Biotransformation of a Novel Antimitotic Agent, I-387, by Mouse, Rat, Dog, Monkey, and Human Liver Microsomes and In Vivo Pharmacokinetics in Mice

Sunjoo Ahn, Jeffrey D. Kearbey, Chien-Ming Li, Charles B. Duke III, Duane D. Miller, and James T. Dalton

Division of Pharmaceutics, College of Pharmacy, The Ohio State University, Columbus, Ohio (S.A., C.M.L., J.T.D.)
Department of Pharmaceutical Sciences, College of Pharmacy, University of Tennessee Health Science Center, Memphis, Tennessee (C.B. D., D.D.M.)
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b) Corresponding author:

James T. Dalton

Address: 3 North Dunlap Street, Memphis, Tennessee, 38163.

Telephone: (901)523-9700.

Fax: (901)844-8078.

E-mail: jdalton@gtxinc.com

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d) A list of nonstandard abbreviations used in the paper.

CL, clearance; Vdss, volume of distribution at steady state; T1/2, terminal half-life; DMSO, dimethylsulfoxide;

AUCinf, The area under the plasma concentration-time curve from time 0 to infinity; Cmax, maximum plasma concentration; Tmax, the time when concentration is Cmax; MRTinf, the mean residence time extrapolated to infinity;

AUMCinf, the area under the first moment curve extrapolated to infinity; F, bioavailability; MRM, Multiple reaction monitoring; EIC, extracted ion chromatogram; EPI, enhanced product ion; TIC, Total ion current
Abstract

I-387, (3-(1H-indol-2-yl)phenyl)(3,4,5-trimethoxyphenyl)methanone, is a novel indole compound with antitubulin action and potent antitumor activity in various preclinical models. I-387 avoids drug resistance mediated by P-glycoprotein and showed less neurotoxicity than vinca alkaloids during in vivo studies. We examined the pharmacokinetics and metabolism of I-387 in mice as a component of our preclinical development of this compound and continued interest in structure-activity relationships for antitubulin agents. Following a 1 mg/kg intravenous (i.v.) dose, non-compartmental pharmacokinetic analysis in plasma showed that clearance (CL), volume of distribution at steady state (Vdss), and terminal half-life (T1/2) of I-387 were 27 ml/min/kg, 5.3 l/kg, and 7 h, respectively. In the in vitro metabolic stability study, half-lives of I-387 were between 10 and 54 min by mouse, rat, dog, monkey and human liver microsomes in the presence of NADPH, demonstrating inter-species variability. I-387 was the most stable in rat liver microsomes and degraded quickly in monkey liver microsomes. Liquid chromatography-tandem mass spectrometry was used in order to identify phase I metabolites. Hydroxylation, reduction of a ketone group, and O-demethylation were the major metabolites formed by the liver microsomes of the five species. The carbonyl group of I-387 was reduced and identified as the most labile site in human liver microsomes. The results of these drug metabolism and pharmacokinetic studies provide the foundation for future structural modification of this pharmacophore to improve stability of drugs with potent anticancer effects in cancer patients.
Introduction

I-387, (3-((1H-indol-2-yl)phenyl)(3,4,5-trimethoxyphenyl)methanone, is a new antitubulin agent with potent in vivo and in vitro antitumor activity in various preclinical cancer models (Figure 1)(Ahn et al.). I-387 destabilizes microtubules by binding to the colchicine binding domain. In addition, I-387 is not a substrate for ABC transporters and maintained potent tumor growth inhibition in xenograft models using cell lines over-expressing P-glycoprotein. This investigational agent is particularly attractive for further development because no significant neurotoxicity was observed during in vivo and in vitro neurotoxicity studies (Ahn et al.). Toward this end, we sought to understand the absorption, distribution, metabolism, and excretion of I-387 as a means to evaluate its toxicity and potency and to optimize the I-387 pharmacophore for better exposure and stability in vivo.

As it is necessary to achieve and maintain effective concentration of drugs at their sites of actions, pharmacokinetic studies to characterize the absorption, distribution, metabolism, and elimination are important in optimization of therapeutic dose and schedule. Although in vivo studies provide definitive data regarding a drug’s disposition in an animal, in vitro studies of drug metabolism provide the opportunity for efficient and rapid characterization of a compound’s metabolic fate in numerous species without the need for synthesis of large quantities of drugs or use of animals. Drug biotransformation is divided into two types of reactions; phase I (hydrolysis, oxidation, and reduction) and phase II (conjugation). The cytochrome P450 enzyme super family plays a dominant role in phase I metabolism and phase II conjugation is caused by uridine diphosphoglucuronosyl transferase, N-acetyl transferase, glutathione S-transferase, sulfotransferase and so on (Schlichting et al., 2000; Sheweita, 2000). Although the isolated perfused liver could give an excellent representation of the in vivo situation, current guidelines for human drug development allows for the use of in vitro systems for metabolism studies, including microsomes, supersomes, cytosol, S9 fraction, cell lines, transgenic cell lines, and primary hepatocytes (www.fda.gov) (Brandon et al., 2003).

In this study, we examined the pharmacokinetics of I-387 in mice following i.v., intraperitoneal (i.p.), and oral (p.o.) dosing in mice. The in vitro metabolic fate of I-387 was investigated in mouse, rat, dog, monkey, and human liver microsomes. Our studies provide the first evidence that i.v. and i.p. administration of I-387 in mice can achieve systemic drug exposure sufficient for in vivo antitumor effect (Ahn et al.). Additionally, we identified a metabolically labile site in the I-387 pharmacophore that can be modified to design more stable drug candidates.
Materials and Methods

Chemicals, Microsomes, and Animals

I-387 and a putative metabolite of I-387 were synthesized using methods similar to those for indole-15 previously described (Ahn et al.). In this study, indole-15 was used as an internal standard. Intermediate 1 was treated with 3,4,5-trimethoxyphenyllithium to afford alcohol 2 (Figure 1A). Alcohol 2 was then oxidized with pyridinium dichromate in dichloromethane at room temperature to form ketone 3, which was deprotected with aqueous sodium hydroxide to afford I-387 (Figure 1A). The putative metabolite, alcohol 4 was also synthesized by de-protection of compound 2 with reflux under basic conditions (Figure 1A). Complete synthetic details are forthcoming in a separate publication.

Pooled liver microsomes from mice, rats, dogs, monkeys, and humans were purchased from Xenotech (Lenexa, KS). Nicotinamide adenine dinucleotide phosphate (NADPH) regenerating system was purchased from BD (Franklin Lakes, NJ). All other chemicals were purchased from Sigma (St. Louis, MO). Male ICR mice (20-25 g) were purchased from Harlan Biosciences (Indianapolis, IN). All animal protocols were approved by the Animal Care and Use Committee at The Ohio State University or the University of Tennessee Health Science Center.

Pharmacokinetic Studies in Mice.

Male ICR mice (5-6 weeks, 20-25 g) were used. For I-387, three doses (1, 5, 15 mg/kg) were administered via the i.v., i.p., and p.o. route. Dosing vehicles were composed of 10% dimethylsulfoxide (DMSO) in PEG300. I.v. doses were administered via the tail vein. Oral doses were administered by gavage. At each time point, three to four mice were euthanized by isoflurane (Baxter Healthcare, Deerfield, IL) and blood samples (up to 600 µl each) were taken from the posterior vena cava. Plasma samples were stored at -20°C prior to analysis.

Sample Preparation for LC-MS/MS Analysis in Mouse Pharmacokinetics

Plasma proteins were precipitated by the addition of acetonitrile (150 µl, containing the internal standard) to 100 µl of mouse plasma. Samples were vortexed and then centrifuged at 8000 g for 10 min. The supernatant was transferred to a clean vial for injection into the mass spectrometer for analysis.

Pharmacokinetic Data Analysis
Mean plasma concentration-time data were analyzed using noncompartmental methods (WinNonlin Version 5.2.1, Pharsight Corporation, Mountain View, CA). The area under the plasma concentration-time curve from time 0 to infinity (AUC_{inf}) was calculated by the trapezoidal rule with extrapolation to time infinity. The terminal T_{1/2} was calculated as ln2/\lambda_z, where \lambda_z was the first order rate constant associated with the terminal (log-linear) portion of the curve. The plasma CL was calculated as Dose/AUC_{inf}. The maximum plasma concentration (C_{max}) and the time when it occurred (T_{max}) were obtained by visual inspection of the plasma concentration-time curve. The apparent V_dss was calculated as CL×MRT_{inf} where MRT is the mean residence time extrapolated to infinity calculated as AUMC_{inf}/AUC_{inf}, where AUMC_{inf} is the area under the first moment curve extrapolated to infinity. The bioavailability (F) was calculated as (AUC_{non-intravenous}×dose_{i.v.})/(AUC_{i.v}×dose_{non-intravenous}).

In Vitro Metabolism Studies

For both phase I and phase I & II metabolism studies, animal microsomes was used as described previously (Li et al.). Briefly, the incubation mixture consisted of 1 mg/ml liver microsomal proteins, 3 mM NADPH, and 0.5 \mu M test compound in 65 mM potassium phosphate buffer (pH 7.4). The concentration of 0.5 \mu M was selected for these studies based on preliminary experiments showing that the K_m value for metabolism was > 10 \mu M. For the phase I & II metabolism, 5 mM uridine diphosphate glucuronic acid (UDPGA), 5 mM D-Saccharolactone, 50 \mu g/ml alamethicin, and 3 mM magnesium chloride were added to the phase I metabolism study mixture. The concentration of methanol (used for dissolving the substrate) was 1 % (v/v). Total volume of the incubation was 200 \mu l and the reaction mixtures were incubated at 37°C. For metabolite identification, the reaction mixture was incubated for 2 hr. To generate the stability curves for I-387 different incubations were stopped at 10, 20, 30, 60, and 90 minutes for analysis of I-387 remaining. All reactions were stopped by the addition of 200 \mu l ice-cold acetonitrile containing an internal standard for quantification. Subsequently, the samples were then centrifuged at 8000 g for 5 min and supernatant was analyzed by LC-MS/MS. The mean peak area ratio of I-387 to internal standard observed at time zero as 100%. Replicate studies were conducted on at least two separate occasions for each species.

LC-MS/MS Analyses

The LC-MS/MS analysis was performed on a 4000 Q trap triple quadrupole/linear ion trap mass spectrometer (Applied Bioscience, Foster City, CA) with a nanospray interface. The nanospray temperature was set...
as 500°C, curtain gas at 30 psi, ion spray energy at 5500 V, Nebulizer gas (gas 1) at 30 and Nano gas (gas 2) at 40 psi. Nitrogen was used as the collision gas. The collision energy was 45 eV. The protonated molecules were investigated. Aliquots of samples were injected into the HPLC system (Model 1100 Series Chemstation, Agilent Technology Co, Santa Clara, CA). Gradient mode was used to achieve separation of the analytes using mixtures of mobile phase A (95% water and 5% acetonitrile with 0.1% formic acid) and mobile phase B (95% acetonitrile and 5% water with 0.1% formic acid) at a flow rate of 300 μL/min.

Samples for pharmacokinetic studies were separated with a Halo 2.1 x 50 mm C18 column (Wilmington, DE) within a runtime of 8 min. Mobile phase A was used at 80% from 0 to 1 min followed by a linearly programmed gradient to 80% of mobile phase B within 1.5 min, and 80% of mobile phase B was maintained for 2 min before a quick ramp to 20% mobile phase B for 30 sec. 20% of mobile phase B and 80% of mobile phase A were continued for another 3 min towards the end of analysis. Multiple reaction monitoring (MRM) mode scanning m/z 388.2 → 192 was used to obtain the most sensitive signals for I-387.

Metabolite separation was performed on an Alltech Alltima HP 2.1 x 100 mm C18 column (Deerfield, IL). Total run time was 15 min. The mobile phase was comprised of 100% of A for the first 1 min and increased to 100% B in a linear gradient over 7 min. 100% of mobile phase B was maintained for 2 min and it quickly returned to 0% for 30 sec. 100% mobile phase A was continued for 4.5 more min until the end of analysis. Q1 full (data was not shown), precursor ion, neutral loss, and enhanced product ion (EPI) scan modes, were utilized to identify metabolites of I-387.
Results

Pharmacokinetics of I-387 in Mice

Doses of 1, 5, and 15 mg/kg were administrated via the i.v., i.p., and p.o. route in order to elucidate the pharmacokinetic parameters of I-387 in ICR mice. Plasma concentrations of I-387 in mice declined in a biexponential manner after i.v. injection, with a terminal T_{1/2} of 4-9 h. The V_{ss} of I-387 was 3.8 to 9.8 l/kg following i.v. administration indicating high tissue binding. The high tissue binding of I-387 is likely a result of the hydrophobic nature of this compound. The estimated log P and clog P values of I-387 were 4.15 and 4.77, respectively (ChemDraw Ultra Version 11.0., CambridgeSoft Corporation, Cambridge, MA). CL slightly decreased with increasing dose for the 1, 5, and 15 mg/kg with values of 27, 21, and 19 ml/min/kg, respectively (Table 1 and Supplemental Figure 1), which is 40-60% of hepatic plasma flow (Davies and Morris, 1993). MRT_{int} increased from 3 and 3.3 h at the 1 and 5 mg/kg doses, respectively, to 8.8 h following the 15 mg/kg dose after i.v. administration (Table 1). After i.p. administration, I-387 was absorbed quickly with T_{max} values of 5-10 min. Systemic exposure following i.p. doses was 65-95% of the analogous i.v. dose (Table 1 and Supplemental Figure 2). Plasma concentrations of I-387 peaked at about 30 min after p.o. administration with absolute bioavailability of 24, 13, and 8 % after doses of 1, 5, and 15 mg/kg, respectively (Table 1 and Supplemental Figure 3). A drug administered by i.p. injection is subject to the same potential hepatic first pass metabolism as oral administration. However, the i.p. bioavailability (65-95%) of I-387 greatly exceeded that of orally administered drug (8-24%), suggesting that the oral bioavailability of I-387 was primarily limited by its poor stability in gastric or intestinal fluids and/or gut wall metabolism or its incomplete absorption due to poor permeability and/or low aqueous solubility. The approximate terminal T_{1/2} (mean T_{1/2} = 7 h) after i.v. administration was similar to that observed after i.p. and p.o. doses (mean T_{1/2} = 7-8 h) (Table 1).

Metabolic Stability of 387 in Mouse, Rat, Dog, Monkey, and Human Liver Microsomes

Metabolic stability was investigated by incubating I-387 with liver microsomes from mouse, rat, dog, monkey, and human. In order to evaluate the metabolism by phase II conjugation, UDPGA was introduced to the microsomal preparation. Half-life was determined with a two parameter single exponential decay curves using SigmaPlot Version 10.0 (Systat Software, Inc., Chicago, IL). Metabolic stability of I-387 in the presence and absence of UDPGA was not significantly different as evidenced by similar half-lives during incubation with phase I
and phase I & II systems (Table 2). This result suggests that the primary metabolic pathway of I-387 predominantly involves phase I enzymes. I-387 was most stable in rat liver microsomes with the longest T\(_{1/2}\) (41.9 min) and degraded quickly in monkey liver microsomes with the shortest T\(_{1/2}\) (15 min) in phase I metabolism. The phase I metabolic stability of I-387 in human liver microsomes (23.6 min) appears to be more similar to those of mouse and dog with half-lives of 36.7, and 29.4 min, respectively than those of the other two species.

**Identification of Metabolites in Human Liver Microsomal Incubation.**

To identify metabolites in human liver microsomes, I-387 was incubated with human liver microsomes in the presence of NADPH for 2 h. Samples were analyzed by LC-MS/MS. Total ion current (TIC) chromatograms of the acetonitrile extracted I-387 in precursor ion and neutral loss scan modes are shown in the Figure 2. The results are described using the terms of A, B, and C rings of I-387 as shown in Figure 1. The product ion spectrum of I-387 (m/z 388.2) results in two prominent fragment ions of m/z 192 and 220 (Figure 3A). Thus, precursor ion of m/z 192 and neutral loss of 167 u scan modes were used to identify metabolites of I-387 and its product fragments. The precursor ion scan of m/z 192 was utilized to identify modifications in the carbonyl group and/or C-ring, while metabolites containing metabolic modifications in rings A or B or the carbonyl could be identified by the neutral loss of 167 m/z.

In addition to unchanged I-387, three metabolites (M1, M2, and M3) were detected after 2 hr incubation in human liver microsomes (Figure 2). In the precursor scan mode, M1 showed the [M + H]\(^+\) ion at m/z 374.7, which was 13.5 amu lower than that of the parent compound (388.2 amu), suggesting the loss of a CH\(_3\) group from the C-ring. M2 observed in neutral loss as well as precursor scan modes showed the [M + H]\(^+\) ion at m/z 390.6, which was 2.4 amu higher than that of the parent compound. These results suggest that the carbonyl group was reduced. M3 showed the [M + H]\(^+\) ion at m/z 404.7, which was 16.5 amu higher than that of the parent compound in the neutral loss scan mode, indicating the addition of an oxygen atom to the A or B ring of parent compound. The fragment ion masses of M1, M2, and M3 obtained in product ion spectrum clearly indicated O-demethylation in the C-ring, reduction of the carbonyl group between rings B and C, and hydroxylation in the A or B ring (Figure 3). The structure of M2 was verified using a synthetic reference standard.

**Metabolites of I-387 in Mouse, Rat, Dog, Monkey, and Human Liver Microsomes.**
To compare its metabolic fate in different species, I-387 was incubated for 2 hr with mouse, rat, dog, and monkey liver microsomes under the same conditions applied to the human liver microsomes. The TIC chromatogram of linear ion trap product ion scan for m/z; 375, 390.5, and 404.5 for each species is shown in Figure 4. Nine metabolites (M1-M9) were observed in mouse, rat, dog, and monkey liver microsomes. Table 3 shows the retention time, [M + H]^+ and major fragment ions of I-387 and its metabolites in the liver microsomal incubations of the five species. In the EIC of m/z 375 and 404.5, additional metabolites (M4, M8, and M9) were observed, with different retention times compared to M1 and M3. M4 yielded the same fragment ion at m/z 192 as M1. Both M8 and M9 generated the characteristic fragment ion at m/z 208, as did M3. In the EIC of m/z 390.5, not only was the metabolite with the reduced carbonyl group (M3) detected but additional metabolites (M5, M6, and M7) were also found. The LC-MS/MS fragmentation pattern of M5, M6, and M7 yielded the characteristic fragment ion at m/z 208, indicating that demethylation and hydroxylation occurred.

The amount of each metabolite was obtained from peak areas in the EIC (Figure 4). The metabolism of I-387 in monkey and human liver microsomes showed similar patterns, with reduction of the carbonyl group and hydroxylation. In dog liver microsomes, hydroxylation was the primary metabolic pathway. Dog liver microsomes generate three different hydroxylated metabolites (M3, M8, and M9). M3 and/or M8 were also observed in the microsomes of other species. O-demethylation was observed to a lesser degree in dog, monkey, and human microsomes. Incubation of mouse and rat liver microsomes with I-387 produced the reduced carbonyl, O-demethylated, and hydroxylated metabolites in similar proportions. Monkey liver microsomes produced similar patterns of metabolism of I-387 as human liver microsomes in vitro. Figure 5 depicts the proposed metabolic pathways of I-387 in the liver microsomes of the five species.
Discussion

Microtubules have been considered to be one of the major and best targets for cancer chemotherapy for decades. In spite of the clinical successes of several antitubulin agents including vinca alkaloids and taxane analogs, toxicity and drug resistance have limited the effectiveness of antimitotic agents in clinical use (Dumontet and Sikic, 1999; Verrills and Kavallaris, 2005; Malik and Stillman, 2008). We identified a novel compound, I-387, which inhibits the in vitro growth of a number of human cancer cell lines with nanomolar IC\textsubscript{50} values. I-387 inhibits tubulin polymerization via the colchicine binding site and circumvents P-glycoprotein-mediated multidrug resistance. An in vivo tumor xenograft study using immunodeficient mice provided compelling evidence of the ability of I-387 to inhibit tumor growth. Furthermore, in vitro and in vivo studies showed that I-387 was less neurotoxic than vinblastine and vincristine, tubulin destabilizers with known neurotoxicity at equi-effective doses.

For the pharmacokinetic studies of I-387 in ICR mice, three doses (1, 5, and 15 mg/kg) of I-387 were administered via the i.v., i.p., and p.o. route. Non-compartmental pharmacokinetic analysis after i.v. injection of I-387 showed that it has a large volume of distribution and low clearance in mice. The relatively long half-life of I-387 after i.v., i.p., and p.o. administration provides the opportunity for less frequent dosing. The bioavailability of I-387 was >65% and <24% after i.p. and p.o. administration, respectively. Therefore, i.p. injection achieved sufficient exposure to I-387 as compared to i.v. injection, without the technical complications or potential morbidity associated with repetitive i.v. or p.o. administration. In the recent xenograft study, 10 mg/kg I-387 was administered via i.p. injection and induced 68% and 76% tumor growth inhibition (Ahn et al.). The pharmacokinetic study showed that plasma concentrations of I-387 (15 mg/kg, i.p.) were maintained > 71 ng/mL for 30 hr, suggesting that systemic drug concentration approaching 50 ng/mL are necessary to achieve in vivo antitumor effect. The low to moderate oral bioavailability of I-387 suggests that additional structural modifications may present a plausible approach to identifying an antitubulin agent that could be administered orally. We recently reported the synthesis and promising in vitro and in vivo anticancer activity of a series of 2-aryl-4-benzoyl-imidazoles with greatly improved aqueous solubility (Chen et al.) as an advance toward this goal.

In vitro metabolic stability in liver microsomes of five different species was examined in the presence of NADPH. I-387 was metabolized mainly via phase I pathways in mouse, rat, dog, monkey, and human liver microsomes. Since I-387 showed similar stability in mouse and dog to human liver microsomes, further efficacy studies in mice and pharmacokinetic studies in dogs might be helpful to predict the effects of I-387 in human
compared to two other species. *In vitro* metabolite identification studies showed that carbonyl reduction, $O$-demethylation, and/or hydroxylation are likely the predominant metabolic pathways for I-387. The positions of hydroxylation and/or demethylation varied between species. Quantitative differences in metabolism were also observed in the liver microsomes of different species. As a result, carbonyl reduction was the most important metabolic pathway in human suggesting that further modification in the carbonyl linkage could improve metabolic stability of this structural pharmacophore in humans.

In conclusion, a promising antitubulin agent, I-387, was slowly cleared, moderately distributed, and moderately metabolized in mice. The metabolic fate of I-387 varied between species. The metabolic identification study indicates that further structural optimization of I-387 in carbonyl linkage could improve metabolite stability while the inclusion of heterocyclic rings instead of the phenyl B-ring linkage may improve aqueous solubility and oral bioavailability. Our study suggests that further structural optimization of I-387 will lead to the development of new orally available antitubulin agents for the treatment of drug-resistant cancer.
Acknowledgments

Sunjoo Ahn, Jeffrey D. Kearbey, Chien-Ming Li, Duane D. Miller, and James T. Dalton are employees of GTx, Inc. and hold stock options in the company.
Authorship Contribution

James T. Dalton, Sunjoo Ahn, Duane D. Miller, and Chien-Ming Li participated in research design. Sunjoo Ahn conducted experiments and performed data analysis. Charles B. Duke III contributed new reagents with Sunjoo Ahn. This manuscript was written by Sunjoo Ahn, Jeffrey D. Kearbey, and James T. Dalton.
REFERENCES


Legends for figures

Figure 1. Synthesis scheme.
A. Synthesis of I-387. Reagents and Conditions: a.) 3,4,5-trimethoxyphenyllithium, Tetrahydrofuran, -78°C, b.) pyridinium dichromate, CH₂Cl₂ c.) aq. NaOH, EtOH, reflux. For better understanding putative structures of I-387 metabolites, we will refer to sections denoted in the figure as the A, B, and C rings. B. The structure of indole-15 (internal standard).

Figure 2. Chromatograms of I-387 incubated with human liver microsomes in precursor ion of m/z 192 and neutral loss of 167 m/z scan modes. Chromatograms of I-387 samples in the precursor ion scan mode (A and B) and in the neutral loss scan mode (C and D) are shown.

Figure 3. Total ion current chromatograms of extracted ions of I-387 parent and metabolites in human liver microsomes using product ion scan mode and their fragmentation patterns.

Figure 4. Total ion current chromatograms of extracted ions of I-387 metabolites in mouse, rat, dog, monkey, and human liver microsomes using product ion scan mode.
The amount of each metabolite was analyzed based on its fragmentation pattern and was shown in right panel; O-demethylation (■), reduction of a ketone group (▲), and hydroxylation in A or B ring (□).

Figure 5. The proposed metabolic schemes of I-387 in the mouse, rat, dog, monkey, and human liver microsomes.
Table 1. Pharmacokinetic parameters of I-387 in male ICR mice after i.v., i.p. and p.o. administration. (n = 3 / dose group)

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</tr>
<tr>
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Table 2. Half life of I-387 in mouse, rat, dog, monkey, and human liver microsomes.
(n=2–3)

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<tr>
<td>Monkey</td>
<td>15 ± 2.3</td>
<td>11 ± 6.9</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>24 ± 2.7</td>
<td>30 ± 3.4</td>
<td></td>
</tr>
</tbody>
</table>
Table 3. Summary of the metabolite profile generated following incubation of I-387 with liver microsomes of mouse, rat, dog, monkey, and human

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention time (min)</th>
<th>[M+H]+</th>
<th>Fragment ions</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-387</td>
<td>11.3-11.4</td>
<td>388</td>
<td>192</td>
</tr>
<tr>
<td>Demethylation in C ring</td>
<td>M1</td>
<td>10.6</td>
<td>375</td>
</tr>
<tr>
<td></td>
<td>M4</td>
<td>10.8</td>
<td></td>
</tr>
<tr>
<td>Reduction of ketone</td>
<td>M2</td>
<td>10.5</td>
<td>390.5</td>
</tr>
<tr>
<td>Demethylation in C ring and hydroxylation in A/B ring</td>
<td>M5</td>
<td>9.5</td>
<td>390.5</td>
</tr>
<tr>
<td></td>
<td>M6</td>
<td>9.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M7</td>
<td>11.4</td>
<td></td>
</tr>
<tr>
<td>Hydroxylation in A/B ring</td>
<td>M8</td>
<td>10</td>
<td>404.5</td>
</tr>
<tr>
<td></td>
<td>M3</td>
<td>10.2-10.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M9</td>
<td>10.6</td>
<td></td>
</tr>
</tbody>
</table>
Fig 1.
Fig 2.

A 0 min

B 120 min

C 0 min

D 120 min

m/z 192

m/z 374.7

m/z 390.6

m/z 404.7

m/z 390.6

m/z 167
Fig 5

I-387

M1, M4
Demethylation

M3, M6, M9
Hydroxylation

M2
Reduction of Ketone

M5, M6, M7
Demethylation and Hydroxylation

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