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NOSCAPINE RECIRCULATES ENTEROHEPATICALLY AND INDUCES SELF-CLEARANCE

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Abstract

Noscapine (Nos), an antitussive benzyloisoquinoline opium alkaloid, is a non-toxic tubulin-binding agent currently in Phase II clinical trials for cancer chemotherapy. While preclinical studies have established its tumor-inhibitory properties in various cancers, poor absorptivity and rapid first-pass metabolism producing several uncharacterized metabolites for efficacy, present an impediment in translating its efficacy in humans. Here we report novel formulations of Nos in combination with dietary agents like capsaicin (Cap), piperine (Pip), eugenol (Eu) and curcumin (Cur) known for modulating Phase I and II drug metabolizing enzymes. *In vivo* pharmacokinetic (PK), organ toxicity evaluation of combinations, microsomal stability and *in vitro* cytochrome P450 (CYP) inhibition effects of Nos, Cap and Pip using human liver microsomes were performed. Single-dose PK screening of combinations revealed that the relative exposure of Nos (2 µg.h/mL) was enhanced by 2-fold (4 µg.h/mL) by Cap and Pip and their plasma concentration-time profiles showed multiple peaking phenomena for Nos indicating enterohepatic recirculation or differential absorption from intestine. CYP inhibition studies confirmed that Nos, Cap and Pip are not potent CYP inhibitors (IC₅₀>1 µM). Repeated oral dosing of Nos, Nos+Cap and Nos+Pip showed lower exposure (C_{max} and AUC_{last}) of Nos on day 7 compared to day 1. Nos C_{max} decreased from 3087 ng/mL to 684 ng/mL and AUC_{last} from 1024 ng.h/mL to 508 ng.h/mL. In presence of Cap and Pip, the decrease in C_{max} and AUC_{last} of Nos was similar. This may be due to potential enzyme induction leading to rapid clearance of Nos as the trend was observed in Nos alone group also. The lack of effect on intrinsic clearance of Nos suggests that the potential drug biotransformation modulators employed in this study did not contribute towards increased exposure of Nos on repeated dosing. We envision that Nos-induced enzyme induction could alter the therapeutic

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efficacy of co-administered drugs, hence emphasizing the need for strategic evaluation of noscapine's metabolism to reap its maximum efficacy.

Graphical abstract

Keywords

CYP inhibition; noscapine; liver microsomes; LC/MS/MS

1. INTRODUCTION

Noscapine (Nos) is an antitussive agent that has been used for decades for its cough-suppressive action (Ke et al., 2000; Ye et al., 1998). Nos is a microtubule-modulating agent and was employed in Phase II clinical development for the therapy of multiple myeloma (Aneja et al., 2010). It is a weak base (pKa 7.8) with poor oral bioavailability thus necessitating administration of relatively high doses (300–450 mg/kg body weight in preclinical models) for optimal therapeutic benefits (Aneja et al., 2007; Ke et al., 2000; Ye et al., 1998). The bioavailability of Nos is around 32% in mice and its rapid first-pass metabolism and shorter half-life (<2 h) due to extensive biotransformation can result in poor translation of its efficacy in humans (Aneja et al., 2007). This is in agreement with previous reports on preclinical pharmacokinetic (PK) parameters of Nos that suggest that this tubulin-binding agent undergoes a rapid metabolism as it peaks in blood as early as 5 min, followed by complete elimination within 4 h of oral administration (Aneja et al., 2007).

Noscapine's metabolism in mice has been shown to result in the formation of at least 20 or more metabolites including a reactive intermediate that undergoes conjugation with glutathione (Fang et al., 2012). While Phase I metabolism of Nos has been shown to result in more than 10 metabolites, Phase II metabolism primarily produces at least 9 glucuronide conjugates (Fang et al., 2012). Several enzymes including cytochrome P450 (1A2, 2C8, 2C9, 2C19, 2D6 3A4, 3A5, UGTs (2B7, 1A1, 1A3, 1A9) and flavin monooxygenases (FMOs) have been shown to catalyze the biotransformation of Nos. It appears that Nos also inhibits/inactivates CYP enzymes resulting in drug-drug interactions when combined with other anti-cancer drugs such as doxorubicin (Fang et al., 2012). In a more recent study, Nos was shown to inhibit hydroxylation of (S)-warfarin by inhibiting CYP2C9 through formation of reactive metabolite (Zhang et al., 2013).

While most drugs typically undergo oxidation or hydrolysis during phase I metabolism, they conjugate to hydrophilic substances, like glucuronic acid, sulfate, and acetyl during phase II metabolism (Blaut et al., 2003; Cummings and Macfarlane, 1991, 1997). During phase I metabolism, various cytochrome P450 (CYP) enzymes play a crucial role in biotransformation of drug molecules. Glucuronidation, sulfation and acetylation are the most important phase II reactions, catalyzed by the uridine diphosphate glucuronosyl transferase (UGT), sulfotransferase (SULT) and N-acetyltransferase (NAT) enzyme. This process produces molecules that are highly hydrophilic and hence can be easily excreted. UGTs are a widely distributed superfamily of enzymes responsible for metabolism of endogenous substrates and xenobiotics to more polar, water-soluble conjugates for elimination (Jancova et al., 2010; Jansen et al., 1992). It is well known that many herbal/dietary agents can interact with drug metabolism processes and can inhibit drug metabolism (Zhang and Lim, 2008).

Thus co-administration of CYP and UGT substrates and/or modulators with drugs is likely to be beneficial for improving pharmacokinetic properties and maintaining sustained drug levels. Although a majority of known CYP/UGT modulators are pharmaceutical compounds, it is noteworthy that several food components and natural products are also substrates or inhibitors of CYP and UGT enzymes (Scheepens et al., 2010; Zhang, 2001). A wide range of studies have reported that a number of flavonoids, including quercetin, tannic acid, benzoin gum, capsaicin, dihydrocapsaicin, eugenol, gallic acid, gallic acid, geraniol, menthol, menthyl acetate, naringenin, all spice berry oil, N-vanillylnonanamide, clove bud oil, peppermint oil, silibinin, piperine, silymarin, epigallocatechingallate and curcumin, inhibit the metabolism of various drugs by interacting with one or more CYP/UGT enzymes (Benet and Wachter, 2003; Gerk et al., 2014; Scheepens et al., 2010). As a result, an inhibitor helps in increasing and maintaining the concentration of a therapeutic drug. For example, co-administration of curcumin with pioglitazone significantly decreased the metabolism of the latter, in normal and diabetic rats (Prasad Neerati, 2012). Similarly, capsaicin and ellagic acid strongly inhibit the rat CYP 2A2, 3A1, 2C11, 2B1, 2B2 and 2C6 (Zhang et al., 1993). Hence, it is important to identify a compound or different classes of compounds that are capable of enhancing bioavailability by inhibiting CYP/UGT enzymes at the intestine and liver and hence reducing the first pass effect.

In the current study, we investigated whether co-administration of Nos with putative CYP/UGT modulating herbal/dietary agents such as capsaicin (Cap), piperine (Pip), eugenol (Eu) and curcumin (Cur) would alter the pharmacokinetics of Nos. We rationalized that these dietary agents may influence the bioavailability of Nos as they might inhibit its extensive biotransformation and increase the overall exposure. Thus the overarching aim of the study was to examine if Nos's rapid first-pass metabolism could be slowed down or inhibited to improve its overall pharmacokinetic parameters.

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

Noscapine (Nos), capsaicin (Cap), piperine (Pip), eugenol (Eu), curcumin (Cur), polyethylene glycol 300 (PEG 300), N-methylpyrrolidone (NMP) and β -glucuronidase were

purchased from Sigma (St. Louis, MO). Acetonitrile (ACN), and methanol (MeOH) were obtained from Fisher Scientific (Pittsburgh, PA). Liver microsomes from male CD-1 mouse, Sprague-Dawley rat, and Beagle dog, and mixed gender human (pool of 50) were procured from XenoTechLLC (Kansas, USA; protein content: 20 mg/mL). Standard substrates and inhibitors used for CYP inhibition assay were procured from Sigma (Bengaluru, India). All the stable labeled internal standard(s) (IS) used for analyzing the CYP inhibition samples were from Toronto Research Chemicals, Canada. NADPH, formic acid, ammonium formate, sodium dihydrogen phosphate and disodium hydrogen phosphate, and dimethyl sulfoxide (DMSO) and were purchased from Sigma (Bengaluru, India). 96-well plates of 1 mL capacity were purchased from Axygen Scientific, USA. Milli-Q[®] water was used for preparation of buffer (Millipore Corporation). All other reagents used in the assay were of analytical grade.

2.2 Formulation Recipe of Nos and Various Dietary Ingredients for in vivo PK studies

Oral bioavailability of Nos is reported to be 30% in mouse and humans. In order to rule out the possibility of Nos's limited solubility being a rate-limiting step in determining its bioavailability in presence of dietary agents, water-soluble co-solvents like N-methyl-2-pyrrolidone (NMP) and polyethylene glycol (PEG300) were used. The main objective was to produce a homogenous solution for dosing. These excipients are considered as safe in rodent pharmacokinetic and toxicokinetic studies. Required amounts of Nos, Cap, Pip, Eu and Cur were weighed and triturated with 10% v/v NMP and 30% v/v PEG 300. The volume was made up with Milli-Q water, vortex mixed and sonicated for 5 min. The dose volume used for dosing was 10 mL/kg.

2.3 Pharmacokinetic Studies of Nos and Dietary Ingredients

Pharmacokinetic studies were performed in male CD-1 mice (NCI, Frederick) following a single oral (PO) administration of Nos, Nos+Cap, Nos+Eu, Nos+Pip and Nos+Cur (at 50 mg/kg Nos and 5 mg/kg dietary ingredients). A clinical study was conducted for Nos, where Nos was given at 50 mg/kg t.i.d. (Olsson et al., 1986). Furthermore, another clinical study involving a dose of 300 mg Nos was conducted by Karlsson et al. Applying a scaling factor of 12.3 and considering 70 kg as body weight, the mouse equivalent dose was determined to be 50 mg/kg (Karlsson et al., 1990). Based on these studies, we employed a dose of 50 mg/kg bw Nos in our study. To avoid masking the effect of Nos, Cap, Eu, Pip and Cur were administered at 5 mg/kg bw, as these modulators are known to act locally at the intestine. Formulations were prepared freshly before dosing. They were assessed for accuracy by LC/MS/MS and were within 20% of nominal concentration on Day 1 and 7. All animals of 8–12 weeks age and 30–40 g body weight were acclimatized for 3 days before dosing. Feed and water was provided *ad libitum* throughout the study period. Animals were marked and housed (three per cage) in polypropylene cages and maintained in controlled environmental conditions with 12 h light and dark cycles. The temperature and humidity of the room was maintained between 22 ± 3°C and 30 to 70%, respectively, and approximately 10–15 fresh air change cycles per hour. All animals were dosed through oral gavage and a sparse sampling design with 3 animals in each group was used to collect blood samples (~200 µL) through retro-orbital plexus at 5 min, 10 min, 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, 12 h and 24 h into K₂EDTA (200 mM, 20 µL/mL of blood) coated tubes. Animals were anesthetized

using 5% isoflurane for 1 min before each sampling point. Plasma was harvested from blood by centrifugation of samples at 8000 g for 10 min. All samples were stored below -60°C until bioanalysis.

All samples were processed by protein precipitation method. An aliquot (50 μL) of plasma sample was added with 20 μL internal standard (IS, 7-hydroxy bromo noscapine, 0.2 $\mu\text{g}/\text{mL}$), 180 μL ACN, and vortex mixed for 3 min. The tubes were centrifuged at 8000 g for 10 min and an aliquot of supernatant was transferred into auto-sampler vials for analysis. The stock solutions of Nos, and 7-hydroxy bromo noscapine were prepared in water at 1.0 mg/mL and 200 ng/mL, respectively. A calibration curve range from 1 ng/mL (LLOQ) to 1000 ng/mL was employed for the quantification of Nos. The calibration curve consisted of blank, blank with internal standard and 10 non-zero calibration standards. The calibration standards were within $\pm 15\%$ of the nominal concentration at all concentrations except LLOQ, which was accepted at $\pm 20\%$ of nominal.

All samples were analyzed using liquid chromatography tandem mass spectrometric method (Agilent 6410 series). The ion spray voltage was set at 3000 V, ionization temperature set as 250°C and drying gas flow rate was 10 L/min. Data acquisition and quantitation were performed using Mass Hunter software (Agilent Technologies). Separation was achieved using HP1100 series LC (Agilent Technologies, Wilmington, DE) equipped with a photodiode array (PDA) detector, using Zorbax reversed-phase SB-C18, 2.1×50 mm, 5.0 μm (Agilent) column. An isocratic elution method was employed to separate Nos and other co-administered compounds using mobile phase A (80%, 0.1% formic acid in water) and mobile phase B (20%, ACN) at a flow rate of 0.25 mL/min and an injection volume of 10 μL . The column oven was maintained at 35°C . The MRM transitions monitored for Nos was m/z 414.2/220.4 and of IS was m/z 478.1/462.4 with Nos eluting at 7.7 min and IS at 14.8 min.

Pharmacokinetic parameters were calculated from the concentration-time data using the non-compartmental analysis tool of Phoenix[®] software (Version 6.3, Pharsight, USA). The area under the concentration time curve (AUC_{last}) was calculated by the linear trapezoidal rule. Following oral administration, peak concentration (C_{max}) and time for the peak concentration (T_{max}) were the observed values.

2.4 CYP Inhibition Assay

All incubations were performed as previously described (Mukkavilli et al., 2014). Stock solutions of Nos, Cap, and Pip (20 mM) were prepared in ACN:DMSO::80:20 mixture and subsequent test dilutions (final concentrations of 100, 50, 25, 12.5, 6.25, 3.125, 1.563, 0.781, 0.390, 0.195 and 0.098 μM , 11 different dilutions) were prepared in ACN:DMSO::80:20. A microsome-buffer-substrate mixture (MBS mix) was prepared for each isozyme by pre-mixing appropriate volumes of sodium phosphate buffer (pH 7.4, 50 mM), microsomes and substrate. MBS mix (179 μL) was transferred to 96-well reaction plate to which 1 μL of inhibitor stock solution was added to achieve the final target inhibitor concentration. The reaction plate was pre-incubated for 10 min at 37°C followed by reaction initiation by addition of 20 μL of 10 mM NADPH solution. The reaction plate was then incubated at 37°C for a predetermined time period following which it was quenched with 200 μL ACN for

all CYPs and 200 μ L 1% formic acid in water:ACN (70:30) for CYP1A2. In all cases, the final incubations after addition of substrate and inhibitor contained 0.1% DMSO (v/v), and the total organic solvent (DMSO and ACN) content was less than or equal to 1% (v/v). The incubations were performed in duplicate for Nos, Cap and Pip along with respective positive control inhibitors.

All samples were processed using protein precipitation method and analyzed by employing positive (for all CYPs) and negative (for CYP2A6, 2C19 and 2E1) ionization mode in liquid chromatography tandem mass spectrometry (API4000, Applied Biosystems, USA). The peak area ratio of analyte (metabolite produced) to deuterated IS of metabolite produced was used for calculations. An isocratic method comprising 5 mM ammonium formate and ACN (40:60) with 0.05% formic acid was used for elution. For CYP2C19, a mobile phase consisting of 5 mM ammonium formate and ACN (30:70) was used. The analytes and IS were retained on BDS Hypersil Phenyl (150 $\times$ 4.6 mm, 5 μ , Thermo, USA) column. A flow rate of 0.5 mL/min (CYP1A2), 0.6 mL/min (CYP2C19, CYP2E1), 0.7 mL/min (CYP2C9), 0.8 mL/min (CYP2A6, CYP3A), 1.0 mL/min (CYP2B6, CYP2C8, CYP2D6) was maintained using Shimadzu Prominence solvent delivery system (LC-20AD). The mobile phase was degassed using degasser (DGU-20A3), samples were loaded into autosampler (SIL-HTc) and the column temperature was maintained at 40 $^{\circ}$ C by column oven (CTO-20A). Injection volumes for the samples were as follows: 5 μ L (CYP1A2, CYP2D6, and CYP3A), 10 μ L (CYP2B6, CYP2C8, and CYP2E1) and 20 μ L (CYP2A6, CYP2C9, and CYP2C19). Data was collected and processed using Sciex Analyst 1.4.2.

The IC₅₀ value was estimated from the percentage reduction in CYP activity at eleven inhibitor concentrations with respect to control. The area ratio of the metabolite in the sample without inhibitor was considered as 100%, and the percentage reduction in the CYP activity at each inhibitor concentration was determined relative to the no-inhibitor area ratio using the following equation:

$$\% \text{CYP activity} = \frac{\text{Area ratio of metabolite at each dilution}}{\text{Area ratio of no-inhibitor controls}} \times 100$$

The non-linear regression model in GraphPad Prism[®] software was used to analyze the percent CYP activity data at different concentrations and the data were fitted to the following equation and IC₅₀ was calculated:

$$Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{1 + 10^{((\text{LogIC}_{50} - X) * \text{Hill coefficient})}}$$

Where, X = Log concentration; Y = Response (% CYP activity)

The data was analyzed using 4PL (parameter logistic model), 3PLFB (bottom fixed), 3PLFT (top fixed), 2PL (top and bottom fixed), and the relative IC₅₀ was with lowest standard error was further manipulation. Absolute IC₅₀ was calculated from this relative IC₅₀ by using the equation below:

$$\text{Absolute IC}_{50} = \frac{\text{Relative IC}_{50}}{\left[\frac{[(\text{top}-\text{bottom})]}{((50-\text{bottom})-1)} \right] \frac{1}{\text{Slope}}}$$

2.5 Microsomal Stability Assay

Microsomes from male species of CD-1 mouse, Sprague-Dawley rat, and Beagle dog, and mixed gender human (pool of 50) were used for assays. Incubations (1 mL) consisted of liver microsomes (0.5 mg/mL), NADPH (2 mM) and 50 mM phosphate buffer (pH 7.4). Following pre-incubation (10 min, 37 °C), reactions were initiated by adding Nos, Nos+cap, Nos+pip. Samples (100 µL) were withdrawn at 0, 5, 15, and 30 min and quenched with 400 µL acetonitrile containing internal standard. Concomitant NADPH-free control incubations were sampled at 0 and 30 min.

All samples were analyzed for Nos using a high performance liquid chromatography (HPLC, Shimadzu Prominence, Japan) tandem mass spectrometric (API4000, Applied Biosystems, USA) method. Positive-ion electron spray ionization mode was used and MRM transitions of m/z 414.0/220.0 for Nos and m/z 326.1/291.1 for midazolam (IS, 100 ng/mL) were monitored. An isocratic HPLC method with a 3.5 min run time was employed for analysis. The mobile phase comprised 10 mM ammonium acetate and acetonitrile 10:90 (v/v) with 0.05% formic acid and the flow rate was 0.5 mL/min. Separation was achieved using Gemini® C18 column (4.6 × 50 mm, 5 µ, Phenomenex, India) maintained at 40 °C employing an injection volume of 5 µL for *in vitro* samples.

The percent remaining for Nos in each sample was determined by considering peak area ratio in the 0 min sample as 100%. The first order decay equation ($A = A_0 e^{-kt}$) was used to estimate elimination rate constant *k* using GraphPad Prism® software. Intrinsic clearance was calculated using the formula

$$CL_{\text{intr}} = k \times \frac{\text{volume of reaction mixture (mL)}}{\text{protein content (mg)}} \times 52.5 \text{ (mg protein/g of liver)}$$

Where, *k* = decay rate constant (min⁻¹).

Statistical Analysis

All the values where applicable are expressed as mean ± standard deviation (SD). ANOVA was used to compare the differences in the exposure from in-life phase studies.

3. RESULTS

3.1 Screening of dietary ingredients that can influence the PK profile of noscapine

Several dietary agents such as capsaicin (Cap), piperine (Pip), eugenol (Eu) and curcumin (Cur) have been previously shown to be effective in inhibiting drug metabolism by altering PK profiles of various marketed drugs like rifampicin, propranolol, nevirapine, theophylline,

colchicine, doxorubicin, celiprolol, and midazolam (Li et al., 2011; Prasad et al., 2004; Reddy and Lokesh, 1994; Shugarts and Benet, 2009; Zhai et al., 2013a; Zhai et al., 2013b; Zhang and Lim, 2008). Thus, we first asked if these dietary agents could influence the PK profile of Nos. To this end, we screened the *in vivo* potential of various Nos+dietary ingredient combinations, where male CD-1 mice were orally dosed with Nos (50 mg/kg), Nos+Cap (50 mg/kg+5 mg/kg), Nos+Eu (50 mg/kg+5 mg/kg), Nos+Pip (50 mg/kg+5 mg/kg), Nos+Cur (50 mg/kg+5 mg/kg). The time taken to reach the maximum plasma concentrations (T_{max}) was less than 15 min for all the groups tested, suggesting rapid absorption (Figure 1). The pharmacokinetic parameters were calculated for various combinations tested (Table I, Figure 2) and it was observed that Nos was rapidly cleared with a half-life of less than 1 h. Upon oral feeding, Nos+Pip achieved the highest C_{max} for Nos (4997 ng/mL) compared to Nos alone (989 ng/mL), followed by Nos+Eu (3926 ng/mL) and Nos+Cap (3731 ng/mL) (Figure 2, Table I). We found that with the inclusion of these dietary agents as adjuvants, we clearly see an increase in the C_{max} values of Nos by 3–5 folds.

An increase in exposure (AUC_{last}) of Nos was found in case of Nos+Cap (~2 fold), Nos+Pip (~2 fold) and Nos+Eu (1.7 fold) combinations (Table I), suggesting possible inhibition of noscapine metabolism as hypothesized. Interestingly, Nos+Cur showed lower exposure (AUC_{last}) compared to the others (Table I, Figure 2). Three out of the four combinations tested, namely Nos+Cap, Nos+Eu, Nos+Pip showed increase in C_{max} compared to Nos+Cur (Table I). In the presence of Eu, T_{max} of Nos remained unaffected. The other dietary agents employed in the study, Cap, Cur and Pip, however delayed the T_{max} of Nos from 5 min to 15 min. This could be due to their affinity to get absorbed in the intestine compared to Nos. This delayed T_{max} with increase in exposure upon combining Nos with Cap, Cur and Pip was an indication of enhanced Nos bioavailability, which was not the case in Nos+Eu combination. Hence, we focused our efforts on combinations of Nos with Cap and Pip.

3.2 Effects of Nos, Cap and Pip on drug metabolizing enzymes

As a next step in the development of these novel binary combinations, it was crucial to evaluate the potential of all the components to affect each other's metabolism. Hence, we next determined if Nos, Cap and Pip could inhibit the drug metabolizing enzymes and thus result in enhanced bioavailability or exposure. To understand possible drug-drug interactions and their relevance in clinical settings, various concentrations of Nos, Cap and Pip were used to screen against CYPs 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4 (Table II). The results thus obtained indicated that Nos, Cap and Pip are not potent inhibitors of any of the tested CYPs with IC_{50} values greater than 3 μ M.

Cap was found to inhibit the activity of CYP1A2 (3.3 μ M), CYP2C8 (6.1 μ M), CYP2C9 (4.1 μ M), CYP2C19 (12 μ M), CYP3A4 (16 μ M, midazolam as substrate), and CYP3A4 (9.7 μ M, testosterone as substrate) (Figure 3, Table II), while Pip only inhibited CYP3A4 (16 μ M, testosterone as substrate) (Table II). Nos showed inhibition of CYP2C8 (9.2 μ M), CYP2C19 (5.4 μ M), and CYP3A4 (20 μ M, testosterone as substrate) (Table II).

Given that CYP-mediated metabolism predominantly occurs in liver and intestine, we next attempted to evaluate the impact of Cap and Pip on *in vitro* microsomal stability of Nos.

Typically, as a golden rule, *in vitro* metabolism studies are performed in pre-clinical and human liver microsomes to determine similarities and differences in extent of biotransformation and choose appropriate species for further lead optimization studies. Hence, microsomal stability of Nos was assessed in mouse (MLM), rat (RLM), dog (DLM) and human (HLM) liver microsomes with and without Cap and Pip. Cap and Pip were tested at 1 μ M and 10 μ M to understand the interaction profile, if any. Intrinsic clearance of Nos remained unaffected by Cap and Pip in MLM on the contrary we found an increase in rate of clearance. Noscapine's rate of metabolism was found to be ~1 to 4-fold higher in case of Nos+Cap and ~2.7 to 3-fold higher in case of Nos+Pip in MLM (Table III). Intriguingly, upon incubation in RLMs, the clearance of Nos decreased with increasing concentrations of Cap and Pip (Table III). Furthermore, in case of DLM and HLM, Pip showed marginal decrease in metabolism of Nos at the highest concentration tested (Table III) and in the presence of Cap, the rate of metabolism of Nos was similar in DLM and HLM. Overall, the rate of metabolism of Nos was observed to be higher in MLM (moderate clearance) followed by RLM and DLM $\approx$ HLM (low clearance) (Table III). These results clearly indicate that Cap and Pip do not have an effect on the clearance profile of Nos in MLM, DLM and HLM with an exception of RLM, where they inhibited Nos metabolism.

CYP inhibition and microsomal stability studies could not explain the increase in Nos exposure following oral administration. Our next logical step was to evaluate if repeated oral administration of Nos+Cap and Nos+Pip lead to accumulation of Nos.

3.3 Repeated dose PK study with Nos+Cap and Nos+Pip combinations

Considering our observations from a single dosing (Figure 1) of Nos+Cap and Nos+Pip where an increase in exposure of Nos along with an increase in T_{max} was observed, we next hypothesized that upon repeated dosing, the exposure of Nos would further increase and/or sustained levels of Nos will be maintained *in vivo*. To this end, male CD-1 mice were dosed with the same dose of Nos, Nos+Cap and Nos+Pip (50 mg/kg Nos and 5 mg/kg Cap/Pip) for seven days. Further, the blood samples collected on day 1 and day 7 of this regimen were processed and quantitated for Nos levels and compared (Figure 4, Table IV).

Intriguingly, our observations indicated that the plasma concentrations of Nos on day 7 were lower in all groups compared to that on day 1. The exposure of Nos on day 7 was found to be half the exposure of day 1 (Figure 4A, Figure 5B, Table IV) and in case of Nos+Cap and Nos+Pip, there was ~5–6 fold decrease in exposure (Figure 4B-C, Figure 5B, Table IV).

Often, in cases where drugs are co-administered with xenobiotics, accelerated clearance of the drugs is observed due to drug-drug/herb interactions (Mohutsky et al., 2010). If one of the co-administered drugs is an inhibitor of the particular CYP enzyme responsible for metabolism of the other drug, it may lead to an increase in the exposure of the latter. Drug-drug interactions may also occur if one drug induces the CYP enzyme responsible for clearance of another drug, leading to therapeutic failure (Mohutsky et al., 2010). There also exists a possibility of auto-induction of the metabolizing enzymes by the drug administered.

Thus considering their marginal CYP inhibition potential (Figure 3), Cap and Pip could have a potential role in the induction of enzymes responsible for the metabolism of Nos.

However, the decreased exposure of Nos in case of continuous administration of Nos alone (Figure 4A, Figure 5, Table IV) also signifies a possibility of Nos inducing the enzymes responsible for its own clearance, i.e., auto-induction.

Interestingly, these results also revealed that Nos undergoes multiple peaking phenomenon on day 1 and day 7, where more than one C_{max} was observed for Nos, Nos+Cap and Nos+Pip combinations (Figures 1 and 4). Upon single dose administration of Nos on day 1, the first C_{max} was achieved at 0.17 h (3087 ± 1232 ng/mL) and a second C_{max} at 0.5 h (966 ± 633 ng/mL). On day 7, the first C_{max} was achieved as early as 0.083 h (507 ± 9 ng/mL) followed by a decrease at 0.17 h. A second C_{max} of 684 ± 31 ng/mL at 0.25 h was observed followed by clearance of Nos from the system. Similarly, in case of Nos+Cap, at 0.083 h a C_{max} of 2624 ± 168 ng/mL was observed followed by decrease in this value at 0.17 h on day 1. And at 0.25 h, a C_{max} value of 3732 ± 730 ng/mL was observed, indicating the recirculation of Nos and potential inhibition of its clearance. However, the C_{max} values were found to have drastically shifted on day 7. The first C_{max} of 831 ± 48 ng/mL was achieved at 0.5 h, followed by a significant decrease in the plasma concentration of Nos. At 1.5 h, an increase in the plasma value of Nos was observed indicating a second C_{max} (409 ± 9 ng/mL). Also, administration of Nos+Pip combination on day 1 resulted in two C_{max} values, one at 0.083 h (3327 ± 148 ng/mL) and the second one at 0.25 h (4997 ± 1344 ng/mL). Similar to day 1, the first C_{max} value on day 7 of Nos+Pip administration was achieved at 0.083 h (744 ± 179 ng/mL), followed by a second C_{max} of 136 ± 2 ng/mL at 1.5 h.

This consistent phenomenon of multiple peaks in case of Nos, Nos+Cap and Nos+Pip thus indicates a possible role of enterohepatic recirculation (EHR) in prolonged existence of Nos in the systemic circulation. Also, as there is a risk of toxic analyte and/or metabolite build up due to continuous co-administration of drugs, the next logical step in our study seemed to be a detailed evaluation of the toxic effects of Nos+Cap and Nos+Pip on blood parameters.

3.4 Non-toxicity of Nos+Cap and Nos+Pip

The induction of hepatic DMEs as observed above, often results in the potential generation of metabolites, which may be toxic. Hence, to evaluate the safety of daily oral administration of Nos+Cap and Nos+Pip combinations, we next tested their possible toxic effects by studying the clinical chemistry parameters.

Also, given the possibility of accumulation of free Nos or its reactive metabolites in various organs, eventually leading to toxicity, we evaluated the effect of these binary combinations on serum biochemical markers like alanine transaminase, alkaline phosphatase, aspartate aminotransferase for hepatic function, and creatinine and electrolytes *viz.* K^+ , Na^+ , Ca^{2+} and Cl^- , for renal function. The results of clinical chemistry parameter evaluation revealed no significant differences between the samples tested from vehicle-treated and Nos+Cap- and Nos+Pip-treated mice after 7 days dosing. Similar values were observed for each parameter tested among all the groups indicating the absence of apparent toxicity.

4. DISCUSSION

The bioavailability of a drug within the body can be a double-edged sword, both a driving force and a concern in drug development. Poor absorption due to low solubility, low permeability and rapid metabolism are major concerns even in the clinic for attesting the usefulness of a potential drug or formulation (Anand et al., 2007; Lin and Lu, 1997). However, the drug metabolism process and the enzymes involved in Phase I (CYP450 enzymes) and Phase II (UGTs, SULTs and NATs) biotransformation also influence the bioavailability of certain drugs (Mohutsky et al., 2010; Soars et al., 2004). Considering the lower bioavailability of Nos, in our current study, we screened novel combinations of Nos and potential CYP and/or UGT modulators to enhance the exposure of Nos and thus resulting in improved bioavailability. Phytochemicals like Cap, Pip, Eu and Cur were employed as potential UGT/CYP modulators, which are often consumed as a part of our daily diet.

From our observations, an increase in the exposure of Nos in case of Nos+Cap and Nos+Pip upon single dose administration indicated a possible inhibition of CYP/UGT enzyme pathway. However, further in-depth studies revealed that Nos, Cap and Pip exhibited a marginal CYP enzyme inhibitory potential. Of all the 3 compounds tested using HLMs with appropriate substrates (Table II), Cap was only found to have inhibitory potential against CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP3A4 (Figure 3) with mean IC_{50} values of 3.3 μ M, 6.1 μ M, 4.1 μ M, 12 μ M, 16 μ M, and 9.7 μ M, respectively (Table II). We believe that Cap and Pip, when in combination with Nos, are resulting in enhanced bioavailability of Nos possibly due to their higher affinity for absorption and drug metabolizing enzymes compared to Nos. Further, Nos is a substrate for phase I enzymes like CYP1A2, 2C8, 2C9, 2C19 and 3A4 and phase II enzymes like UGT2B7, UGT1A1, UGT1A3, and UGT1A9. It is possible that, when administered in combination with Nos, the metabolites of Cap and Cur may be inhibiting Nos's biotransformation by these drug-metabolizing enzymes. Further, an increase in the rate of metabolism of Nos in the presence of Cap and Pip was observed with MLM. These data clearly suggest that the increase in exposure of Nos observed during primary screening (Figure 1, Table I) upon single dose administration could not be solely due to the CYP enzyme inhibition by Cap and Pip. Furthermore, the microsomal stability studies of Nos using MLM revealed that Cap and Pip are not inhibitors of clearance of Nos. However, the inhibitory effect was observed marginally in RLM, DLM and HLM.

Further evaluation of PK parameters upon repeated dosing of Nos+Cap and Nos+Pip combinations resulted in an intriguing observation, where the exposure was actually decreased by ~5 to 6 fold (Figures 4 and 5) on day 7 along with diminished C_{max} values compared to day 1. This observation was contrary to our hypothesis that repeated dosing of the potential combinations would result in an increase in the exposure of Nos and hence improve the bioavailability. Intriguingly, multiple dosing of Nos resulted in a similar scenario, where on day 7, exposure of Nos decreased by half of that observed on day 1 (Figure 4). This decrease in the C_{max} values on day 7 irrespective of the combinations could be due to induction of enzymes responsible for Nos metabolism. Also, in a clinical study performed by Karlsson et al, various doses of Nos, i.e., 100, 200 and 300 mg/kg dose for human studies (Karlsson et al., 1990). In this study, a 9-fold increase in AUC was observed

for a 3-fold increase in dose. The mean $\pm$ SD values of AUC/Dose ($\text{h} \cdot \mu\text{g} \cdot \text{l}^{-1} \cdot \text{mg}^{-1}$) reported were 2.19 ± 1.69 for 100 mg, 3.97 ± 2.87 for 200 mg, 6.57 ± 3.86 for 300 mg. For 100 mg dose, the highest AUC/Dose is $3.88 \text{ h} \cdot \mu\text{g} \cdot \text{l}^{-1} \cdot \text{mg}^{-1}$ and for 300 mg dose the lowest AUC/Dose value is $2.71 \text{ h} \cdot \mu\text{g} \cdot \text{l}^{-1} \cdot \text{mg}^{-1}$, clearly indicating the possibility of variations in the AUC values within the dose groups. These data thus provide a plausible explanation to our observations in the current study.

The increase in C_{max} and AUC_{last} values of Nos on day 1 as opposed to day 7 can further be attributed to be due to CYP inhibition or a likely absorption interaction with Cap and Pip, which leads to a transient increase in the free form, but an induction of CYP metabolizing enzymes by day 7 could be facilitating clearance of Nos. Alternatively, we also speculate that on day 1, Cap and Pip have higher affinity for DMEs compared to Nos, and thus get cleared out of the system comparatively quickly. Such interactions probably occurring at the absorption site could be leading to the slower absorption of Nos and thus resulting in an increase in T_{max} as observed (Figure 4, Supp. Table III). It is reported in the literature that CYP1A1/2, CYP2C8/9/19, CYP2D6, CYP3A4/5/7, UGT1A1/3/9, and UGT2B7 and FMO1 are responsible for the metabolism of Nos, resulting in 10 Phase I metabolites and 11 conjugate metabolites (Fang et al., 2012). In the event that noscapine induces the activity of any of these CYP/UGT enzymes, it is likely that noscapine will enhance its own metabolism and hence elimination. It is logical to reason that co-administration of drugs that are substrates of specific CYP/UGT enzymes induced by Nos may even result in therapeutic failure and/or alteration of efficacy owing to the fact that the Nos-induced CYP/UGT enzymes can metabolize the co-administered drug at a much quicker rate. However, further studies are warranted to confirm the ability of noscapine to induce drug-metabolizing enzymes.

In essence, the loss of free Nos on day 7 strongly indicates the biotransformation of Nos at that point in time resulting in metabolite formation. While, as a rule of the thumb, most metabolites and conjugated forms are inactive and are meant for elimination from the body, in some cases, the nature of a drug metabolite also affects the drug efficacy (Fang et al., 2012; Lopus et al., 2010). In such cases, accumulation of an active metabolite in the targeted organ site would result in enhanced efficacy (Drayer, 1976; Fang et al., 2012). On the contrary, such accumulation in very high amounts irrespective of the activity may result in toxicity (Drayer, 1976). A recent report has shown that Nos metabolism results in the production of both inactive and active metabolites (Fang et al., 2012). In fact, it is likely that active metabolites may be more potent than the parent free drug. Our data suggest a possibility of accumulation or circulation of active metabolites on day 7 upon repeated dosing of Nos, Nos+Cap and Nos+Pip (Figure 4), which requires further evaluation. Given that the concentration of beta-glucuronidase is well-known to be more in tumor cells compared to normal cells (Bosslet et al., 1998; Murdter et al., 1997), it is likely that glucuronide conjugates of Nos or its metabolites enter tumor cells, get deconjugated due to high activity of beta-glucuronidase and those enhanced cellular levels of free Nos or its metabolites might underlie drug efficacy as benchmarked by tumor regression effects. Further our clinical chemistry and hematology parameters showed that Nos dosing for 7 days did not affect any of the critical blood or tissue parameter, suggesting that Nos or its metabolite(s) did not have any adverse effects for at least 7 days.

Our data show that Nos was observed to exhibit multiple peaking phenomenon on day 1 (Figure 4), where more than one $C_{\max}$ peaks were observed. Multiple peaking patterns are often associated with EHR (Davies et al., 2010), or differential absorption following oral administration from the intestine. Glucuronide conjugates formed due to the activity of UGT enzymes often undergo elimination via biliary excretion, get de-conjugated in the gut and reabsorbed leading to prolonged exposure of the drug (Cummings and Macfarlane, 1997; Jancova et al., 2010; Jansen et al., 1992; Mohutsky et al., 2010; Ritter, 2000; Scheepens et al., 2010). This observation further strengthens our interpretation of lack of UGT inhibition in the presence of Cap and Pip, as the glucuronidation process is obvious for the EHR to take place (as observed in Figure 4). The reentry of glucuronide conjugates formed possibly due to induction of UGT activity by Nos, may perhaps underlie the reappearance of Nos for prolonged periods. Furthermore, a recent investigation demonstrating the presence of Nos, its conjugates and metabolites in feces (Fang et al., 2012) support our observations of EHR. While those indications of Nos undergoing EHR are becoming evident, we are the first to report a possible EHR mechanism of Nos, encouraging further investigation.

In conclusion, our study highlights that the potential drug metabolizing enzyme modulators that were employed in our study did not aid in increasing the exposure and enhancing bioavailability of Nos. The increase in exposure of Nos on Day 1 could be due to interaction of Cap and Pip with UGT enzymes during the absorption phase and/or elimination phase. Cap and Pip also undergo biotransformations involving DMEs including CYPs/UGTs and it may be possible that their affinity is more compared to Nos for these enzymes leading to observed increase in $T_{\max}$ for Nos. However, *in vitro* UGT inhibition experiments involving mouse liver (MLM) and intestinal (MIM) microsomes with Cap and Pip did not reveal significant inhibitory effects on the rate of metabolism of Nos (Figure 7). Our observations of possible enterohepatic recirculation of Nos and induction of self-clearance by Nos upon repeated dose administration are compelling and generate grounds for further investigation into the biotransformation process of Nos and efficacy of its metabolites. Furthermore, upon modulating the multiple peaking phenomenon, futuristic dosing regimen of Nos can be designed. Although our studies present an essential advancement in integrative medicine, systematic *in vitro* enzyme induction, *in vivo* pharmacokinetic and pharmacodynamic studies are required to validate the findings of our study and design better regimens for Nos, a non-toxic promising anticancer drug in clinical trials.

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ABBREVIATIONS

ACN	Acetonitrile
Cap	Capsaicin
Cur	Curcumin
CYP	Cytochrome P450

DLM	Dog liver microsomes
DMSO	Dimethylsulfoxide
Eu	Eugenol
IC₅₀	Inhibitory concentration 50%
HLM	human liver microsomes
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LLOQ	Lower limit of quantitation
MBS	Microsomes buffer substrate mix
MLM	mouse liver microsomes
NADPH	β -Nicotinamide Adenine Dinucleotide 2'-Phosphate
NMP	N-methylpyrrolidone
Nos	Noscapine
PK	Pharmacokinetics
RLM	rat liver microsomes

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Figure 1. Plasma concentration-time profile of Nos (50 mg/kg) with various dietary ingredients (5 mg/kg) following oral administration
(A) Nos+Cap, (B) Nos+Eu, (C) Nos+Pip, (D) Nos+Cur. Values and error bars shown in the graphs represent mean $\pm$ SD.

Figure 2. Oral PK parameters of Nos and dietary ingredients

(A) Peak plasma concentration of Nos (C_{max}), (B) area under the curve (AUC_{last}). Error bars represent $\pm$ SD values.

Figure 3. Evaluation of CYP inhibition potential of Nos, Cap, Pip against respective positive control inhibitors

(A) CYP1A2 (α -naphthoflavone, α -Nap), (B) CYP2A6 (tranylcypromine, Tra) (C) CYP2B6 (ticlopidine, Tic) (D) CYP2C8 (quercetin, Que), (E) CYP2C9 (sulfaphenazole, Sul), (F) CYP2C19 (N-3-benzylirinanol, N-3-Ben), (G) CYP2D6 (quinidine, Qui), (H) CYP2E1 (tranylcypromine, Tra), (I) CYP3A4^a (ketoconazole, Ket), (J) CYP3A4^b (ketoconazole, Ket).

Figure 4. Nos exhibits pronounced multiple peaking phenomenon in Nos+Cap and Nos+Pip on day 1 and showed lower exposure on day 7

(A) Nos (B) Nos+Cap and (C) Nos+Pip. Values and error bars shown in the graphs represent mean $\pm$ SD.

Figure 5. Exposure of Nos+Cap and Nos+Pip combinations

(A) Peak plasma concentration of Nos (C_{max}), (B) Area under the curve (AUC_{last}) were quantitated in plasma samples collected at various time points upon oral administration of Nos, Nos+Cap, Nos+Pip on day 1 and day 7. The dose of Nos administered was 50 mg/kg while the dietary agents were dosed at 5 mg/kg. Error bars represent $\pm$ SD values.

Figure 6. Clinical chemistry parameters of Nos+Cap and Nos+Pip

Levels of (A) albumin and bilirubin, (B) the biomarkers of liver, alkaline phosphatase (ALP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (C) blood urea nitrogen, and (D) lactate dehydrogenase (LDH), creatinine kinase were comparable for both the test combinations and control. Values and error bars shown in the graphs represent mean $\pm$ SD.

Figure 7. Nos+Cap and Nos+Pip show higher metabolism in the liver as compared to intestine Levels of Nos in (A) mouse liver microsomes (MLM), (B) mouse intestinal microsomes (MIM) were compared for rate of metabolism in case of Nos+Cap and Nos+Pip. The rate of metabolism for Nos alone is MLM>MIM. Nos has the potential to undergo extensive first pass metabolism at intestine and liver before reaching systemic circulation. Values and error bars shown in the graphs represent mean and SD, respectively.

Table I

PK parameters of noscapine following oral gavage administration of Nos, Nos+Cap, Nos+Eu, Nos+Pip and Nos+Cur in CD-1 mice (dose Nos: 50 mg/kg bw; Cap, Eu, Pip, Cur: 5 mg/kg bw).

Parameter	Tmax (h)	Cmax (ng/ml)	AUC _{last} (ng*h/ml), PO
Nos	0.08	988.69	1922.75
Nos+Cap	0.25	3731.60	3986.44
Nos+Eu	0.08	3916.25	3344.44
Nos+Pip	0.25	4997.20	3957.26
Nos+Cur	0.17	2025.28	1550.09

Table II

Half-minimal concentrations (IC_{50}) values of Nos, Cap and Pip against various CYP450 enzymes compared to their respective positive controls (substrates).

IC ₅₀ (μM)										
Compound	CYP 1A2	CYP 2A6	CYP 2B6	CYP 2C8	CYP 2C9	CYP2 C19	CYP 2D6	CYP 2E1	CYP3 A4 ^a	CYP3 A4 ^b
Noscapine	>100, >100	>100, >100	>100, >100	10, 8.4	18, 24	5.0, 5.7	>100, >100	>100, >100	19, 20	27, 28
Capsaicin	3.1, 3.5	>100, >100	26, 24	6.3, 5.9	3.7, 4.5	12, 11	68, 68	>100, >100	15, 17	11, 8.3
Piperine	35, 38	>100, >100	87, 90	84, 78	54, 54	25, 22	>100, >100	>100, >100	58, 50	16, 16
Positive control	0.007, 0.009	0.09, 0.10	0.06, 0.12	0.67, 0.64	0.65, 0.71	0.65, 0.61	0.15, 0.16	6.8, 5.2	0.015, 0.016	0.027, 0.027

^a Midazolam,

^b testosterone, <1 μM is considered as potent inhibition

Table III
Intrinsic clearance of Noscapine in the presence of Cap and Pip

Levels of Nos in mouse (MLM), rat (RLM), dog (DLM) and human (HLM) liver microsomes, were compared for rate of metabolism in presence of Cap and Pip (n=2)

Compound and inhibitor	Intrinsic Clearance (mL/min/g liver)			
	MLM	RLM	DLM	HLM
Noscapine (10 µM)	6.4	4.0	1.9	1.8
Noscapine (10 µM) + Capsaicin (1 µM)	26.2	3.0	1.6	1.5
Noscapine (10 µM) + Capsaicin (10 µM)	7.4	1.3	1.7	1.8
Noscapine (10 µM) + Piperine (1 µM)	17.7	2.5	1.4	1.8
Noscapine (10 µM) + Piperine (10 µM)	19.6	2.0	1.1	1.1

Table IV

Comparison of PK parameters of noscapine following oral gavage administration of Nos+Cap, Nos+Pip and Nos in CD-1 mice (dose Nos: 50 mg/kg bw; Cap, Pip: 5 mg/kg bw) on Day 1 and Day 7.

Day 1			
Parameter	T _{max} (h)	C _{max} (ng/mL)	AUC _{last} (ng*h/mL)
Nos	0.17	3087.37 ±1232.27	1023.76
Nos+Cap	0.25	5883.81 ±688.74	4551.26
Nos+Pip	0.17	4035.96 ±657.62	2570.28
Day 7			
Parameter	T _{max} (h)	C _{max} (ng/mL)	AUC _{last} (ng*h/mL)
Nos	0.25	684.04 ±31.77	507.74
Nos+Cap	0.50	831.30 ±48.50	774.68
Nos+Pip	0.08	743.76 ±179.49	549.07