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

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List of abbreviations:

ABT 1-aminobenzotriazole
AUC area under the plasma drug concentration versus time curve
AUC/AUC predicted ratio of \textit{in vivo} exposure of (S)-warfarin with vs. without coadministration of noscapine
cDNA complementary DNA
C\textsubscript{max} maximal plasma concentration
C\textsubscript{max,u} unbound maximal plasma concentration
CYP cytochrome P450
DDI drug-drug interactions
FA fraction of CYP2C9 activity resulting from the combination of mutated alleles
f\text{int}(CYP2C9) the extent of CYP2C9-mediated metabolism in overall (S)-warfarin elimination
f\text{u} free fraction of noscapine in pooled human serum
HLMs human liver microsomes
HPLC high-performance liquid chromatography
[I]\textsubscript{in vivo} inhibitor concentration at the enzyme active site
IC\textsubscript{50} half maximal inhibitory concentration
INR international normalized ratio
k\text{deg}(CYP2C9) first-order rate constant of in vivo degradation of CYP2C9
K\text{I} enzyme-inhibitor constant
K\text{I,u} reversible inhibition constant adjusted by the free fraction of noscapine in pooled HLMs
K\text{I} inactivator concentration required for half the maximal inactivation rate
K\text{I,u} unbound K\text{I} adjusted by the free fraction of noscapine in pooled HLMs
k\text{inact} maximal inactivation rate
K\text{m} substrate concentration that yields a half-maximal velocity
k\text{obs} observed rate of inactivation
LC-MS/MS  liquid chromatography-tandem mass spectrometry
MBI    mechanism-based inactivation
MI     metabolite-intermediate
S, I   concentrations of substrate and inhibitor, respectively
TDI    Time-Dependent Inhibition
v, V_max velocity and maximal velocity of the enzymatic reaction, respectively
VTE    venous thromboembolism
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 co-administered.
Introduction

Noscapine (Figure 1), a non-addictive phthalideisoquinoline 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 PC3 human prostate cancer in nude mouse models (Barken et al., 2010). Noscapine is currently undergoing phase I/II trials for non-Hodgkin’s lymphoma or hematological malignancies treatment (Aneja et al., 2007), and combination chemotherapy regimens with noscapine are being investigated for human non-small lung cancer and triple negative breast cancer (Chougule et al., 2011a; Chougule et al., 2011b). As an anticancer agent, noscapine is used at 6- to 15-fold higher doses than as a cough suppressant [MedInsight Research Institute, http://www.pcref.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 C-C cleavage, O-demethylation, monohydroxylation and demethylation reactions (Tsunoda and Yoshimura, 1981). Several cytochrome P450 (CYP) 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 its 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 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 (DDI) with
warfarin when administrated 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 therefore expected. However, the magnitude and underlying mechanisms of noscapine-warfarin interaction remain unknown.

Warfarin is administrated as a racemic mixture and undergoes stereoselective metabolic clearance in the human liver. (S)-warfarin (Figure 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 benzbromarone, sulfamethizole, and progesterone was greater with (S)-warfarin than phenytoin, tolbutamide and diclofenac was 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 to 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 noscapine remains to be characterized specifically for warfarin considering its narrow therapeutic index.
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 \textit{in vitro} inhibition kinetics using pooled HLMs and complementary DNA (cDNA)-expressed CYP2C9. Furthermore, we examined the effects of CYP2C9 genotype on the inhibitory potency of noscapine to guide warfarin dosage adjustment. The extent of the \textit{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), 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), .2 (R144C), and .3 (I359L) coexpressing human NADPH-CYP reductase and human cytochrome b5 were obtained from BD Biosciences (Woburn, MA). Formic acid (ACS grade), acetonitrile and methanol (Optima grade) were purchased from Fisher Scientific (Pittsburgh, PA). All other reagents were of high-performance liquid chromatography (HPLC) 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 NADPH-regenerating system (5 mM isocitric acid, 0.2 unit/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 min, the reactions were started by addition of NADP\(^+\) (1mM) and further incubated for another 20 min. The reactions were then terminated by adding 100 µL ice-cold acetonitrile containing mebendazole (1 µM) as internal standard and kept on ice for 30 min, followed by centrifugation at
16,100 g for 15 min 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 by using GraphPad Prism 5 software (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 NADPH-regenerating system for 20 min. 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 noscapine at different concentrations (0-25 µM), and the concentration of 7-hydroxywarfarin was measured. The K$_i$ values 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:

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

(eq. 1)

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

(eq. 2)

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

(eq. 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 \( (V_{\text{max}}) \). The inhibition type was determined from the Lineweaver-Burk plot, 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 pre-incubated with CYP2C9.1, CYP2C9.2, or CYP2C9.3 (400 pmol/ml) for 30 min at 37°C with 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 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 regeneration system for the measurement of residual CYP2C9 activity.

**Determination of \( K_i \) and \( k_{\text{inact}} \).** Noscapine (0-50 µM) was pre-incubated with pooled HLMs (1 mg/ml) or recombinant CYP2C9 enzymes (400 pmol/ml) in NADPH-regenerating system. At different time points (0-30 min), 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 \( K_m \) values obtained from different enzyme sources (Hutzler et al., 2009; Liu et al., 2012). The observed rates of inactivation (\( k_{\text{obs}} \)) were determined from the slopes of residual CYP2C9 activity vs.
time plot. Inactivation kinetic parameters (k_{\text{inact}} and K_I) were calculated using nonlinear regression of the data according to eq. 4.

\[
\frac{k_{\text{obs}}}{k_{\text{obs}[I=0]+k_{\text{inact}}[I]}} = \frac{[I]}{K_I+[I]} \tag{4}
\]

where [I] represents the initial concentrations of noscapine in the inactivation preincubations; k_{\text{obs}[I=0]} is the apparent inactivation rate constant measure in the absence of noscapine; k_{\text{inact}} is the theoretical maximal inactivation rate; K_I is the inactivator concentration required for half the maximal inactivation rate; and unbound K_I (K_{I,u}) is K_I multiplied by f_{u,m}, where f_{u,m} 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 pre-incubated with ABT (1 mM), noscapine (50 µM), sulfaphenazole (10 µM), or vehicle (control) in NADPH-regenerating system for 20 min 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; cut-off 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 14,000 g for 10 min, collected by a reverse spin at 1000 g for 2 min, 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,m}** Single-Use Rapid Equilibrium Dialysis plate from Thermo Scientific-Pierce Biotechnology (Rockford, IL) with cutoff molecular weight 8000 Da was used. HLMs (0.1 mg/ml or 1 mg/ml) solutions (100 µl) containing noscapine (0.3-50 µM) were placed into the sample chamber and 300 µL 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 microsomes 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 sec and chilled on ice for 30 min, then centrifuged at 16,100g for 15 min 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 a LC-MS/MS method as previously described with slight modifications (Liu et al., 2012). An Agilent 1200 HPLC interfaced with Applied Biosystems Qtrap 3200 equipped with an electrospray ion source was used. Chromatographic separation was carried out with a Waters XTerra MS C18 column (2.1×50 mm, 3.5 μm; Waters Corporation, Milford, MA). Mobile phase was delivered 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 min, held constant for 1 min, and then restored to the initial composition. 7-Hydroxywarfarin was detected by MS/MS (323/176 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 min at a 200 μl/min flow rate. The elution was then ramped linearly to 90% mobile phase B over 1.5 min, maintained for 5 min, and followed by a return to initial conditions. Multiple reaction monitoring for noscapine (MS/MS 414/220 amu) was performed in positive ionization mode, and phenytoin (MS/MS 253/182 amu) was employed as the internal standard.
Spectral Analysis of Metabolite-Intermediate (MI) 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 min of pre-incubation 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 500 to 400 nm wavelength light (readings at 1 nm intervals) at 1, 2, 4, 8, and 16 min. Once MI complex formation had ceased, the incubation was quenched by adding a few crystals of dithionite to each cuvette. Following a 3 min 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 taken to determine the amount of residual CYP capable of binding CO. The amount of CO-bound CYP was quantified by applying a molar extinction coefficient of 91 cm⁻¹mM⁻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 metabolic intermediate (MI) complex formation was captured by measuring the absorbance at 458 nm and 490 nm from 0 min to 20 min and plotting the increase in difference in absorbance over time. The concentrations of MI complexes was approximately calculated by dividing the absorbance difference at 458 nm and 490 nm by the molar extinction coefficient of 65 cm⁻¹mM⁻¹ (Manno et al., 1988). The initial rates of MI complex formation (k) and maximal concentration of MI complex (MICmax) were calculated by fitting the data to the standard monoexponential function MICt = MICmax (1 - e⁻kt), where MICt 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 co-administration of noscapine were predicted based on reversible inhibition (eq. 5) or TDI mechanism (eq. 6) (Brown et al., 2005; Grimm et al., 2009).

\[
\frac{AUC_i}{AUC} = \frac{1}{1 - f_m(CYP2C9) + \frac{f_m(CYP2C9)}{1 + \frac{[I]_{\text{invivo}}}{K_{i,u}}}}
\]  

(5)

\[
\frac{AUC_i}{AUC} = \frac{1}{1 + \frac{f_m(CYP2C9)}{k_{\text{inact}} \times [I]_{\text{invivo}}} + (1 - f_m(CYP2C9))}
\]  

(6)

$AUC_i/AUC$ is the predicted ratio of *in vivo* exposure of (S)-warfarin with vs. without co-administration of noscapine; $k_{\text{deg (CYP2C9)}}$ is the first-order rate constant of *in vivo* degradation of CYP2C9 [0.00026 min⁻¹ (Obach et al., 2007)]; $K_{i,u}$ is $K_i$ adjusted by the free fraction of noscapine in 0.1 mg/ml pooled HLMs (= $K_i \times f_u$); $k_{\text{inact}}$ is the theoretical maximal inactivation rate; and $K_{i,u}$ is the unbound inhibitor concentration at 1 mg/ml microsomes yielding 0.5 times $k_{\text{inact}}$. $[I]_{\text{invivo}}$ represents the inhibitor concentration at the enzyme active site. The unbound $C_{\text{max}}$ at steady state (defined as $f_u \times C_{\text{max}}$, $C_{\text{max},u}$) was used for $[I]_{\text{invivo}}$ because $C_{\text{max},u}$ yields the most accurate predictions of drug-drug interactions (Obach et al., 2007) and the use of total $C_{\text{max}}$ typically leads to overprediction of DDI risk (Fujioka 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 mg, 200 mg and 300 mg noscapine tablet led to the $C_{\text{max}}$ values of 0.31 µM, 0.95 µM, and 2.1 µM, respectively (Karlsson et al., 1990). A recent clinical study with the dosage of 50 mg demonstrated that the noscapine concentration at 4 hours after dosing ranged from 0.02-0.19 µM (Rosenborg et al., 2010). Based on all these previous data, $C_{\text{max}}$ values ranging from 0.02-2.1 µM were selected to estimate the $AUC_i/AUC$ ratio.
The extent of CYP2C9-mediated metabolism in overall (S)-warfarin elimination ($f_{m(CYP2C9)}$) was estimated by using the following equations (Ito et al., 2005).

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

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

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

$$f_{m(CYP2C9)}^{XM} = 1 - \frac{(1 - f_{m(CYP2C9)}^{EM}) CL^{EM}}{CL^{XM}}$$  \hspace{1cm} (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 0-0.92 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 $[I]_{in \, vivo}$ 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).

$$Dose \, \text{Adjustment Factor}_{Genotype} = \frac{AUC^{EM}}{AUC^{XM}} = 1 - f_{m(CYP2C9)}^{EM} (1 - FA)$$  \hspace{1cm} (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.

\[
Dose \text{ Adjustment Factor} = Dose \text{ Adjustment Factor}_{\text{Genotype}} \times \frac{AUC}{AUC_i} \tag{11}
\]

Results

Inhibition of (S)-warfarin 7-hydroxylation by Noscapine. To determine the (S)-warfarin concentration employed 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 \(\mu\)M and 6.9 ± 0.2 pmol/min/mg protein, respectively (Figure 2A). In the subsequent experiments using 2.5 \(\mu\)M (S)-warfarin, noscapine inhibited CYP2C9-mediated (S)-warfarin 7-hydroxylation with an IC\(_{50}\) of 6.5 ± 0.7 \(\mu\)M in pooled HLMs (Figure 2B). In cDNA-expressed enzyme preparations of CYP2C9.1, the IC\(_{50}\) value was 2.6 ± 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 \(\mu\)M and 4.9 ± 0.7 \(\mu\)M, respectively). As shown in Figure 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 \(\mu\)M (in pooled HLMs) and 1.0 ± 0.1 \(\mu\)M (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 by using (S)-warfarin as the probe drug. The resulting inactivation kinetics was best fit to the standard hyperbolic equation (Figure 4), with $k_{\text{inact}}$ of 0.041 min$^{-1}$ and $K_I$ of 6.8 μM. The estimated $k_{\text{inact}}/K_I$ 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 pre-incubated with noscapine (or vehicle) in the presence (or absence) of NADPH for 30 min, 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 to the activity in the absence of NADPH) was used as a cut-off 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% (Figure 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, $p<0.05$) (Figure 5A). Similar results were also observed when inactivation kinetics was compared among different CYP2C9 variants (Figure 5B through 5D). CYP2C9.1 was inactivated by noscapine with $K_I$ of 4.4 μM and $k_{\text{inact}}$ of 0.013 min$^{-1}$. Although $K_I$ values for CYP2C9.2 (5.7 μM) and CYP2C9.3 (2.6 μM) were similar to that of CYP2C9.1, their $k_{\text{inact}}$ values increased noticeably (0.033 min$^{-1}$ for CYP2C9.2 and 0.022 min$^{-1}$ 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)-warfarin 7-hydroxylation rates. ABT (a mechanism-based inactivator of
CYPs) 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)-warfarin 7-hydroxylase activity after ultrafiltration, whereas >30% recovery was detected with
sulfaphenazole (Figure 6). Together, these results indicate that noscapine-mediated CYP2C9 inhibition is
irreversible and that similarly to ABT, noscapine inhibits CYP2C9 by mechanism-based inactivation
(MBI).

Spectral Analysis of MI Complex Formation. In order 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 (Figure 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
(Figure 7B). Based on the data for the first 30 sec of reaction, the initial rate of MI complex formation
was calculated to be 0.0133 dAbs/µM CYP/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 sum, all these spectra data combined together
suggested that MBI of (S)-warfarin 7-hydroxylation by noscapine was mainly due to the formation of MI
complex with CYP2C9.

Prediction of In Vivo (S)-Warfarin-Noscapine Interactions. The clinical impact of CYP2C9
inhibition by noscapine 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 non-specific binding to 0.1 mg/ml ($f_{uu,m} = 97 \pm 3\%$) and 1.0 mg/ml ($f_{uu,m} = 22 \pm 1\%$) HLM proteins, respectively, yielding the adjusted values ($K_{ii, u}$ of 4.5 $\mu$M and $K_{ii, u}$ of 1.5 $\mu$M). AUC changes upon noscapine co-administration were predicted for varying $f_{m(CYP2C9)}$ and noscapine concentrations. Values of $f_{m(CYP2C9)}$ ranging from 0 to 0.92 reflect the differential CYP2C9 enzyme activity of CYP2C9 variants (Table 1; see materials and methods for details), while noscapine concentrations ranging from 0.02 to 2.1 $\mu$M reflect $C_{max}$ obtained from a typical oral dose of noscapine for antitussive or anticancer effects. Figure 8 shows 3-dimensional (and contour) plots for the estimated $AUC_i/AUC$ ratios when noscapine inhibits CYP2C9 activity only by MBI (Figure 8A) or competitive inhibition mechanism (Figure 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 (Figure 8A) but a minimal increase (~3%) by the competitive inhibition mechanism (Figure 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 (Figure 8C and 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 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, the direct in vitro evidence on 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 the 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<10 μM) (Figure 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 difference in inhibitory mechanism was 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 the access of (S)-warfarin to the substrate-binding site in the CYP2C9 active
pocket, but not that of diclofenac, 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 IC₅₀ value for CYP2C9.3 was 1.9-fold higher than that for CYP2C9.1 (Figure 2). This result is in good agreement with a previous study in which fluconazole Kᵢ 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 (Figure 4). Removal of free noscapine and its metabolites from the HLM incubation by using ultrafiltration did not reverse noscapine-mediated inhibition of (S)-warfarin metabolism, indicating irreversibility of the inhibition (Figure 6). Our spectral analysis further demonstrated a time-dependent increase in the maximum absorbance at 458 nm (Figure 7A), suggesting the formation of MI complex by noscapine. This is reasonable because noscapine has a methylenedioxyphenyl group incorporated into its structure (Figure 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 CYP variants (Polasek and Miners, 2007; Orr et al., 2012). To verify that formation of reactive metabolite(s) of noscapine by CYP2C9 is responsible for the MBI by noscapine, the kinetics of CYP2C9 inhibition were characterized by 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/K_i) for CYP2C9.2 and CYP2C9.3 than for CYP2C9.1. This suggests that formation of 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 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 noscapine depletion rates are significantly different among these enzymes.

Noscapine 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 noscapine while that of MBI could be significant (Figure 8). Our study predicted that co-administration of noscapine 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 co-administered with noscapine (as an antitussive drug) and warfarin (Ohlsson et al., 2008). The typical dose of noscapine as a cough suppressant (50 mg) produces a C_{max} of ~0.5 µM (Haikala et al., 1986; Olsson et al., 1986). This concentration would require up to 70% decrease in warfarin dose in both EM and IM patients but little change in PM patients, especially in carriers of CYP2C9*3/*3 (Figure 8 and Table 1).

Our estimation is based on the previously reported plasma C_{max} after a single oral dose of noscapine ranging from 50 to 300 mg, but a higher C_{max} may be achieved clinically. In fact, the dosage of
off-label use of noscapine as an anticancer agent ranges from 1,000 to 2,250 mg per day divided in 3 doses [MedInsight Research Institute, http://www.pcref.org/MedInsight%20-%20PCREF%20Noscapine%20Review.pdf]. Furthermore, noscapine is known to exhibit a nonlinear pharmacokinetic behavior in humans (Karlsson et al., 1990); a 3-fold increase in dose (from 100 mg to 300 mg) leads to a 9-fold rise in AUC. Thus, the extent of pharmacokinetic interactions between warfarin and noscapine may be greater than what was estimated in this study, indicating that careful monitoring of INR will be required when noscapine is combined with warfarin.

In conclusion, we have shown that noscapine 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 noscapine provided a basis to predict genotype-dependent changes in warfarin exposure upon various concentrations of noscapine. Significant changes in warfarin dosage are required due to the MBI of CYP2C9 by noscapine. These findings should shed light on better understanding and prediction of drug-drug interaction involving noscapine and CYP2C9.
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Authorship Contributions

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

Conducted experiments: N. Zhang and Seguin.

Performed data analysis: N. Zhang and Seguin.

Wrote or contributed to the writing of the manuscript: N. Zhang, Y.-Y. Zhang, Seguin, Kunze and Jeong.
References


Footnotes

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Legends to figures

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

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

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

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

Figure 5. NADPH-dependent inactivation of (S)-warfarin 7-hydroxylation by noscapine (15 μM) in recombinant CYP2C9 (A). Kinetic plots demonstrating observed rates of inactivation ($k_{\text{obs}}$) by noscapine (0-50 μM) in recombinant CYP2C9.1 (B), CYP2C9.2 (C), and CYP2C9.3 (D).
Figure 6. Effects of ultrafiltration on the time- and NADPH-dependent inhibitory potential of noscapine towards CYP2C9-mediated (S)-warfarin 7-hydroxylation.

Figure 7. Spectral evidence for the formation of an MI complex with CYP2C9.1 and noscapine (50 µM) (A) and the time course for complex formation (B).

Figure 8. 3-Dimensional and contour plots for the calculated AUC/AUC ratios with \( f_{\text{m(CYP2C9)}} \) and \( C_{\text{max}} \) of noscapine in plasma resulted from MBI (A) or competitive inhibition (B). Suggested dose adjustment factors based on the range of noscapine plasma \( C_{\text{max}} \) values and patients of different genotypes (C).
TABLE 1

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

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Fraction of Dose Expected to be Cleared by CYP2C9</th>
<th>Magnitude of the Potential (S)-Warfarin-Noscapine Interactions</th>
<th>Dose Adjustment Factor&lt;sup&gt;c&lt;/sup&gt; Based Solely on Genotypes</th>
<th>Dose Adjustment Factor&lt;sup&gt;c&lt;/sup&gt; Based on Both Genotypes and Inhibitory Effects of Noscapine</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2C9*1/*1</td>
<td>0.82-0.92&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.12-7.36</td>
<td>1.00</td>
<td>0.14-0.88</td>
</tr>
<tr>
<td>CYP2C9*1/*2</td>
<td>0.69-0.86</td>
<td>1.09-5.20</td>
<td>0.83</td>
<td>0.16-0.74</td>
</tr>
<tr>
<td>CYP2C9*1/*3</td>
<td>0.66-0.85</td>
<td>1.09-4.96</td>
<td>0.60</td>
<td>0.12-0.53</td>
</tr>
<tr>
<td>CYP2C9*2/*2</td>
<td>0.44-0.75</td>
<td>1.06-3.38</td>
<td>0.72</td>
<td>0.21-0.65</td>
</tr>
<tr>
<td>CYP2C9*2/*3</td>
<td>0.23-0.66</td>
<td>1.03-2.63</td>
<td>0.44</td>
<td>0.17-0.40</td>
</tr>
<tr>
<td>CYP2C9*3/*3</td>
<td>0-0.13</td>
<td>1.00-1.14</td>
<td>0.20</td>
<td>0.18-0.19</td>
</tr>
</tbody>
</table>
a All simulation were performed at noscapine $C_{\text{max}}$ values range from 0.02 µmol/l to 2.1 µmol/l, and the fraction of wild-type CYP2C9 activity (FA) for each mutant were obtained directly from literature (Castellan et al., 2013). b Values were estimated from mass balance data as described previously (Kunze and Trager, 1996). c Dose adjustment ratios were predicted at the upper most value of estimated $f_{m(CYP2C9)}$. 
Fig. 1

Noscapine

(S)-Warfarin
S-Warfarin (μM) vs. 7-Hydroxywarfarin formation rate (pmol/min/mg pro) for CYP2C9.1, CYP2C9.2, CYP2C9.3, and Pooled HLMs.

A: (S)-Warfarin (μM) vs. 7-Hydroxywarfarin formation rate (pmol/min/mg pro).
B: Noscapine (μM) vs. 7-Hydroxywarfarin formation rate (% of control).

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Fig. 3

A

![Graph showing 7-Hydroxywarfarin formation rate vs. 1/(S)-Warfarin, µM](image)

B

![Graph showing 7-Hydroxywarfarin formation rate vs. Noscapine, µM](image)

C

![Graph showing 7-Hydroxywarfarin formation rate vs. 1/(S)-Warfarin, µM](image)

D

![Graph showing 7-Hydroxywarfarin formation rate vs. Noscapine, µM](image)
Fig. 5

A) 7-Hydroxywarfarin formation rate (pmol/min/nmol CYP).

B) 

\[ k_{ob} \] (Observed rate constant) vs. Noscapine concentration (µM).

C) 

\[ k_{ob} \] (Observed rate constant) vs. \( K_{obs}/\text{Noscapine} \).

D) 

\[ k_{ob} \] (Observed rate constant) vs. \( K_{obs}/\text{Noscapine} \).
Fig. 7

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