Enzyme-Transporter Interplay in the Formation and Clearance of Abundant Metabolites of Faldaprevir Found in Excreta but not in Circulation

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ABSTRACT

Faldaprevir is a hepatitis C virus protease inhibitor that effectively reduces viral load in patients. Since faldaprevir exhibits slow metabolism in vitro and low clearance in vivo, metabolism was expected to be a minor clearance pathway. The human [14C] absorption, distribution, metabolism, and excretion study revealed that two monohydroxylated metabolites (M2a and M2b) were the most abundant excretory metabolites in feces, constituting 41% of the total administered dose. To deconvolute the formation and disposition of M2a and M2b in humans and determine why the minor change in structure [the addition of 16 atomic mass units (amu)] produced chemical entities that were excreted and were not present in the circulation, multiple in vitro test systems were used. The results from these in vitro studies clarified the formation and clearance of M2a and M2b. Faldaprevir is metabolized primarily in the liver by CYP3A4/5 to form M2a and M2b, which are also substrates of efflux transporters (P-glycoprotein and breast cancer resistance protein). The role of transporters is considered important for M2a and M2b as they demonstrate low permeability. It is proposed that both metabolites are efficiently excreted via bile into feces and do not enter the systemic circulation to an appreciable extent. If these metabolites permeate to blood, they can be readily taken up into hepatocytes from the circulation by uptake transporters (likely organic anion transporting polypeptides). These results highlight the critical role of drug-metabolizing enzymes and multiple transporters in the process of the formation and clearance of faldaprevir metabolites. Faldaprevir metabolism also provides an interesting case study for metabolites that are exclusively excreted in feces but are of clinical relevance.

Introduction

Chronic hepatitis C is a major clinical concern worldwide. It is one of the leading causes of liver transplantation (Gonzalez, 2010) and the second most common cause of hepatocellular cancer (Perz et al., 2006). Faldaprevir (BI 201335, FDV) (Fig. 1) is a potent and selective hepatitis C virus (HCV) NS3/4A protease inhibitor that has achieved high sustained viral response rates in treatment-naïve and treatment-experienced patients with chronic genotype 1 infection when used in combination with pegylated interferon alfa-2a and ribavirin (Sulkowski et al., 2013a,b).

In the [14C]absorption, distribution, metabolism, and excretion (ADME) study in humans, faldaprevir was the predominant drug-related material in plasma, representing >97.9% of all drug-related material (Chen et al., 2014). Faldaprevir was almost exclusively excreted in bile, with 98.7% of the radioactivity recovered from feces and negligible urinary excretion (0.11%). Unchanged faldaprevir constituted 52% of fecal radioactivity in humans. The most abundant fecal metabolites were two monohydroxylated metabolites, M2a and M2b (Fig. 1), which represented 22% and 20% of fecal radioactivity (22% and 19% of the dose), respectively. There were five additional very minor phase 1 fecal metabolites.

Since faldaprevir exhibits slow metabolism in vitro and low clearance in vivo (Duan et al., 2012) and minimal metabolites were detected in human plasma, the substantial presence of two hydroxylated metabolites (M2a and M2b, combined 41% of the dose) in human feces was unexpected (Chen et al., 2014). Some phase 2 metabolites, such as glucuronides, are efficiently effluxed to the gastrointestinal tract after their formation in the gut or liver and are not detectable in plasma (Hjelle and Klaassen, 1984; Sfakianos et al., 1997; Zamek-Gliszczynski et al., 2006). However, this phenomenon is unusual for phase 1 metabolites, with one case reported for another material (Zamek-Gliszczynski et al., 2006). Faldaprevir is metabolized primarily in the liver by CYP3A4/5 to form M2a and M2b, which are also substrates of efflux transporters (P-glycoprotein and breast cancer resistance protein). The role of transporters is considered important for M2a and M2b as they demonstrate low permeability. It is proposed that both metabolites are efficiently excreted via bile into feces and do not enter the systemic circulation to an appreciable extent. If these metabolites permeate to blood, they can be readily taken up into hepatocytes from the circulation by uptake transporters (likely organic anion transporting polypeptides). These results highlight the critical role of drug-metabolizing enzymes and multiple transporters in the process of the formation and clearance of faldaprevir metabolites. Faldaprevir metabolism also provides an interesting case study for metabolites that are exclusively excreted in feces but are of clinical relevance.

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ABBREVIATIONS: ADME, absorption, distribution, metabolism, and excretion; B/P, blood-to-plasma ratio; BSA, bovine serum albumin; CLint, intrinsic clearance; CLint passive, intrinsic clearance of passive diffusion; CLint uptake, intrinsic clearance of sinusoidal uptake; Cmax, maximum plasma concentration; DDI, drug-drug interactions; DME, drug-metabolizing enzymes; Fp, fraction absorbed after oral administration; Fu, the fraction of drug escaping metabolism in the liver; Fu, the fraction of drug escaping metabolism in the intestine; fL, free fraction of drug in liver; fL, free fraction of drug in plasma; HCV, hepatitis C virus; HBSS, Hank’s balanced salt solution; HLM, human intestinal microsomes; HLC, human liver cytosol; HLM, human liver microsomes; IVIVC, in vitro-in vivo correlation; kmax, first-order absorption rate constant; LC-MS/MS, high-performance liquid chromatography-tandem mass spectrometry; Papp, effective permeability in human; Qc, blood flow through enterocytes; Qb, hepatic blood flow; Qs, hybrid term of the villous blood flow (Qvill) and permeability clearance (CLp); rCYP, recombinant human cytochrome P450; [S]unbound substrate concentrations in the liver; [S]unbound substrate concentrations in the intestine.
Faldaprevir, were synthesized at Boehringer Ingelheim Pharmaceuticals, Inc. (Ridgefield, CT). NADPH was purchased from Sigma-Aldrich (St. Louis, MO). Fresh pooled heparinized human plasma was obtained from Biologic Specialty Suppliers. Caco-2 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). HCV genotype 1a (strain H77) and genotype 1b (strain HCV40) NS3/4A heterodimeric proteins were purified from baculovirus-infected Sf21 insect cells. The microsomes, cytosol, rCYPs, and HCV proteins were stored at −80°C until used. All other reagents and solvents were of analytical grade or higher purity and were obtained from commercial suppliers.

Protein Binding and Blood-to-Plasma Ratio
Teflon dialysis cells and dialysis membranes (Spectra/Por) with 12,000–14,000 molecular weight cutoff (Spectrum, Rancho Dominguez, CA) were used for equilibrium dialysis to determine the binding of [14C]faldaprevir in pooled human plasma, 4% human serum albumin, HLM (0.5 mg/ml), and HIM (0.5 mg/ml). Concentration ranges of [14C]faldaprevir tested were 1.1–46 μM for plasma protein binding and 0.3–115 μM for albumin binding to cover therapeutic concentrations. [14C]faldaprevir concentrations were 0.5–100 μM for microsomal binding to cover the concentrations used in the incubations with HLM and HIM described as follows. For each matrix and [14C]faldaprevir concentration tested, five individual dialysis cells were prepared and rotated at 20 rpm using a Spectrum dialysis cell rotor in a water bath maintained at 37°C for 4 hours. At the end of the incubation period, the contents of each side were transferred to scintillation vials and processed for liquid scintillation counting. Faldaprevir blood to plasma ratio was determined by measuring the total radioactivity in plasma and whole blood after spiking [14C]faldaprevir at two clinically relevant concentrations (0.03 μM and 17 μM) to fresh human blood. All vials were incubated at 37°C with gentle agitation and samples were collected at 20, 40, and 60 minutes from each vial. The radioactivity in whole blood (after oxidizing) and plasma was analyzed by liquid scintillation counting. The experiment was run in duplicate.

Stability of Faldaprevir in Fecal Sample during Storage and Extraction
Fresh fecal human sample (2.89 g) from a healthy male subject was weighed and homogenized with 3.2 ml of water. Faldaprevir stock solution was spiked to the fecal homogenate at a final concentration of 0.37 mM to mimic faldaprevir’s concentration in the gastrointestinal tract. The sample was vortexed and kept at room temperature for 4 hours and then stored at −20°C for 3 days. A fraction of the spiked fecal homogenate (1.40 g) was centrifuged at 13,000 rpm for 10 minutes. After the supernatant was separated, the pellet was extracted three times with 1.2 ml of 5% acetic acid in acetonitrile. The extracts were combined with the supernatant and then mixed with 1 ml of methanol containing 0.2% formic acid. After centrifugation, the supernatant was separated, evaporated to dryness under a stream of nitrogen gas at 35°C, and then reconstituted in 0.35 ml of methanol with 0.2% formic acid. After centrifugation at 13,000 rpm for 3 minutes, the supernatant was submitted for analysis by LC-MS/MS to monitor the formation of M2a and M2b.

Incubation with Human Gut Content
Individual fecal samples from two healthy male subjects were collected and mixed with Dulbecco’s phosphate buffered saline containing 20 mM glucose to reach a concentration of 0.1 g/ml. The mixture was then homogenized and centrifuged at 500 g for 5 minutes at 4°C to remove debris. The supernatant was transferred to each well and preincubated for 5 minutes at 37°C. Reactions were initiated by the addition of faldaprevir (final concentration 50 μM) to each well and conducted under either aerobic or anaerobic conditions (Li et al., 2012) at 37°C. Final organic solvent concentration in each reaction was 1%. At 0, 10, 20, 40, and 60 minutes, a 50-μl aliquot was transferred out and processed by protein precipitation with organic solvent. The samples were analyzed by LC-MS/MS to monitor the formation of M2a and M2b.

Incubation with HLM, HLC and rCYPs
Faldaprevir (50 μM) was incubated with HLM, HLC, rCYP1A2, rCYP2B6, rCYP2C8, rCYP2C9, rCYP2C19, rCYP2D6, rCYP3A4, or rCYP3A5 at a protein concentration of 1 mg/ml in 50 mM potassium phosphate buffer (pH 7.4). All samples were preincubated for 5 minutes, and reactions were initiated by the addition of NADPH (2 mM) for incubations with HLMs and rCYPs. For developmental drug (Zamek-Gliszczynski et al., 2013). Why did a minor change in structure (i.e., the addition of a mass of 16 to a molecular weight of 870 Da) produce chemical entities (M2a and M2b) that were excreted rather than released into the blood? To address this question, it is important to understand the role of drug metabolizing enzymes (DMEs) and transporters in the formation and clearance of M2a and M2b. To deconvolute the formation and disposition of M2a and M2b in humans, multiple in vitro test systems were used. Reaction phenotyping studies revealed that both M2a and M2b were formed primarily via CYP3A4/5. A logical question to address was whether these metabolites are formed in liver and/or intestine. Liver and intestine are also the main organs involved in drug-drug interactions (DDIs), and so M2a and M2b may play a role in DDIs if formed in these organs. Also, as the liver is the target organ for HCV treatment, it was important to assess the antiviral activity of M2a and M2b and consider whether these metabolites could contribute toward efficacy. In addition, efflux and uptake transporter studies were performed to define more clearly the role of transporters in the disposition of M2a and M2b. Slow in vitro metabolism of faldaprevir created unique challenges for describing the complete fate of M2a and M2b. An additional challenge in understanding faldaprevir disposition was that animals are not satisfactory surrogates to study faldaprevir metabolism in humans, since the formation of M2a and M2b is significantly lower in animals (Ramsden et al., 2014a). This finding necessitated in vitro investigations with human-derived preparations.

These investigations are also in alignment with regulatory requirements to identify the clearance pathways and contributions of enzymes and transporters to drug disposition (FDA, 2012; EMA, 2012). Current regulatory guidance focuses primarily on circulating metabolites, with little consideration for fecal metabolites. Faldaprevir metabolism provides an interesting case study for metabolites, which are exclusively excreted in feces, but are of clinical relevance.

Materials and Methods

Chemicals, Reagents, and Other Materials
Faldaprevir, IN79158 (M2a) and IN79157 (M2b), d7-faldaprevir and [14C]faldaprevir were synthesized at Boehringer Ingelheim Pharmaceuticals, Inc. (Ridgefield, CT). Materials and Methods.

Figure 1. Chemical structures of faldaprevir, M2a, and M2b.
incubations with cytosol, faldaprevir was added to initiate the reaction, and no NADPH was used. Final organic solvent concentration in each reaction was 1%. At different time points up to 60 minutes, a 50-μl aliquot was transferred out and processed by protein precipitation with organic solvent. The samples were analyzed by LC-MS/MS to monitor the formation of M2a and M2b.

Kinetic Determination of the Formation of M2a and M2b by HLM and HIM

Faldaprevir, at total concentrations ranging from 0.363 to 100 μM, was incubated with HLM and HIM at a protein concentration of 0.5 mg/ml. The reactions were initiated by the addition of NADPH at a final concentration of 2 mM. Organic solvent was kept at 1% in all incubations. At specified time points up to 60 minutes, a 50-μl aliquot was transferred out and processed by protein precipitation with organic solvent. The samples were analyzed by LC-MS/MS to monitor the formation of M2a and M2b.

Permeability of Faldaprevir, M2a, and M2b through Caco-2 Cells and their Interactions with Efflux Transporters as Substrates

Caco-2 cells were seeded to collagen-coated, 12-well polycarbonate membranes in Costar Transwell plates (1.13 cm2 insert area, 0.4-μm pore size; Corning Life Sciences, Corning, NY) at a density of 60,000 cells/cm2. The plates were placed in a humidified incubator (37°C, 5% CO2), and the culture medium was changed every other day until the monolayers reached confluence. To initiate the study, the donor compartment was rinsed with the incubation solution [Hanks’ balanced salt solution (HBSS) containing 10 mM HEPES, 15 mM D-glucose, and 1% BSA was added to replace the aliquot]. Then, 1 ml (or 0.5 ml) of fresh HBSS containing 5% BSA, to HepatoPac and fibroblast only plates in the absence and presence of an inhibitor, rifamycin SV (100 μM), was added to the donor compartment, and 1.5 ml (or 0.5 ml) of HBSS [containing 10 mM HEPES, 15 mM D-glucose and 1% bovine serum albumin (BSA)] was added to the receiver compartment. The bidirectional permeability assessment of faldaprevir, M2a, and M2b was performed in the incubator in triplicate in each apical-to-basolateral (AP-to-BL) and BL-to-AP direction in the absence and presence of inhibitors of efflux transporters (valspodar at 1 μM, Ko143 at 2 μM, or MK-571 at 30 μM). For receiver samples, aliquots (300 μl) were taken at four time points up to 120 minutes, and the same volume of fresh HBSS containing 10 mM HEPES, 15 mM D-glucose, and 1% BSA was added to replace the aliquot. For donor samples, aliquots (20 μl) were taken at the selected time points without replacement. Concentrations of faldaprevir, M2a, and M2b were determined by LC-MS/MS.

Liver Uptake of M2a and M2b into Human Hepatocytes

The HepatoPac model from a female Caucasian donor was prepared by Hepregen Corporation as described previously (Khetani and Bhatia, 2008). Cryopreserved human hepatocytes were seeded with 5000 cells/well and cultured for 9 days before being used. Reactions were initiated by adding M2a or M2b (0.01–100 μM), in fresh HepatoPac incubation medium (65 μl) containing 5% BSA, to HepatoPac and fibroblast only plates in the absence and presence of an inhibitor, rifamycin SV (100 μM). The plates were cultured in an incubator with 10% CO2 and 99% relative humidity at 4°C or 37°C. At each time point, the medium was removed from each well, transferred to a 96-well glass fiber filter plate; and processed by protein precipitation with organic solvent. The samples were analyzed by LC-MS/MS to monitor the levels of M2a and M2b.

A separate study with human HepatoPac was performed to evaluate the inhibition of M2a and M2b uptake into hepatocytes after their formation from faldaprevir. The study was initiated by adding faldaprevir at a final concentration of 0.3 μM [free concentration at 0.003 μM, relevant to free therapeutic concentrations (Ramsden et al., 2014b)], in fresh HepatoPac incubation medium (65 μl) containing 5% BSA, to HepatoPac plates in the absence and presence of an inhibitor, rifamycin SV (100 μM). At each time point (0, 0.5, 1, 2, 4, 8, 12, 24, 48, 72, and 96 hours), both medium and lysate samples were collected as described above and analyzed by LC-MS/MS to determine the levels of faldaprevir, M2a, and M2b.

Activity Assessment of M2a and M2b

Inhibition potency of M2a and M2b, in comparison with faldaprevir, against the NS3/4A serine proteases of HCV genotypes 1a and 1b was determined using a fluorigenic assay performed with the NS3/4A heterodimer protease genotype 1a and 1b as described previously (White et al., 2010). Briefly, the reaction mixture comprised 0.5 mM NS3/4A serine proteases of HCV genotypes 1a or 1b, 5 μM fluorigenic depsipeptide substrate anthranilyl-DidiVPAbu[C(O)-O]AMY(3-NO2)TW-OH and inhibitor in a reaction buffer composed of 50 mM Tris-HCl (pH 8.0), 0.25 M sodium citrate, 0.01% n-dodecyl-β-D-maltoside, 1 mM tris(2-carboxyethyl)phosphine (TCEP) plus 5 mM dimethylsulfoxide (DMSO). The reaction mixtures were incubated at 23°C for 60 minutes (genotype 1a NS3/4A protease) or 45 minutes (genotype 1b NS3/4A protease) and terminated by the addition of 1 M morpholinethane-sulfonic acid (MES) (pH 5.8). Fluorescence of the N-terminal product anthranilyl-DidiVP-Abu was measured using a POLARStar Galaxy plate reader (BMG Labtech Inc., Cary, NC). Calculated percent inhibition at each inhibitor concentration was then used to determine the median 50% inhibition concentration (IC50) (Assay Explorer v3.2; Synmex Technologies).

Liver Uptake Assay

Liver uptake of M2a and M2b into human hepatocytes was monitored by an LC-MS/MS system under the same conditions as used for metabolite identification in the human [14C] ADME study. This system consisted of an Agilent 1200 high-performance LC (HPLC) system (Palo Alto, CA) with a Thermof LTQ Orbitrap XL mass spectrometer (Thermo Scientific, San Jose, CA) equipped with an Advion Triversa NanoMate ion source (Advion Biosystems, Ithaca, NY). Metabolite separation was achieved using a reverse-phase HPLC column (Phenomenex Gemini C18, 3.0 μm, 50 x 2.1 mm, 1.7 μm) or Thermo Hypersil GOLD column (Thermo Scientific, San Jose, CA) attached to either a CTC PAL autosampler (Leap Technologies, Carrboro, NC)-Agilent 1200 UPLC system or a Waters Acquity UPLC system (Milford, MA). Mobile phases included 5% acetonitrile in water as the aqueous phase and 95% acetonitrile in water as the organic phase. Each mobile phase was 0.1% formic acid. Column used included Waters Acquity UPLC BEH C18 column (50 x 2.1 mm, 1.7 μm) or Thermo Hypersil GOLD column (50 x 2.1 mm, 1.9 μm) and were maintained at 50 or 45°C, respectively. The MRM transitions were 869→422 for faldaprevir, 885→422 for M2a and M2b, and 876→429 for d3-faldaprevir using positive ionization mode.

Data Analysis

Determination of CLint at Estimated Hepatic Substrate Concentrations.

Km and Vmax values for the formation of M2a and M2b from faldaprevir during storage and sample extraction was monitored by an LC-MS/MS system under the same conditions as used for metabolite identification in the human [14C] ADME study. This system consisted of an Agilent 1200 high-performance LC (HPLC) system (Palo Alto, CA) with a Thermof LTQ Orbitrap XL mass spectrometer (Thermo Scientific, San Jose, CA) equipped with an Advion Triversa NanoMate ion source (Advion Biosystems, Ithaca, NY). Metabolite separation was achieved using a reverse-phase HPLC column (Phenomenex Gemini C18, 3.0 μm, 50 x 2.1 mm column; Torrance, CA). Mobile phase A consisted of 40 mM ammonium acetate in water, adjusted to pH 5.5 with formic acid, and mobile phase B consisted of acetonitrile. The gradient conditions were: 100% A for 5 minutes, then 15% B over 15 minutes, to 75% B over 40 minutes, to 100% B over 5 minutes, and held at 100% B for 5 minutes. The HPLC flow rate was 0.7 ml/min.

The levels of faldaprevir, M2a and M2b in other in vitro assays were quantified by LC-MS/MS using a 4000 Qtrap (AB Sciex, Thornhill, ON, Canada) attached to either a CTC PAL autosampler (Leap Technologies, Carboro, NC)-Agilent 1200 UPLC system or a Waters Acuity UPLC system (Milford, MA). Mobile phases included 5% acetonitrile in water as the aqueous phase and 95% acetonitrile in water as the organic phase. Each mobile phase contained 0.1% acetic acid. Columns used included Waters Acquity UPLC BEH C18 column (50 x 2.1 mm, 1.7 μm) or Thermo Hypersil GOLD column (50 x 2.1 mm, 1.9 μm) and were maintained at 50 or 45°C, respectively. The MRM transitions were 869→422 for faldaprevir, 885→422 for M2a and M2b, and 876→429 for d3-faldaprevir using positive ionization mode.
including maximum unbound plasma concentration (eq. 2), maximum unbound hepatic inlet concentration (eq. 3) (Ito et al., 2002), or maximum unbound plasma concentration times a liver enrichment factor (eq. 4). The liver enrichment factors at clinically relevant doses of 120 mg daily (QD) and 240 mg QD were estimated to be 31.5 and 21.9, respectively, based on the concentration differences in culture medium and cell lysate determined in the human HepatoPac model (Ramsden et al., 2014b). 

The free fraction of drug in liver (fu,liver) in eq. 7 was estimated using eq. 10, which considered the difference in drug ionization and binding proteins in plasma and in liver. The Qf in eq. 10 corresponds to the ratio of fraction of unoxidized drugs in extracellular plasma (pH 7.4) to intracellular tissue water (pH 7.0). Faldaprevir is an ampholyte, with acidic pKₐ of 5.7 and basic pKₐ of 3.1 (data on file, Boehringer Ingelheim Pharm. Inc.). At pH 7.4 and 7.0, the basic ionization group is mostly un-ionized. F1 value was therefore calculated to be 0.410 based on the ratio of fraction of ionized species of the acidic ionization group at pH 7.4 vs. pH 7.0. The PLR in eq. 10 refers to the ratio of binding protein concentration in the plasma to the whole liver. Faldaprevir extensively binds to serum albumin (see Results). Thus, a value of 13.3 was used according to Poulin et al. (2012b).

Estimate of Gut Metabolism. The Qgut model was used to scale the in vitro clearance to intestinal clearance using eqs. 11 and 12 (Yang et al., 2007b):

\[
Q_{gut} = Q_{df} + CL_{perm} + CL_{int,uptake}
\]

In this model, \(Q_{gut}\) is the free fraction of faldaprevir in the gut and was assumed to be equal to 1 (Yang et al., 2007b), and \(F_g \cdot M2a + M2b\) is the fraction of drug escaping metabolism through the formation of M2a and M2b in the intestine. The \(Q_{gut}\) in eq. 12 is a hybrid term of the villous blood flow \(Q_{villi}\) and permeability clearance \(CL_{perm}\) (eq. 11). The input value for \(Q_{villi}\) was 300 ml/min. The \(CL_{perm}\) is calculated as the net cylindrical area of the small intestine (A) times the effective permeability in human (Pgf). The input value for A was 0.66 m², CLint,M2a + M2b in eq. 12 is the sum of intrinsic clearance values for the formation of M2a and M2b in gut. The CLint,M2a + M2b in gut was scaled by two methods: 1) based on average CYP3A abundance (50 pmol/mg of protein) (Gertz et al., 2010) and total intestinal CYP3A content (70.5 nmol) (Paine et al., 1997), and 2) based on intestinal microsomal recovery (3 mg/g of intestine and the total intestine weight of 2100 g/70 kg, human) (Soars et al., 2002).

Results

Stability of Faldaprevir in Human Feces. Faldaprevir was spiked into fresh human fecal samples, followed by storage and sample extraction, which mimicked the conditions used for the processing of fecal samples in the \(^{14}C\) human ADME studies. No M2a and M2b were found in these samples.

Formation of M2a and M2b in Various Enzyme Systems. Faldaprevir was incubated with fresh human feces under aerobic and anaerobic conditions. Neither M2a nor M2b was detected after the incubations. Similarly, M2a and M2b were not found after incubation with HLC. M2a and M2b were detected in the human liver microsomal incubation samples.

Formation rates of M2a and M2b by rCYP isoforms (rCYP1A2, rCYP2B6, rCYP2C8, rCYP2C9, rCYP2C19, rCYP2D6, and rCYP3A4) were determined. The contribution of each cytochrome to the formation of M2a and M2b was determined based on rates of metabolite formation after normalization by relative liver content of each cytochrome. The normalized contributions by rCYP3A4 were 94% and 97%, respectively, for M2a and M2b. Minimal contribution was observed in incubations with CYP2C8, and no formation of M2a and M2b was observed with other CYP isoforms (Fig. 2).

The relative contribution of CYP3A4 and CYP3A5 to the formation of M2a and M2b was assessed using recombinant enzymes (208 pmol/ml) in a separate study, rCYP3A4 and rCYP3A5 formed M2a at comparable rates, whereas the formation rate of M2b was 27-fold higher in incubations with rCYP3A4 compared with rCYP3A5 (Table 1). Based on the relative content of CYP3A4 and CYP3A5...
in subjects with different CYP3A5 genotypes (Lin et al., 2002), the expected relative contributions of CYP3A4 and CYP3A5 to the formation of M2a and M2b and the relative ratio of the formation of M2a and M2b were calculated specifically for each CYP3A5 genotype (Table 1). CYP3A5 has a minimal contribution to the formation of M2b, and its contribution to the formation of M2a varies based on CYP3A5 genotype.

Considering the slow in vitro metabolism of faldaprevir, a high faldaprevir concentration (50 μM) and high protein concentrations were used in these in vitro systems to ensure sufficient turnover to form the metabolites. This faldaprevir concentration is 10-fold higher than steady-state plasma Cmax of faldaprevir (4.18 μM in phase 2 trial 1220.5, data on file, Boehringer Ingelheim Pharmaceuticals, Inc.) after oral dosing of faldaprevir at 120 mg QD in patients but was still relevant to therapeutic concentrations at the higher dose since there is nonlinearity in pharmacokinetics (steady-state plasma Cmax of faldaprevir was 23.9 μM after oral dosing of faldaprevir at 240 mg QD in patients in phase 2 trial 1220.5; data on file, Boehringer Ingelheim Pharmaceuticals, Inc.). As for the cytochrome phenotyping study, since there was almost no turnover with recombinant cytochromes other than rCYP3A4/5 at a faldaprevir concentration of 50 μM, significant turnover with CYPs other than CYP3A4/5 at lower substrate concentrations would not be expected.

**Kinetics of the Formation of M2a and M2b.** Faldaprevir was incubated with HLM or HIM at 0.5 mg/ml protein concentration, and the formation of M2a and M2b over time was measured. The fractions of unbound faldaprevir (fu) in the HLM and HIM incubations were measured by equilibrium dialysis at the same protein concentration (0.5 mg/ml). The average fu of faldaprevir was determined to be 0.275 using the fu of 0.002 after dosing with faldaprevir 120 mg QD and 240 mg QD, respectively, in separate studies, which is related to weak to moderate microsomal binding. The kinetic profiles for M2a and M2b formation by HLM and HIM are presented in concentration-velocity plots (Fig. 3, A–D). Although the kinetic profiles for M2a and M2b formation resemble a typical substrate inhibition kinetic profile, the empirical substrate inhibition model did not accurately describe the kinetic data, suggesting that the decrease in velocity at high substrate concentrations may be due to other mechanism(s). A time and faldaprevir concentration-dependent loss of CYP3A4 enzyme activity was observed in a separate study, which is related to weak to moderate inactivation of CYP3A4 by faldaprevir (data on file, Boehringer Ingelheim Pharmaceuticals, Inc.).

Based on these findings, the kinetic analysis for parameter estimation was then performed with the 5-minute incubation data sets, as these were the least affected by time-dependent inhibition of CYP3A4. Data points that displayed substrate inhibition (decreasing velocity with increasing substrate concentration) were also eliminated from further kinetic analysis since these points were likely affected by faldaprevir-mediated CYP3A4 inactivation. The kinetic plots for M2a and M2b by HLM and HIM are shown in Fig. 4, A and B, respectively using only the 5-minute incubation data and after removing free concentrations above 8 μM to minimize the impact of CYP3A4 inactivation. The kinetic data fitted the Michaelis-Menten equation, and the kinetic parameters generated are shown in Table 2. Intrinsic clearance, organ clearance (CLh,B and CLg), and the fraction of drug-escaping metabolism (Fe and Fp) were estimated using multiple estimated hepatic/intestinal concentrations and multiple in vitro to in vivo correlation (IVIVC) models. The results are shown in the supplemental data (Supplemental Table S-1 to S-3) and are summarized in Table 3. The unbound fraction of faldaprevir in plasma (fu,p) was determined to be 0.002 by equilibrium dialysis using pooled human plasma and remained constant over a range of faldaprevir concentrations (1.1–46 μM). Binding of faldaprevir to human serum albumin was high, with fu determined to be 0.003–0.008 over a wide range of faldaprevir concentrations (0.3–115 μM). Blood-to-plasma ratio (B/P) of faldaprevir was determined in vitro by measuring the total radioactivity in human plasma and whole blood. Blood cell partitioning was not dependent on faldaprevir concentration, with an average B/P ratio of 0.613. The other parameters used in the calculations of intrinsic clearance, organ clearance, and the fraction of drug-escaping metabolism in liver and intestine are listed in the Materials and Methods section and contained in the supplemental material.

The free steady-state Cmax ([S]h,a) was 0.00836 and 0.0478 μM using the fu of 0.002 after dosing with faldaprevir 120 mg QD and 240 mg QD, respectively, in phase 2 trials (Supplemental Table S-1). Other estimated liver concentrations ([S]h,b) ranged from 0.0127–1.05 μM (Supplemental Table S-1). These concentrations were all

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**Fig. 2.** Relative contribution (%) of seven major cytochromes to the formation of M2a and M2b using rCYPs.

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**TABLE 1**

Comparison of the contribution of CYP3A4 versus CYP3A5 to the formation of M2a and M2b

<p>| Metabolite Formation rate in rCYP3A4: Metabolite Formation Rate in rCYP3A5 (at the Same Cytochrome P450 Content, 206 pmol/ml) |</p>
<table>
<thead>
<tr>
<th>M2a</th>
<th>M2b</th>
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<tr>
<td>M2a</td>
<td>1:4:1</td>
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<tr>
<td>M2b</td>
<td>27:1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CYP3A5 Allelic Variants</th>
<th>Frequency (Lin et al., 2002)</th>
<th>Contribution of CYP3A4-CYP3A5, Normalized by Relative Content of CYP3A4 and CYP3A5 in Subjects with Different CYP3A5 Allelic Variants</th>
<th>Ratio of Formation Rates for M2a Versus M2b, Normalized by Relative Content of CYP3A4 and CYP3A5 in Subjects with Different CYP3A5 Allelic Variants</th>
</tr>
</thead>
<tbody>
<tr>
<td>*1/*1</td>
<td>1.7%</td>
<td>30: 70</td>
<td>89: 11</td>
</tr>
<tr>
<td>*1/*3</td>
<td>22%</td>
<td>58: 42</td>
<td>96: 4</td>
</tr>
<tr>
<td>*3/*3</td>
<td>77%</td>
<td>97: 3</td>
<td>99.8: 0.2</td>
</tr>
</tbody>
</table>
below 8 μM (the highest in vitro free concentration in Fig. 4), supporting the removal of higher concentrations in generating kinetics. The concentration of faldaprevir in enterocytes ([S]g) was estimated to be 7.22–92.0 μM after 120 mg and 240 mg QD dosing (Supplemental Table S-1), which are at the high end of the curve or greater than the concentration range used to generate kinetics for the formation of M2a and M2b in HIM. Therefore, the decrease in the velocity at the higher concentrations in the HIM incubations may not be captured in the calculation of CLint, M2a + M2b in the gut, resulting in an overestimation of CLg, M2a + M2b. However, even with this overestimation, the calculated CLg,M2a + M2b was still very low compared with intestinal blood flow. Therefore, the overestimation is not expected to impact the conclusion.

**Permeability and Interaction with Efflux Transporters of M2a and M2b and Comparison with Faldaprevir.** The absorptive permeability (apical to basolateral) and efflux transport (basolateral to apical) of M2a, M2b and faldaprevir were evaluated using Caco-2 cell monolayers and are shown in Fig. 5. The absorptive permeability values for M2a and M2b were similar at approximately 0.14 × 10⁻⁶ cm/s and 0.16 × 10⁻⁶ cm/s, and were lower than the efflux permeability values of approximately 5.8 × 10⁻⁶ cm/s and 4.2 × 10⁻⁶ cm/s, respectively. These permeability values were considerably lower than those for faldaprevir which were approximately 11 × 10⁻⁶ cm/s and 61 × 10⁻⁶ cm/s for absorptive and efflux permeability, respectively.

The impact of specific inhibitors of transport was evaluated. Valspodar, Ko143, and MK571 were used as specific inhibitors of P-gp, BCRP, and MRP2, respectively. The efflux ratios of both metabolites were significantly reduced by inhibitors of P-gp (valspodar) and BCRP (Ko143), indicating that both metabolites are substrates of P-gp and BCRP. Consequently, the efflux ratios of both metabolites were further reduced by a mixture of valspodar and Ko143. MK571, an MRP2 inhibitor, together with valspodar and Ko143, had no minimal impact on the efflux ratios of M2a and M2b, suggesting that they are not substrates of MRP2.

Faldaprevir is subject to efflux by P-gp since valspodar partially reduced the efflux transport of faldaprevir. The addition of Ko143 and MK571 on top of valspodar had limited impact on the efflux of faldaprevir. However, the efflux ratios did not normalize to ~1 even in the presence of inhibitors of all three efflux transporters, suggesting possible involvement of other efflux transporters.

**Active Uptake of M2a and M2b in Hepatocytes.** The active uptake of M2a and M2b was determined using the human HepatoPac model in the presence and absence of rifamycin SV (100 μM) and at 37°C versus 4°C by measuring intracellular levels of the test compounds over 60 minutes (Fig. 6, A and B). The passive permeability was minimal for M2a and M2b at all time points as determined at 4°C (with or without rifamycin SV). Significant active uptake was observed for M2a and M2b at 37°C compared with 4°C, and the uptake of M2a and M2b was markedly inhibited by rifamycin SV at 37°C by 15- and 17-fold, respectively.

In a separate study, the impact of rifamycin SV inhibition on the formation of M2a and M2b from faldaprevir was also evaluated (Fig. 7). In the absence of rifamycin SV, both M2a and M2b were detected in the medium and lysate, with M2b levels slightly higher than M2a. In the presence of rifamycin SV, the total amounts of M2a and M2b formed decreased on average by ~60% over 24 hours, and neither of the metabolites was detected in the lysate. In the same study, faldaprevir uptake was inhibited to a similar extent (~60%) on average over the experimental time course (through 24 hours).

**Activity of M2a and M2b against NS3/4A Protease.** M2a and M2b were tested in the genotype 1a and 1b protease assays using the same method described previously (White et al., 2010). The activity of faldaprevir was also measured in parallel for comparison. Both
metabolites were more potent inhibitors of genotype 1 NS3/4A proteases than faldaprevir. M2a had IC50 values of 1.5 and 1.7 nM, and M2b had IC50 values of 3.6 and 2.2 nM for genotype 1a and 1b enzymes, respectively (Table 4).

Discussion

Human [14C]ADME studies can provide a thorough assessment of the metabolism and excretion of compounds in drug development. Recent technological advances provide confidence in metabolite profiling via nonradiolabeled methods, generally applied to plasma and urine samples (Nedderman et al., 2011; Obach et al., 2012). These approaches have meant that there should be few surprises from human [14C]ADME studies. The human [14C]ADME study for faldaprevir revealed the unexpected presence of two abundant hydroxylated metabolites in feces that were not detected in the systemic circulation (Chen et al., 2014). It was therefore important to identify the enzymes and tissues responsible for the metabolism of faldaprevir to understand the formation and distribution of these metabolites.

Using recombinant cytochromes, CYP3A4 was shown to be almost exclusively responsible for the formation of M2a and M2b, with a very minor contribution from CYP2C8 (Fig. 2). Both CYP3A4 and CYP2C8 have an expansive active site (Yano et al., 2004; Scott and Halpert, 2005; Schoch et al., 2008) capable of accommodating large molecules such as faldaprevir (870 Da). Formation of M2a and M2b by additional mechanisms (e.g., faldaprevir degradation in feces or metabolism by gut bacteria), although unlikely, was evaluated and excluded.

CYP3A4 and CYP3A5 have overlapping substrate specificities (Lamba et al., 2002), and both enzymes can produce M2a and M2b (Table 1). CYP3A5 expression is highly variable in humans, with readily detectable levels in 25 to 30% of the population and very low or undetectable levels in the remainder (Wrighton et al., 1990; Paine et al., 1997; Wrighton et al., 2000). Normalizing the formation of M2a and M2b by the relative content of CYP3A4 and CYP3A5 in subjects with different CYP3A5 genotypes (Lin et al., 2002) indicated that M2a is formed primarily by CYP3A4 with substantial contribution by CYP3A5 only in subjects with the CYP3A5 genotype *1*1 (~1.7% of population) (Table 1). M2b was mostly formed by CYP3A4 with negligible contribution from CYP3A5. Interestingly, in the most abundant CYP3A5 genotype (*3/*3), the formation of M2b is slightly higher than M2a (1.5-fold; Table 1). Overall, CYP3A5 polymorphisms are not expected to significantly impact the variability of faldaprevir exposure.

Since CYP3A4 and CYP3A5 are extensively expressed in liver and intestine, it was important to determine whether M2a and M2b are formed in the intestine and then excreted directly into feces, or they are formed in the liver. The intestine is an important site for CYP3A-dependent first-pass metabolism (Paine et al., 1997). For example, extraction ratios for the prototypical CYP3A substrate, midazolam, are comparable in liver and intestine (Paine et al., 1996; Thummel et al., 1996). The in vitro kinetics of faldaprevir metabolism did not fit to a substrate inhibition model (Fig. 3) and was complicated by the fact that faldaprevir is a weak to moderate inactivator of CYP3A4. Using the shortest incubation time (5 minutes) and lower unbound substrate concentrations to minimize the impact of CYP3A4 inactivation, the formation of M2a and M2b fitted to Michaelis-Menten kinetics with similar apparent Km values (Table 2). The CYP3A4 activity of the pooled HLM and HIM lots used in our studies was average for humans (testosterone-6β-hydroxylation, 5300 and 1260 pmol/min/mg, respectively). By comparison, the Vmax values for M2a and M2b were much lower (<34 pmol/min/mg for HLM and <7 pmol/min/mg for HIM). Intrinsic clearances for formation of M2a and M2b in the liver and intestine were calculated after the incorporation of estimated hepatic and intestinal concentrations of faldaprevir (Table 3).

**Table 2**

<table>
<thead>
<tr>
<th></th>
<th>M2a</th>
<th>M2b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$ (μM)</td>
<td>$V_{max}$ (pmol/min/mg of protein)</td>
</tr>
<tr>
<td>HLM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment 1</td>
<td>4.30 (0.274)$^a$</td>
<td>25.1 (0.823)</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>3.70 (0.172)</td>
<td>23.8 (0.538)</td>
</tr>
<tr>
<td>Average</td>
<td>4.00</td>
<td>24.5</td>
</tr>
<tr>
<td>HIM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment 1</td>
<td>4.74 (0.528)</td>
<td>4.59 (0.299)</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>4.62 (0.701)</td>
<td>4.27 (0.345)</td>
</tr>
<tr>
<td>Average</td>
<td>4.68</td>
<td>4.43</td>
</tr>
</tbody>
</table>

HIM, human intestinal microsomes; HLM, human liver microsomes.

$^a$Numbers in parentheses are SEs of estimates.
Typically, the well stirred model is used for estimation of hepatic clearance (Houston, 1994). The Poulin method showed better accuracy (Poulin et al., 2012a) compared with other in vitro-in vivo extrapolation methods for hepatic clearance estimation, especially for low-clearance compounds highly bound to albumin. Faldaprevir fits this profile. This method incorporates a correction factor for a higher fraction of unbound drug in the liver, resulting in higher metabolic CL. Because faldaprevir is a substrate of uptake transporters, a modification of the well stirred model, incorporating active uptake, was also used (Webborn et al., 2007). These modified models were preferred because they incorporated important properties of faldaprevir (high protein binding and liver uptake) and resulted in higher hepatic clearance compared with the standard well stirred model (Table 3).

Fg was estimated with a "Qgut" model (Table 3), which retains the form of the well stirred model, but the flow term (Qgut) is a hybrid of both permeability through the enterocyte membrane and villous blood flow, which captures the fact that lower permeability would increase the compound’s first-pass exposure to DMEs in gut. The Qgut model showed significantly improved predictive accuracy for Fg over the well-stirred model (Yang et al., 2007b).

The formation of M2a and M2b is expected to be very slow in both liver and intestine based on low CLh,B and CLg (hepatic and gut intrinsic clearances). Table 3 shows the summary of intrinsic clearance (CLint), organ clearance, and the fraction of drug escaping metabolism through the formation of M2a and M2b in liver and intestine.

### Table 3

<table>
<thead>
<tr>
<th>Organ</th>
<th>Clinical dose</th>
<th>CLint, M2a (µl/min/mg of protein)</th>
<th>CLint, M2b (µl/min/mg of protein)</th>
<th>CLint, M2a+M2b (µl/min/mg of protein)</th>
<th>CLg, M2a+M2b (ml/min per intestine)</th>
<th>Fg, M2a+M2b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>120 mg QD</td>
<td>5.75-6.11</td>
<td>8.01-8.52</td>
<td>13.8-14.6</td>
<td>3.94-4.24 (well stirred model)</td>
<td>0.986-0.998</td>
</tr>
<tr>
<td></td>
<td>240 mg QD</td>
<td>4.85-6.05</td>
<td>6.77-8.43</td>
<td>11.6-14.5</td>
<td>3.25-4.19 (well stirred model)</td>
<td>0.986-0.998</td>
</tr>
<tr>
<td>Intestine</td>
<td>120 mg QD</td>
<td>0.0874-0.372</td>
<td>0.133-0.582</td>
<td>0.220-0.954</td>
<td>0.307-4.94</td>
<td>0.823-0.989</td>
</tr>
<tr>
<td></td>
<td>240 mg QD</td>
<td>0.0458-0.205</td>
<td>0.0693-0.315</td>
<td>0.115-0.520</td>
<td>0.167-2.93</td>
<td>0.895-0.994</td>
</tr>
</tbody>
</table>

QD, daily.

*Estimates are shown as a range for each dose because four estimated hepatic concentrations were used to estimate intrinsic clearance and three in vitro-in vivo correlation models were applied to estimate organ clearance. The detailed calculations are included in Supplemental Table S-1 and S-2.

*Estimates are shown as a range for each dose because intrinsic clearance in intestine was scaled using two estimated intestinal concentrations and two methods. The detailed calculations are included in Supplemental Table S-1 and S-3.

Fig. 5. Transport of M2a (A), M2b (B) and faldaprevir (C) in the absence and presence of inhibitors. Val, valspodar; Ko, Ko143; MK, MK571; ER, efflux ratio. (n = 3)
intestinal blood flow) and high fraction of drug escaping hepatic and intestinal metabolism (Table 3). This is also consistent with low oral clearance of faldaprevir in humans (oral blood clearance at steady state, CL<sub>B,Fss</sub>, from the phase 2 study 1220.5 was approximately 67.4 ml/min after 120 mg QD dosing and 19.7 ml/min after 240 mg QD dosing, data on file, Boehringer Ingelheim Pharmaceuticals, Inc.). Using the modified well-stirred models, CL<sub>h,B</sub> was estimated to be significantly higher than CL<sub>g</sub>. In addition, faldaprevir has a long half-life in human (20 hours), taking 7 days to excrete 85% of the dose (Chen et al., 2014). Therefore, faldaprevir is expected to be slowly metabolized in the liver to form M2a and M2b over several days and as such first-pass intestinal metabolism is minimal.

Accumulation of faldaprevir in the liver (Ramsden et al., 2014a,b) raises questions about possible contributions of uptake and efflux transporters (Shitara et al., 2005, 2006). Compared with faldaprevir, M2a and M2b demonstrated considerably lower (10-fold) absorptive and efflux transport in Caco-2 cells (Fig. 5). M2a and M2b are good substrates of P-gp and BCRP with efflux ratios greater than 26 (Fig. 5, A and B). The passive permeability of M2a and M2b into hepatocytes was also negligible (Fig. 6). The active uptake of M2a and M2b into hepatocytes was largely inhibited by rifamycin SV, suggesting that they are substrates of OATPs (Vavricka et al., 2002). Rifamycin SV has also been shown to inhibit NTCP (Mita et al., 2006). Therefore, the possibility that M2a and M2b are substrates of NTCP cannot be excluded. Incubation of faldaprevir with human HepatoPac resulted in very high intracellular levels of M2a and M2b (Fig. 7). This enrichment is likely an artifact because the metabolites are initially formed in cells. After that, they are excreted (passively or actively) into medium at low levels and are then more efficiently taken back into hepatocytes by uptake transporters, resulting in artificially high hepatocyte enrichment values. Consistent with this hypothesis, in the presence of rifamycin SV, the levels of M2a and M2b in incubations decreased and the metabolites were no longer detectable in hepatocytes (Fig. 7). Faldaprevir uptake was inhibited by ~60% in the same study, contributing to the lower formation of M2a and M2b. The absence of M2a and M2b in cell lysate, in the presence of rifamycin SV, is likely a combination of inhibition of reuptake of M2a and M2b and lower formation. By contrast, in vivo, M2a and M2b, once formed in liver, would be efficiently removed from the cells via bile and excreted. Therefore, the levels of M2a and M2b in the liver in vivo are expected to be low, which is also consistent with the lack of circulating levels of M2a and M2b in blood.

M2a and M2b are pharmacologically active (2- to 4-fold more potent than faldaprevir in HCV genotype 1a and 1b NS3/4A protease inhibition assays; Table 4). However, the contribution of M2a and M2b to the clinical efficacy should be limited considering their expected low exposure resulting from slow formation and efficient efflux into bile. Since faldaprevir is predominantly metabolized by CYP3A in the liver, its exposure may be affected by concomitant medications that inhibit or induce hepatic CYP3A. Based on our findings, inhibition of CYP3A in the gut is unlikely to change faldaprevir exposure. Administration of faldaprevir with an inducer (efavirenz) (Kiser et al., 2013) or an inhibitor (itraconazole, data unpublished) of CYP3A produced moderate DDIs. Thus, although faldaprevir has a very low metabolic clearance, metabolism is still an important clearance pathway.

In summary, faldaprevir is metabolized primarily in the liver by CYP3A4/5 to form two abundant metabolites, M2a and M2b. With

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**TABLE 4**

Summary of activity of M2a and M2b against NS3/4A protease genotype 1a and 1b and comparison with faldaprevir (n = 3)

<table>
<thead>
<tr>
<th>Compound</th>
<th>1a</th>
<th>1b</th>
</tr>
</thead>
<tbody>
<tr>
<td>M2a</td>
<td>1.5 ± 0.3</td>
<td>1.7 ± 0.4</td>
</tr>
<tr>
<td>M2b</td>
<td>3.6 ± 1.4</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>Faldaprevir</td>
<td>6.1*</td>
<td>6.1 ± 0.3</td>
</tr>
</tbody>
</table>

*Average of two determinations (7.7 and 4.4 nM), no S.D. calculated.
low permeability, M2a and M2b are not expected to diffuse freely from the liver. Efflux transporters (P-gp and BCRP) facilitate the excretion of M2a and M2b into bile. If low levels of metabolites permeate to blood, they can be readily taken back up into hepatocytes by uptake transporters (likely OATPs). In addition, as a result of the low permeability, the absorption of M2a and M2b from the intestine is expected to be limited. The combination of these processes explains the absence of these metabolites in the systemic circulation. Overall, understanding the fate of M2a and M2b has been helpful to explain or predict clinical DDIs with faldaprevir as a victim drug and qualify their possible contribution to the overall antiviral activity. Fecal metabolites are generally overlooked due to a lack of clinical impact. Our studies demonstrate that some fecal metabolites could be of clinical relevance and may warrant further evaluation.

Acknowledgments

The authors thank Monica Keith-Luzzi for conducting the assays to determine plasma protein binding, blood to plasma ratio, and microsomal binding; Yanping Mao and Dr. Lin-Zhen Chen for evaluating stability of faldaprevir in feces during storage and extraction; Songping Zhao and Dr. Peter White for evaluating activity of M2a and M2b; and Dr. Timothy S. Tracy for scientific advice.

Authorship Contributions

Participated in research design: Li, Zhou, Ramsden, Taub, Tweedie. Conducted experiments: Zhou, Ramsden, O’Brien, Xu. Contributed new reagents or analytic tools: Busacca, Gonnella. Performed data analysis: Li, Zhou, Ramsden, O’Brien, Xu, Taub. Wrote or contributed to the writing of the manuscript: Li, Ramsden, Tweedie.

References


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Effect of Enzyme-Transporter Interplay on FDV Metabolism


