text{warfarin } 7\text{-hydroxylation in pooled HLMs (A and B) and recombinant CYP2C9.1 (C and D). Ctrl, control.**
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 \( \mu M \) reflect \( C_{\text{max}} \) obtained from a typical oral dose of noscapine for antitussive or anticancer effects.

Fig. 4. Time- and concentration-dependent inhibition of (S)-warfarin 7-hydroxylation by noscapine (0–50 \( \mu M \)) 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 \( (k_{\text{obs}}) \) was determined by linear regression analysis of the natural logarithm of the percentage of activity remaining versus preincubation time data (A). \( K_I \) and \( k_{\text{inact}} \) were calculated by nonlinear regression analysis of the \( k_{\text{obs}} \) versus noscapine concentration data according to the equation described in Materials and Methods (B).

Fig. 5. NADPH-dependent inactivation of (S)-warfarin 7-hydroxylation by noscapine (15 \( \mu M \)) in recombinant CYP2C9 (A). Kinetic plots demonstrating observed rates of inactivation \( (k_{\text{obs}}) \) by noscapine (0–50 \( \mu M \)) in recombinant CYP2C9.1 (B), CYP2C9.2 (C), and CYP2C9.3 (D).
Figure 8 shows three-dimensional (and contour) plots for the estimated AUC/AUC ratios when noscapine 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 noscapine is predicted to cause a 7-fold increase in the exposure of (S)-warfarin by MBI (Fig. 8A) but a minimal increase (3%) by the competitive inhibition mechanism (Fig. 8B). The dose adjustment factor was then estimated for warfarin based on the combined effects of noscapine-mediated inhibition and CYP2C9 genotypes on warfarin exposure (Fig. 8C; Table 1). The coadministration of noscapine 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 noscapine 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). Noscapine 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 noscapine-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 noscapine-mediated inhibition remains unknown. The objective of this study was to investigate the inhibitory mechanisms of (S)-warfarin metabolism by noscapine and examine the effects of common genetic polymorphisms of CYP2C9 on the extent of noscapine-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, noscapine was found to be a competitive inhibitor of CYP2C9-mediated (S)-warfarin 7-hydroxylation in both HLMs and recombinant enzyme systems (Ki<sub>10</sub> μM) (Fig. 3). Interestingly, when diclofenac 4'-hydroxylation was used as the probe reaction, noscapine 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, noscapine 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 noscapine, the IC<sub>50</sub> 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<sub>i</sub> 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 noscapine is likely due to differences in the conformation of the active site and the orientation of inhibitor binding (Kumar et al., 2006). Taken together, noscapine inhibits CYP2C9-mediated (S)-warfarin metabolism via a competitive
inhibition mechanism, and the inhibitory profiles exhibit substrate- and genotype-dependent variation.

Our study revealed that noscapine is also a mechanism-based inhibitor of CYP2C9, exhibiting time- and NADPH-dependent inhibition (Fig. 4). Removal of free noscapine and its metabolites from the HLM incubation using ultrafiltration did not reverse noscapine-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 noscapine. This is reasonable because noscapine 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 noscapine by CYP2C9 is responsible for the MBI by noscapine, 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 noscapine was 2-fold more efficient (i.e., kinact/KI) for CYP2C9.2 and CYP2C9.3 than for CYP2C9.1. This suggests that formation of a reactive metabolite(s) of noscapine may be greater from CYP2C9.2 and CYP2C9.3. In fact, it was previously shown that a metabolite of noscapine 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

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Fraction of Dose Expected to be Cleared by CYP2C9 (fm(CYP2C9))</th>
<th>Magnitude of the Potential (S)-Warfarin-Noscapine Interactions (AUC/AUC)</th>
<th>Dose Adjustment Factorb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Based Solely on Genotypes</td>
<td>Based on Both Genotypes and Inhibitory Effects of Noscapine</td>
</tr>
<tr>
<td>CYP2C9*1/*1</td>
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<td>1.12–7.36</td>
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<tr>
<td>CYP2C9*1/*2</td>
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<td>1.09–5.20</td>
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<tr>
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<td>1.09–4.96</td>
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<tr>
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<td>1.06–3.38</td>
<td>0.72</td>
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<tr>
<td>CYP2C9*2/*3</td>
<td>0.23–0.66</td>
<td>1.03–2.63</td>
<td>0.44</td>
</tr>
<tr>
<td>CYP2C9*3/*3</td>
<td>0–0.13</td>
<td>1.00–1.14</td>
<td>0.20</td>
</tr>
</tbody>
</table>

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

Dose adjustment ratios were predicted at the upper most value of estimated fm(CYP2C9).

Inhibition of (S)-Warfarin Metabolism by Noscapine

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

All simulations were performed at noscapine Cmax 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).

Table 1

Figure 8.

Three-dimensional and contour plots for the calculated AUC/AUC ratios with fm(CYP2C9) and Cmax of noscapine in plasma resulting from MBI (A) or competitive inhibition (B). Suggested dose adjustment factors based on the range of noscapine plasma Cmax values and patients of different genotypes (C).


Inhibition of (S)-Warfarin Metabolism by Noscapine

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