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Hepatic metabolism of MK-0457, a potent Aurora kinase inhibitor: Interspecies comparison and role of human cytochrome P450 and flavin-containing monooxygenase

J. E. BALLARD, T. PRUEKSARITANONT, C. TANG

Department of Drug Metabolism

Merck Research Laboratories

West Point, PA 19486, USA

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Running Title: *In vitro* metabolism of MK-0457 in hepatic preparations.

Correspondence: Jeanine E. Ballard, Department of Drug Metabolism, WP75A-203, Merck Research Laboratories, West Point, PA 19486, USA
Tel (215) 652-1398, Fax (215) 652-2410,
Email: jeanine_ballard@merck.com

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Abbreviations: CYP, cytochrome P450; FMO, flavin-monooxygenase; K_m, apparent Michaelis constant; K_s, substrate inhibition constant; K_i, inhibition constant; V_{max}, maximum reaction velocity; LC-MS/MS, liquid chromatography coupled with tandem mass spectrometry.

ABSTRACT

MK-0457, an Aurora kinase inhibitor in development for the treatment of cancer, was evaluated for its *in vitro* metabolism in different species. This compound primarily underwent *N*-oxidation and *N*-demethylation in human, monkey, dog, and rat liver preparations. However, *N*-demethylation was less significant in dogs. The formation of minor metabolites varied with species, but all metabolites generated in human hepatocytes were observed in animals. Results of immunoinhibition, selective chemical inhibition, thermal inactivation and metabolism by recombinant CYPs and FMOs strongly suggest that CYP3A4 and FMO3 comparably contributed to MK-0457 *N*-oxidation in human liver microsomes, where the reaction conformed to Michaelis-Menten kinetics. These studies indicate a major role of CYP2C8 in the *N*-demethylation reaction, while CYP3A4 only made a minor contribution. However, significant substrate inhibition was observed with MK-0457 *N*-demethylation, at high substrate concentrations (>10 μM) in human liver microsomes relative to the anticipated therapeutic exposure. A multi-enzyme metabolic pathway such as this may mitigate the potential of drug interactions in clinical treatment with MK-0457.

Introduction

MK-0457 is a potent and selective Aurora kinase inhibitor in clinical development for the treatment of solid tumors and hematopoietic cancers. Aurora kinases are involved in the regulation of chromosome segregation and cytokinesis during mitosis, and overexpression of these proteins is likely to contribute to tumorigenesis (Katayama et al., 2003; Jiang et al., 2006). Inhibition of Aurora kinases disrupts progression of the cancer cell cycle and blocks proliferation *in vitro*, while noncycling cells remain unaffected. Rodent *in vivo* studies have shown that MK-0457 inhibits tumor growth, resulting in regression (Harrington et al., 2004). MK-0457 is the first kinase inhibitor to exhibit clinical activity in patients with T315I BCR-ABL mutated chronic myeloid leukemia or acute lymphcytic leukemia (Giles et al., 2007).

To gain an understanding of the species differences and similarities in the disposition of MK-0457 for the purpose of predicting human disposition and determining appropriate species for toxicology studies, metabolism studies were conducted in rat, dog, monkey, and human liver preparations. For the purpose of predicting potential drug-drug interactions, the drug-metabolizing enzymes involved in the biotransformation of MK-0457 in human liver microsomes were identified. In the present studies, the role of cytochrome P450 (CYP) and flavin-containing monooxygenase (FMO), two major enzyme systems that catalyze oxidative metabolic reactions, have been investigated using a combination of *in vitro* reaction phenotyping strategies. These methods include the use of (1) monoclonal antibodies for immuno-inhibition of specific CYP families, (2) selective chemical inhibitors, (3) cDNA-expressed CYP and FMO isoforms, and (4) thermal inactivation of FMO enzymes. Our results indicate that metabolism of MK-0457 in human liver microsomes was mediated by multiple drug-metabolizing enzymes and the resulting metabolite profile was similar to those in monkeys and rats.

Materials and Methods

Chemicals and Biologics. MK-0457 was synthesized at Merck Research Laboratories (MRL, Banyu, Japan). Its desmethyl- and *N*-oxide metabolites (VRT-171335 and VRT-750074) were provided by Vertex Pharmaceuticals Incorporated (Cambridge, MA), and [^{14}C]MK-0457 (52 mCi/mmol) was prepared by ViTrax (Placentia, CA). Quercetin, sulfaphenazole, quinidine, ketoconazole, bupropion, NADPH, $MgCl_2$, and phosphate buffer were purchased from Sigma-Aldrich (St. Louis, MO). Preparations of pooled human liver microsomes (from 10 male donors) were purchased from XenoTech LLC (Lenexa, KS). Immuno-inhibitory monoclonal antibodies selective for CYP isoforms (anti-CYP1A2, anti-CYP2C, anti-CYP2D6, and anti-CYP3A) and microsomes prepared from baculovirus-infected insect cells containing cDNA-expressed CYP enzymes (CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, and 3A5) were provided in-house (MRL, West Point, PA). Microsomes prepared from baculovirus-infected insect cells containing cDNA-expressed FMOs 1, 3, and 5 were purchased from BD Gentest (Woburn, MA). Viable hepatocytes were isolated from fresh beagle dog, cynomolgus monkey and sprague-dawley rat tissue using a modification of the classical two-step collagenase perfusion technique (Li et al., 1992). Fresh human hepatocytes were purchased from In Vitro Technologies (Baltimore, MD), and two batches of cryopreserved cynomolgus monkey hepatocytes were purchased from CellzDirect (Pittsboro, NC).

Incubations with hepatocytes. Incubations of hepatocytes (1 million cells/mL) with [^{14}C]MK-0457 (1 or 10 μM) were performed with continuous shaking (80 times/min) and a gentle flush of gases (O_2/CO_2 95%/5%) at 37ºC. Reactions were terminated with one half volume of acetonitrile and centrifuged to remove cell pellets prior to radiochromatography.

Incubations with human liver microsomes, recombinant human CYPs and FMOs . Incubations of human liver microsomes (1 mg protein/mL) with [^{14}C]MK-0457 (1 or 10 μM) were carried out in phosphate buffer (0.1 M, pH 7.4) containing 1 mM $MgCl_2$. The reactions were initiated by the addition of 1 mM NADPH and terminated with acetonitrile following 30-min or 60-min incubation at 37ºC. Samples were centrifuged to remove precipitated protein and the resulting supernatant was subject to radiochromatography. For other *in vitro* studies with MK-0457 (1 or 10 μM, and 0.5 to 50 μM for metabolite formation kinetics), the incubation mixtures (0.25 ml) contained the same components except for respective enzyme preparations (0.1 mg/mL human liver microsomal protein, equivalent to 50.5 pmol/mL cytochrome P450; 20 pmol/ml recombinant human CYP; 0.2 mg/mL FMO). For thermal inactivation studies, human liver microsomes were preincubated at 50ºC for 2 min in the absence of NADPH to inactivate FMO enzymes, and control reactions were preincubated at 37ºC for 2 min. Following termination of the reaction, an aliquot of 0.5 μM bupropion was added as an internal standard. Samples were centrifuged, and the resulting supernatant was analyzed via LC-MS/MS. Assays were performed in triplicate under linear conditions with respect to protein concentration and incubation time.

Immuno-inhibition. Human liver microsomes (0.025 mg) were preincubated (at room temperature for 5 min) with anti-CYP antibody or non-specific IgG prepration (5 μL). The ratio of microsomal protein-to-antibody volume was chosen to give a maximal inhibitory effect. Afterwards, the samples were diluted with the buffer containing $MgCl_2$ and MK-0457 and reactions were initiated by the addition of 1 mM NADPH. Assays was performed in triplicate.

Chemical inhibition. Known chemical inhibitors selective for CYP2C8 (quercetin), CYP2C9 (sulfaphenazole), CYP2D6 (quinidine), and CYP3A (ketoconazole) were used to perform

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inhibition experiments. The concentration range of quercetin (1.2, 4, and 12 μM), sulfaphenazole (0.15, 0.5, and 1.5 μM), quinidine (0.03, 0.1, and 0.3 μM), and ketoconazole (0.015, 0.05, and 0.15 μM) was chosen based on the K_i values of previous reports (Bourrie et al., 1996; Rahman et al., 1994). Stock solutions of chemical inhibitors were prepared in 50% acetonitrile/water (v/v). The final concentration of acetonitrile in the incubation mixture was 1.5% (v/v). Control incubations, without chemical inhibitor, contained the same volume of acetonitrile, and did not exhibit enzyme inhibition compared to control incubations containing < 1% acetonitrile. Chemical inhibition experiments were performed in triplicate at MK-0457 concentrations of 1 and 10 μM and were determined using human liver microsomes.

Qualitative MS-Radiochromatography. [^{14}C]MK-0457 and its metabolites were separated on a Synergi Hydro-RP column (4.6 x 150 mm, 4 μm, Phenomenex, Torrance, CA) using a Rheos 4000 binary pump (LEAP Technologies, Carrboro, NC) with a flow rate of 1 mL/min. The aqueous mobile phase (solvent A) consisted of 10% acetonitrile in 0.02% acetic acid (pH adjusted to 9.0 using triethylamine), while the organic phase (solvent B) consisted of acetonitrile. The initial mobile phase was composed of 5% of solvent B, which was maintained for 3 min and increased to 15% over a period of 0.5 min, and then further increased to 25% in 11.5 min, to 40% in 10 min, and finally to 70% over 0.5 min. This value was held for 2 min before returning to 5% of solvent B for column equilibration for 2 min prior to the next injection. The post-column flow rate was split 1:9, with 100 μL/min directed to the LCQ ion trap and 900 μL/min to the radiochemical director. Post-column radiochemical detection was performed using a Packard C515 flow scintillation analyzer (PerkinElmer Life Sciences, Meriden, CT) with a 500 μL liquid flow cell and Packard scintillation cocktail (Ultima-Flo™M) running at 3 mL/min. Mass spectrometry data were collected on the Finnigan LCQ ion trap system (Finnigan, San Jose, CA)

equipped with an electrospray ionization (ESI) source operating in positive mode under optimized conditions (voltage, 4.5 kV; sheath gas, 70; auxillary gas, 10; capillary temperature, 220^{o}C).

Quantitative LC-MS/MS. Quantitative analysis of two major metabolites of MK-0457 (desmethyl metabolite VRT-171335 and *N*-oxide VRT-750074) was accomplished by LC-MS/MS analysis. Separation was achieved on a Synergi Polar-RP column (2.0 x 50 mm, 4 µm, Phenomenex, Torrance, CA) using PE 200 binary pumps. Solvent A consisted of 0.02% aqueous acetic acid and 10% acetonitrile, adjusted to pH 9.0 with NH_4OH, and solvent B of 90% acetonitrile in water. The mobile phase was delivered at a flow rate of 0.5 mL/min with a linear increase of solvent B from 5% to 95% over 0.6 min and a hold at 95% for 1.4 min, followed by equilibration for an additional 1.5 min. LC-MS/MS was performed on a Sciex Model API 3000 triple quadrupole mass spectrometer (Concord, Ontario, Canada) interfaced to the column eluent via a Sciex turbospray probe operated at 450ºC. Operating conditions common to both analytes were IS voltage (5000), NEB gas (8), CUR gas (12), and CAD gas (4). Other parameters were optimized by infusion of a mixture of all analytes, using selected reaction monitoring (SRM) experiments in positive ionization mode to monitor transitions of 451>383 for VRT-171335 and 481>394 for VRT-750074. The calibration curve (0.001 – 5 µM) was constructed using the ratio of the analyte peak area to the internal standard peak area. The limit of quantification for this assay was 0.005 µM for both metabolites.

Data analysis. The apparent enzyme kinetic parameters were determined by fitting the reaction velocities versus substrate concentrations to equation (1) or equation (2) (KaleidaGraph 3.52, Synergy Software, Reading PA), which describe Michaelis-Menten kinetics alone or Michaelis-Menten kinetics coupled with uncompetitive substrate inhibition, respectively.

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$$v = \frac{V_{\max} \cdot S}{K_m + S} \qquad (1)$$

$$v = \frac{V_{\max} \cdot S}{K_m + S(1 + S / K_s)} \qquad (2)$$

Where v, S, V_{max}, K_m and K_s are the velocity of reaction, substrate concentration, the maximum reaction velocity, apparent Michaelis constant and substrate inhibition constant, respectively.

Results and Conclusions

Figure 1 outlines the metabolites of MK-0457 generated in human, cynomolgus monkey, dog, and rat hepatocytes. All species gave rise to two major metabolites (Figure 2), namely, *N*-oxide (VRT-750074) and desmethyl products (VRT-171335), although the desmethyl metabolite was much less significant in dogs. Consistent with the findings in human hepatocytes, MK-0457 was metabolized to its *N*-oxide and desmethyl metabolites in a preparation of pooled male human liver microsomes (Figure 3). Other minor metabolites were also observed in hepatocytes, including M1 (human, cynomolgus and rat), M2 (all species tested), and a glucuronide conjugate M3 (human, cynomolgus and rat). Structural confirmation of major metabolites (VRT-750074 and VRT-171335) was achieved using synthesized authentic standards, and identification of M3 as a glucuronide conjugate was based on mass change (data not shown). All of the metabolites detected in human hepatocytes were observed in rat and monkey hepatocytes as well. Also, VRT-750074 and VRT-171335 were the major metabolites formed *in vivo* in rats and monkeys following administration of MK-0457(data not shown). However, in dog hepatocytes, *N*-desmethyl formation was low and M1 as well as the glucuronide conjugate M3 were not observed. The *in vitro* species differences observed in the metabolite profile of MK-0457 suggest that monkey and rat may potentially be better models than dog for the assessment of toxicological risks associated with MK-0457 metabolites.

Since *N*-demethylation and *N*-oxidation were the primary metabolic pathways of MK-0457 in human liver preparations, metabolizing enzymes involved in these reactions were characterized. Monoclonal antibodies, thermal inactivation, chemical inhibitors, and recombinant enzymes were employed to estimate the relative contribution of CYP and FMO

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isoforms involved in MK-0457 metabolism in human liver microsomes. The findings are described below.

Immuno-inhibition was first approached to provide a quick assessment of relative importance of metabolizing enzymes. It was found that anti-CYP2C antibodies markedly inhibited the formation of *N*-desmethyl metabolite VRT-171335 (85 ± 9 and 70 ± 8% inhibition at 1 and 10 μM, respectively), and inclusion of anti-CYP3A antibody led to further inhibition (92 ± 7% inhibition at both MK-0457 concentrations). The result suggests that the reaction was primarily mediated by members of the CYP2C subfamily with a minor contribution from CYP3A enzymes. Further studies with recombinant CYPs showed that CYP2C8 was capable of catalyzing the reaction at the highest rate (1.69 ± 0.18 and 13.63 ± 0.93 pmol/min/pmol CYP at 1 and 10 μM MK-0457), followed by CYP3A4 (0.41 ± 0.04 and 1.97 ± 0.18 pmol/min/pmol CYP at 1 and 10 μM MK-0457). Moreover, of all the inhibitors tested, only quercetin significantly inhibited VRT-171335 formation (~ 80% inhibition at 12 μM). Although recombinant CYP2D6 was able to catalyze this reaction efficiently (0.22 ± 0.02 and 1.83 ± 0.14 pmol/min/pmol CYP) compared with other isoforms (< 0.025 and 0.25 pmol/min/pmol for CYP1A2, 2A6, 2B6, 2C9, 2C19, 2E1, and 3A5 at 1 and 10 μM MK-0457), the contribution of this isoform *in vivo* is anticipated to be low based on the low abundance of CYP2D6 (~2% of total CYP content) compared to CYP2C8 (~8%) and 3A4 (~20-30%) (Lasker et al., 1998; Shimada, 1994; Rodrigues, 1999; Shimada et al., 1999). In agreement, anti-CYP2D6 antibodies and quinidine failed to inhibit the reaction. Collectively, *N*-demethylation of MK-0457 in human liver microsomes is primarily catalyzed by CYP2C8, with a minor contribution from CYP3A4.

On the other hand, the formation of *N*-oxide VRT-750074 was moderately inhibited by antibodies selective for CYP3A (25 ± 6 and 48 ± 7% inhibition at 1 and 10 μM MK-0457,

respectively) and by a combination of antibodies against CYPs 2C and 3A (45 ± 12 and 60 ± 8% inhibition at 1 and 10 μM MK-0457, respectively). The results suggest that the reaction was only partially catalyzed by the CYP3A and, to a minor extent, CYP2C subfamilies. Consistently, recombinant CYP3A4 and CYP2C8 displayed the highest and the 2nd highest metabolic rates (0.20 ± 0.02 and 1.17 ± 0.18 pmol/min/pmol CYP for CYP3A4, 0.036 ± 0.003 and 0.33 ± 0.05 pmol/min/pmol CYP for CYP2C8 at 1 and 10 μM MK-0457) among the isoforms tested (< 0.06 pmol/min/pmol CYP for the rest of isoforms at 10 μM MK-0457). In addition, moderate inhibition (~ 50%) was achieved by ketoconazole (0.15 μM) and quercitin (12 μM). The partial inhibition of MK-0457 *N*-oxidation observed in the immuno-inhibition study elicited further investigation into the enzyme(s) responsible outside the cytochrome P450 family. A role for flavin-containing monooxygenases was anticipated based on their known ability to catalyze *N*-oxidation reactions (Cashman, 1995). This hypothesis was first investigated using the heat lability of FMOs compared to CYPs in human liver microsomes (Grothusen et al., 1996). It was found that pre-incubation of human liver microsomes at 50ºC for 2 min (to inactivate FMOs) inhibited VRT-750074 formation by 44 ± 5% (1 μM MK-0457) and 66 ± 4% (10 μM MK-0457), while VRT-171335 formation was not significantly altered by the same treatment. Next, the specific isoform involved was elucidated using recombinant FMO enzymes. Among the three FMO isoforms tested, FMO1 and FMO3 displayed good catalytic activity for the formation of the *N*-oxide VRT-750074 (1832 ± 7 and 1919 ± 83 pmol/min/mg protein for FMO1 and FMO3 at 10 μM MK-0457), but FMO5 showed a ~ 10-fold lower activity (110 ± 15 pmol/min/mg protein at 10 μM MK-0457). While FMO1 and FMO3 were both capable of efficiently catalyzing the *N*-oxidation reaction, the absence of FMO1 in adult human livers excludes its role in MK-0457 *N*-oxidation. Supported by another fact that the hepatic abundance of FMO3 is

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similar to CYP3A4 in humans **(**Cashman, 2002; Haining et al., 1997), it is conceivable that FMO3 appreciably contributes to MK-0457 *N*-oxidation in human liver microsomes.

Another interesting difference between these two major metabolic pathways in human liver microsomes is that *N*-oxidation conformed to typical Michaelis-Menten formation kinetics (K_m and V_{max} values of 6.6 μM and 499 pmol/min/mg), while *N*-demethylation kinetics appeared to comprise of substrate inhibition as indicated by the decreased formation rate as substrate concentration exceeded ~ 10 μM (Figure 4). This observation was reproduced with recombinant human CYP2C8 (Figure 4). The reduced rate of *N*-demethylation cannot be the result of substrate precipitation at higher concentrations since it did not occur with recombinant CYP3A4 under the same conditions. The possibility of generation of an inhibitory metabolite was ruled out as well, because the product VRT-171335 is not a potent CYP2C8 inhibitor (IC50 > 20 μM). However, different from other cases of substrate inhibition (Lin et al., 2001; Shou et al., 2000) where substrate inhibition constants (K_s) are greater than the K_m, fitting the current data to equation 2 containing a substrate inhibition component gave rise to K_s values of 5.6 and 5.0 μM for human liver microsomes and recombinant CYP2C8, respectively. These values are significantly lower than the respective K_m (24 and 64 μM, respectively) for MK-0457 *N*-demethylation. Attempts to fit the data with a more complex model containing α and β terms (Shou et al., 2000) led to a similar result. Apparently, better understanding of this unusual kinetic feature of CYP2C8-mediated reactions would be an interesting subject for future investigation. However, this phenomenon is not expected to have any clinical significance for MK-0457 as the inhibition occurs at substrate concentrations well above the anticipated therapeutic exposure (≤ 1 μM).

In summary, MK-0457 metabolism in human hepatic preparations is characterized by two major pathways, namely, *N*-demethylation and *N*-oxidation, which are well mirrored in rat and cynomolgus monkey *in vitro* systems. The former pathway in humans is primarily catalyzed by CYP2C8 with a minor contribution of CYP3A4, while CYP3A4 and FMO3 appear to be equally important to the latter reaction. This multiple enzyme mediated metabolism may attenuate the potential of drug interactions in clinical treatment with MK-0457, regardless of the known inter-individual variability in enzyme abundance and activity (Shimada, 1994; Shimada et al., 1999; Bosch et al., 2006; Cashman, 2002) of these three enzymes as well as CYP3A4 vulnerability to induction and inhibition (Guengerich, 1999; Lin and Lu, 2001).

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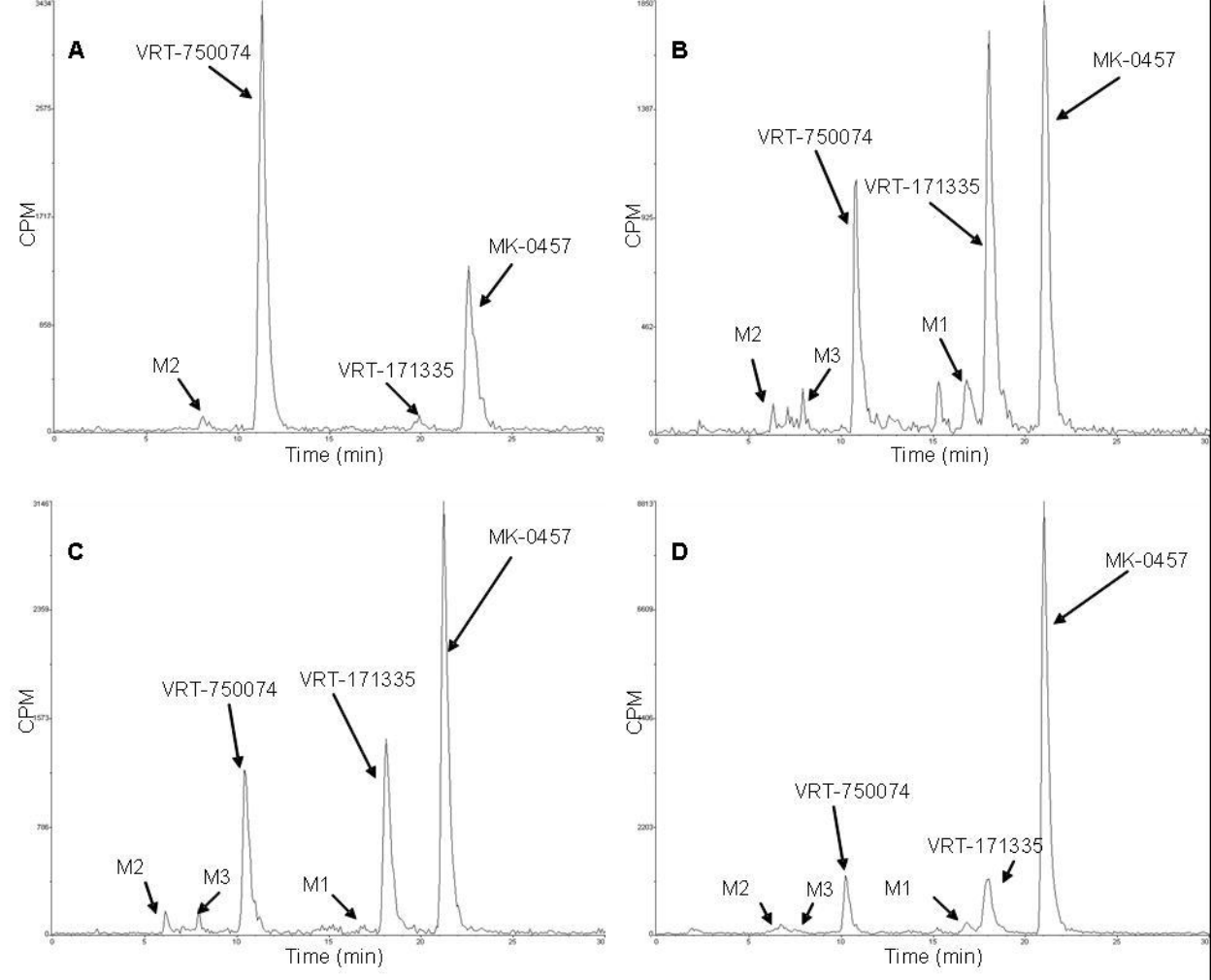
Legends for Figures

Figure 1. Representative radiochromatograms of MK-0457 and metabolites extracted from dog (DHp; A), cynomolgus monkey (cMHP; B), human (HHp; C), and rat (RHp; D) hepatocyte incubates. Incubations were carried out with 10 μM [^{14}C]MK-0457 at 37^{o}C for 30 minutes (DHp, cMHp, HHp) or 120 minutes (RHp) using 1 million cells/mL.

Figure 2. Structures of MK-0457 and major metabolites formed in suspensions of rat (RHp), dog (DHp), cynomolgus monkey (cMHp) and human heptocytes (HHp) as well as human liver microsomes (HLM) supplemented with NADPH.

Figure 3. Representative radiochromatograms of MK-0457 and metabolites extracted from human liver microsome incubates. Incubations were carried out at 37^{o}C with 1 μM [^{14}C]MK-0457 for 30 minutes (A) and 10 μM [^{14}C]MK-0457 for 60 minutes (B) using 1 mg/mL protein concentration.

Figure 4. Formation kinetics of *N*-desmethyl product VRT-171335 over MK-0457 concentration range of 0.5 - 50 μM in human liver microsomes (solid circle) and recombinant CYP2C8 (open square). The kinetic parameters obtained by fitting the data to Equation 2 give rise to V_{max}, K_m and K_s of 4950 pmol/min/mg protein, 24 μM and 5.6 μM for human liver microsomes. The corresponding values for recombinant CYP2C8 are 129 pmol/min/pmol CYP, 64 μM and 5.0 μM.


A
VRT-750074
MK-0457
M2
VRT-171335
CPM
Time (min)
B
MK-0457
VRT-750074
VRT-171335
M2
M3
M1
CPM
Time (min)
C
MK-0457
VRT-750074
VRT-171335
M2
M3
M1
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Time (min)

Figure 1

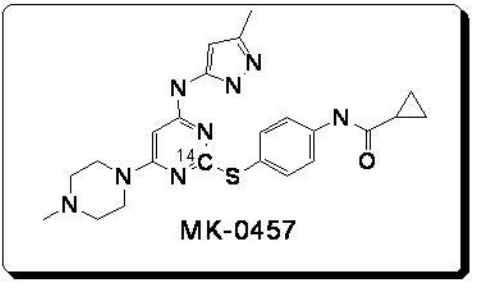
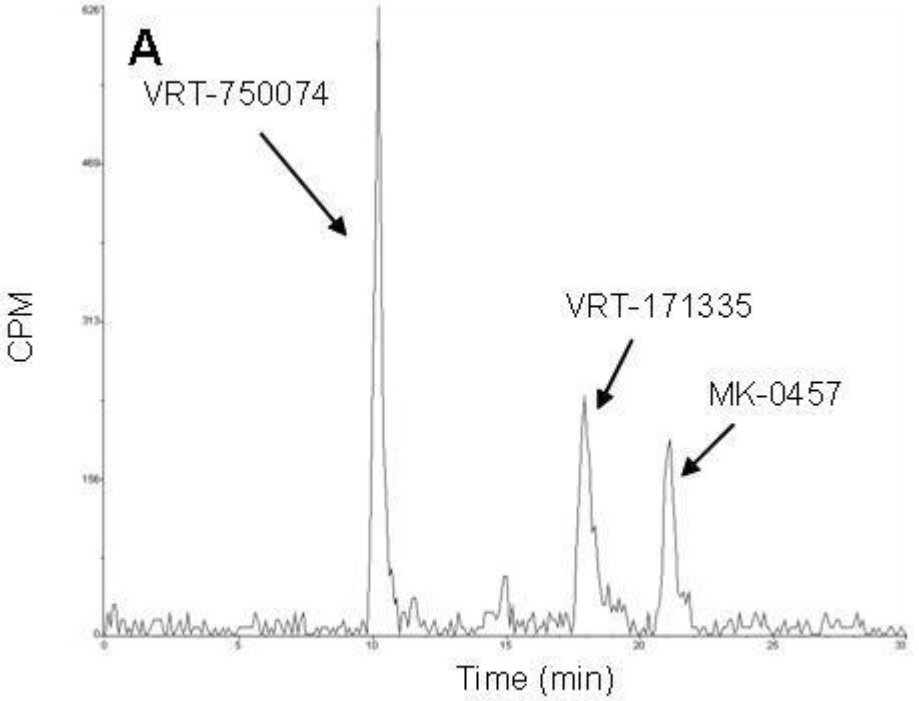

MK-0457
CYP3A4
FMO3
CYP2C8
CYP3A4
VRT-7500074
(DHp, HHp,cMHp, RHp, HLM)
VRT-171335
(DHp, HHp,cMHp, RHp, HLM)

Figure 2



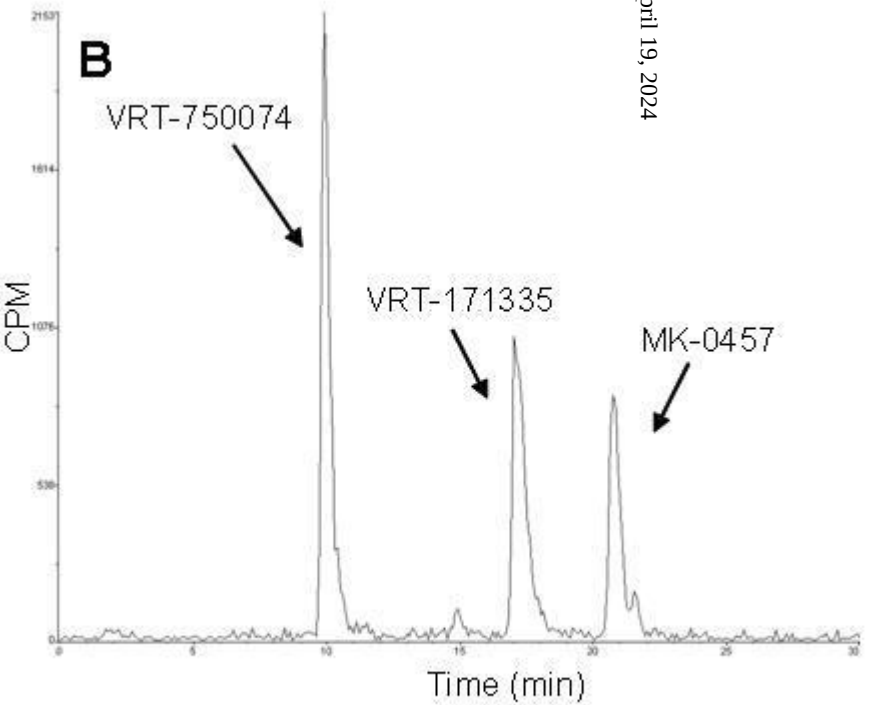


Figure 3

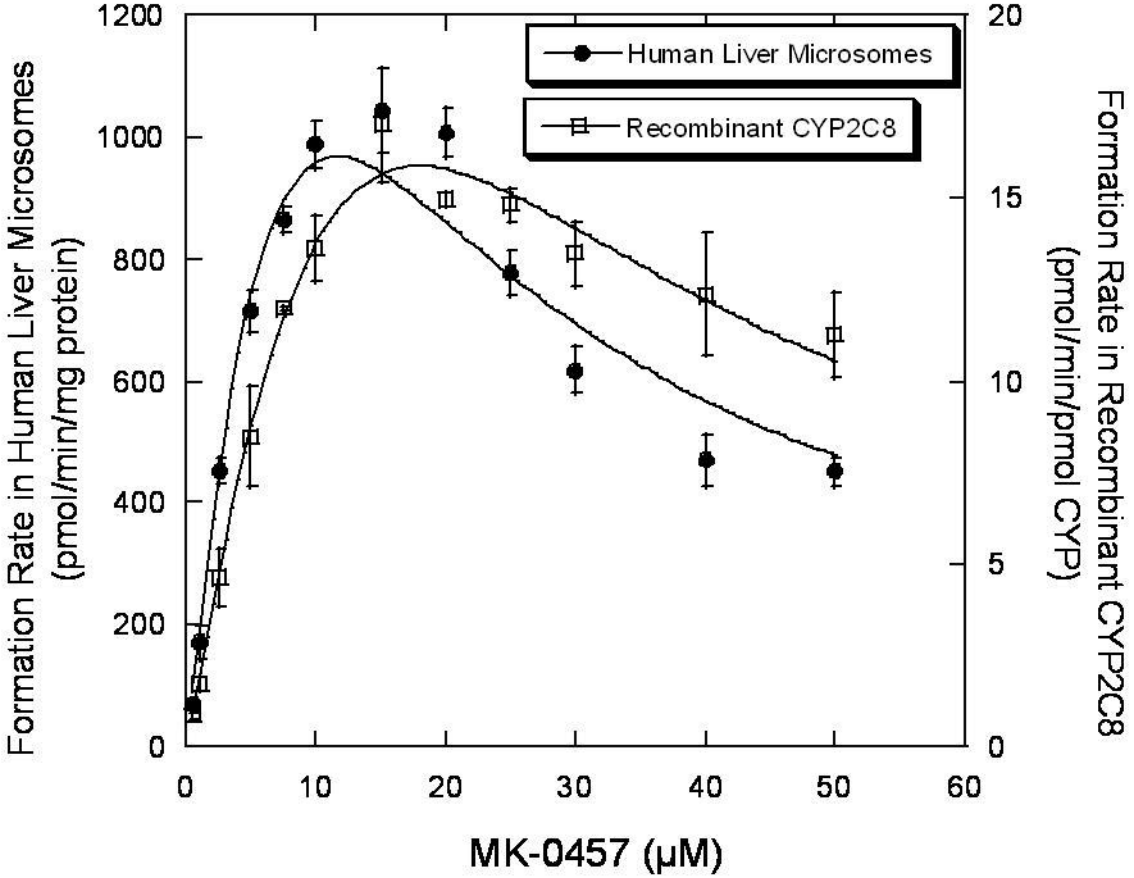

Human Liver Microsomes
Recombinant CYP2C8
Formation Rate in Human Liver Microsomes (pmol/min/mg protein)
Formation Rate in Recombinant CYP2C8 (pmol/min/pmol CYP)
MK-0457 (μM)
0
200
400
600
800
1000
1200
0
5
10
15
20
0
10
20
30
40
50
60

Figure 4