ABSTRACT

Recent studies showed that P-glycoprotein (P-gp) increases the portal bioavailability (FG) of loperamide by sparing its intestinal first-pass metabolism. Loperamide is a drug whose oral absorption is strongly attenuated by intestinal P-gp-mediated efflux and first-pass metabolism by cytochrome P450 3A4 (CYP3A4). Here the effect of the interplay of P-gp and Cyp3a in modulating intestinal first-pass metabolism and absorption was investigated for another Cyp3a/P-gp dual substrate amprenavir, which is less efficiently effluxed by P-gp than loperamide. After oral administration of amprenavir, the portal concentrations and FG of amprenavir were approximately equal in P-gp competent and P-gp deficient mice. Mechanistic studies on the effect of P-gp on Cyp3a-mediated metabolism of amprenavir using intestinal tissue from P-gp competent and P-gp deficient mice (Ussing-type diffusion chamber) revealed that P-gp-mediated efflux caused only a slight reduction of oxidative metabolism of amprenavir. Studies in which portal concentrations and FG were measured in P-gp competent and P-gp deficient mice whose cytochrome P450 (P450) enzymes were either intact or inactivated showed that intestinal first-pass metabolism attenuates the oral absorption of amprenavir by approximately 10-fold, whereas P-gp efflux has a relatively small effect (approximately 2-fold) in attenuating the intestinal absorption. Cumulatively, these studies demonstrate that P-gp has little influence on the intestinal first-pass metabolism and FG of amprenavir and that intestinal P450-mediated metabolism plays the dominant role in attenuating the oral absorption of this drug.

Introduction

The bioavailability of all orally administered drugs is influenced by the physical barrier properties of the intestinal epithelium and the biochemical barriers that are present within the enterocytes. Cytochrome P450 3A4 (CYP3A4) and P-glycoprotein (P-gp), which are both highly expressed in the intestinal epithelium, contribute to its barrier properties via oxidative first-pass metabolism and apical (AP) efflux, respectively. Dufek et al. (2013) recently addressed the controversial question of whether P-gp works synergistically with CYP3A to increase oxidative metabolism within the intestinal epithelium (Gan et al., 1996; Cummins et al., 2002, 2004; Benet et al., 2004) or to decrease the intestinal first-pass metabolism by reducing intracellular concentrations through AP efflux (Tam et al., 2003; Knight et al., 2006; Knight, 2007). Dufek et al. (2013) chose loperamide as a probe substrate to investigate the interaction of P-gp and Cyp3a during oral absorption in mice because it undergoes extensive first-pass metabolism (Kim et al., 2004), and P-gp–mediated AP efflux of loperamide is highly efficient in the mouse intestine with an absorption quotient (AQ) = 0.90, i.e., an approximately 90% reduction in the AP to basolateral (BL) permeability (see Eq. 3 in the Materials and Methods for the definition of AQ). Hence, the following hypothesis was tested: P-gp–mediated efflux should decrease intestinal first-pass metabolism of loperamide, thus increasing the absorption of intact loperamide into the portal circulation [i.e., gut bioavailability (FG)] and attenuating or reversing the negative impact of AP efflux of the drug. Our recent studies provided support for this hypothesis (Dufek et al., 2013). Furthermore, it was shown that the effect of P-gp on intestinal first-pass metabolism was dose dependent, such that P-gp–mediated efflux caused the greatest increase in the FG at doses that produced intestinal concentrations near the apparent Km for Cyp3a-mediated metabolism (Dufek et al., 2013). This was the first report of P-gp in the intestinal epithelium contributing to an increase, instead of a decrease, in the FG of any drug.

The loperamide studies provided an example of P-gp/CYP3A (Cyp3a) interactions at one end of the spectrum, when the AP efflux and intestinal first-pass metabolism are both very efficient. However, it remains to be seen what impact P-gp would have on the intestinal first-pass metabolism and the FG of a drug if the efficiency of efflux by P-gp is not as high as it is for loperamide. To address this question, a second dual substrate of P-gp and Cyp3a, amprenavir, was investigated using the same experimental models and designs that were used for the loperamide studies (Dufek et al., 2013). Amprenavir (Agenerase; GlaxoSmithKline, Research Triangle Park, NC), a human immunodeficiency virus type 1 protease inhibitor that is primarily metabolized by CYP3A4 (Polk et al., 1999; Tréluyer et al., 2003), was selected for the present study because P-gp is less efficient in reducing
the absorptive permeability of amprenavir (approximately 50% in Caco-2 cell model) (Polli et al., 1999) than of lopereamide. Amprenavir exhibited low and highly variable oral bioavailability, presumably because of poor solubility as well as the combined effect of P-gp-mediated efflux in the intestine and oxidative first-pass metabolism (Decker et al., 1998; Polli et al., 1999; Fung et al., 2000; Noble and Gaia, 2000; Tréfyuer et al., 2003), and was replaced in 2007 by its prodrug, fosamprenavir (Lexiva; GlaxoSmithKline) (Furfine et al., 2004; Arvieux, 2005).

The studies reported here show that amprenavir, like lopereamide, is subject to extensive intestinal first-pass metabolism. Surprisingly, the findings in this study also show that the F<sub>i</sub> of amprenavir is nearly the same in P-gp competent and P-gp deficient mice, suggesting that the oral absorption of intact amprenavir, in contrast to that of lopereamide, is not influenced by intestinal P-gp. Thus, the dominant effect of intestinal first-pass metabolism masks the modest decrease in amprenavir intestinal absorption caused by P-gp-mediated efflux.

Materials and Methods

Materials. Samples of amprenavir ((3S)-oxolan-3-yl N-[(2S,3R,4S)-3-hydroxy-4-[N-(2-methylpropyl)(4-aminobenzene)sulfonamido]-1-phenylbutan-2-yl(carbanate) mesylate and [3H]-amprenavir mesylate (5 Ci/mmol) were generously provided by GlaxoSmithKline. The following reagents were purchased from the indicated sources: troleandomycin, sodium hydroxide, sodium bicarbonate, Krebs bicarbonate ringer (KBR), formic acid, and ammonium formate (Sigma Aldrich, St. Louis, MO), 1α-aminobenzotriazole (ABT) (Santa Cruz Biochemicals, Santa Cruz, CA), acetonitrile, 95% ethanol (EtOH), methanol, and ethyl acetate (Fisher Scientific, Pittsburgh, PA), and tetramethyl ammonium perchlorate and trifluoroacetic acid (WVR, Radnor, PA). All reagents were of analytical grade or higher and triple deionized water was used in all experimental procedures.

Animals. Male CF-1 (P-gp competent) and mdr1a1<sup>-/-</sup> (P-gp deficient) mice (Charles River Laboratories, Wilmington, MA) were maintained under a 12-hours day/night schedule with lights on at 8:00 AM under the care of Division of Laboratory Animal Medicine, the University of North Carolina at Chapel Hill, in accordance with approved protocols from the Association for Assessment and Accreditation of Laboratory Animal Care of the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee. All experiments were carried out in compliance with the Animal Welfare Act public laws.

Metabolism of [3H]-amprenavir by Mouse Intestinal Tissue. The harvesting and preparation of intestinal tissue from P-gp competent and P-gp deficient mice was performed as described by Dufek et al. (2013). Briefly, intestinal tissue was harvested from mice, immediately mounted in the diffusion chamber, and the integrity of the epithelial tissue was confirmed by ensuring that the trans epithelial electrical resistance was ≥250 Ω·cm<sup>2</sup>. The intestinal tissue was preincubated for 30 minutes with KBR buffer, or with KBR buffer containing 100 μM of the Cyp3a inhibitor troleandomycin in studies conducted to measure the intestinal permeability of amprenavir, in the AP and BL chambers. After the equilibrium period, the buffer in the BL chamber was replaced with 3 ml fresh KBR buffer and the buffer in the AP chamber was replaced with [3H]-amprenavir (0.5 μCi/ml; 5, 10, 20, 50, 100, 200, or 400 μM) in KBR buffer containing 0.5% EtOH (n = 3 mice with four intestinal tissues from each mouse for each concentration). For the studies conducted to measure the intestinal permeability of amprenavir, the experimental buffer in the AP chamber contained 100 μM troleandomycin and 10 μM [3H]-amprenavir (0.5 μCi/ml). After incubation for 90 minutes at 37°C, 1-ml samples from the donor and the receiver compartment were extracted with 2 ml ethyl acetate. After agitation for 10 minutes, samples were centrifuged at 4500 × g at 4°C for 10 minutes. The organic phase was removed and placed in a microcentrifuge tube. The aqueous phase was washed with 1 ml ethyl acetate two additional times, and the organic phases were combined. The pooled organic phase was evaporated at ambient temperature under nitrogen gas for 20 minutes and reconstituted in 100 μl mobile phase (see below). Samples were transferred to glass micro-inserts (National Scientific, Rockwood, TN) and placed in autosampler vials (National Scientific). Intestinal tissues were processed as follows to extract intact [3H]-amprenavir and its [3H]-metabolites. The intestinal tissues were washed 10 times in ice-cold KBR buffer, homogenized in 200 μl 50:50 KBR and 2 N NaOH with a sonic dismembrator for 30 seconds, and vortexed with 0.5 ml ethyl acetate for 1 minute. The homogenates were centrifuged at 9000 × g for 10 minutes at 4°C, and the supernatants were collected; washing with ethyl acetate was repeated two additional times. The organic supernatants were combined and then evaporated under nitrogen gas for 10 minutes, reconstituted in 0.5 ml chromatographic mobile phase (see below), and stored at −80°C until samples were analyzed by high-performance liquid chromatography (HPLC).

Samples were quantified with a HP1050 HPLC system (Hewlett Packard, Santa Clara, CA) consisting of a radial autosampler and a quaternary solvent delivery pump that was coupled to a Perkin Elmer Fluo 500 TR Series Radioanalytical Flow Analyzer (Perkin Elmer, Waltham, MA). Samples were injected (70 μl) at ambient temperature onto a Zorbax Eclipse XDB C8 column, 150 × 4.6 mm, with a 5 μm particle size stationary phase (Agilent, Santa Clara, CA). The mobile phase consisted of 10 mM tetramethyl ammonium perchlorate and 0.1% trifluoroacetic acid-acetonitrile (65:35 v/v) (Tréfyuer et al., 2003). The isocratic elution was performed at a flow rate of 1 ml/min and the eluent was mixed inline in the radiomatic flow scintillation detector cell with Perkin Elmer Flow Scint Scintillation fluid at 2 ml/min flow rate. [3H]-Amprenavir eluted at 16.2 minutes. The five metabolites that eluted prior to amprenavir were labeled M1-M5 based on order of elution [see Fig. 1 for chemical structures as reported by Tréfyuer et al. (2003)]. The M1 (5.0 minutes), M2 (8.3 minutes), M3 (13.4 minutes), M4 (13.9 minutes), and M5 (14.9 minutes) metabolites eluted at the retention time indicated in the parentheses. The HPLC system was controlled using Agilent Chemstation software (Agilent) and the radiometric signal for [3H]-amprenavir and its [3H]-metabolites were acquired and their respective peak areas calculated using Flow-One Radiodetection software (Perkin Elmer).

Blood to Plasma Ratio of Amprenavir. Blood was collected from P-gp competent mice in 10-U heparinized centrifuge tubes and immediately mixed to prevent clotting. [3H]-amprenavir (0.1 μCi/ml) was added to whole blood (1 ml) to achieve a 10 nM total concentration of amprenavir and was gently shaken at 37°C for 30 minutes. After shaking, 100 μl of the whole blood sample was separated into two groups: whole blood samples or plasma samples. Whole blood samples (n = 4) were directly aliquoted (55 μl) into glass scintillation vials (Fisher Scientific). Plasma samples (n = 4) were collected by centrifuging 100 μl aliquots of the whole blood sample at 9000 × g for 10 minutes and then the plasma (55 μl) was removed and placed in glass scintillation vials. All samples were solubilized with 10 ml Cytoscin Scintillation Cocktail (Fisher Scientific) and measured using a liquid scintillation counter (1600 TR Liquid Scintillation Analyzer; Packard Instrument Company, Downers Grove, IL).

Oral Absorption of Amprenavir: Portal and Jugular Vein Sampling. Mice had either their portal or jugular veins cannulated as described by Dufek et al. (2013). Amprenavir was administered at doses of 0.42 and 2.1 mg/kg by oral gavage in 0.3 ml of saline (0.9% NaCl) with 2% EtOH to P-gp competent or P-gp deficient mice that had their jugular or portal vein cannulated. The 0.3 ml oral bolus of amprenavir, which is the maximum volume that can be orally administered to mice, was selected to produce approximate intestinal lumen concentrations of 100 and 500 μM for the 0.42 and 2.1 mg/kg doses of amprenavir, respectively. P-gp competent (n = 5) and P-gp deficient (n = 5) mice with portal vein cannulas had blood samples (20 μl) withdrawn through the portal cannula at 15, 30, 60, 120, 180, and 240 minutes after the oral administration of amprenavir (0.42 and 2.1 mg/kg). Systemic blood samples (20 μl) from jugular vein cannulated P-gp competent (n = 5) and P-gp deficient (n = 5) mice were withdrawn though the jugular vein cannula at 15, 30, 60, 120, 180, and 240 minutes after amprenavir dosing (0.42 mg/kg only). Each portal or jugular vein blood sample was replaced with an equal volume of 10 U heparinized saline. The blood samples were placed in heparinized microcentrifuge tubes (Fisher Scientific) and stored on ice until the completion of the experiment. After centrifugation at 9000 × g at 4°C for 10 minutes, plasma was collected and the samples were stored at −80°C. For cytochrome P450 (P450) inhibition studies, 50 mg/kg ABT in 0.3 ml saline with 2% EtOH was dosed to mice by oral gavage 3 hours prior to dosing of amprenavir. Then, an additional 50 mg/kg ABT was coadministered to the mice with 0.42 mg/kg amprenavir at the start of the study.
After the completion of each experiment, 50 μl ice-cold acetonitrile, containing ritonavir (55 nM) as the internal standard, was added to 10 μl of each portal or systemic plasma sample. Samples were vortexed for 1 minute and centrifuged at 9000 × g at 4°C for 10 minutes. Amprenavir was quantified in portal and systemic plasma samples using liquid chromatography–tandem mass spectrometry. The metabolites of amprenavir were not quantified in portal- or jugular-plasma samples. The liquid chromatography–tandem mass spectrometry system consisted of LC10-ADVP quaternary pumps (Shimadzu, Kyoto, Japan) that were fitted with a CTC-PAL autosampler (LEAP Technologies, Carrboro, NC) and a Sciex API-4000 triple-quadrupole mass spectrometer (Applied Biosystems, Foster City, CA). An Aquasil C18, 50 × 2.1 mm, with a 5 μm particle size (Thermo-Scientific, Waltham, MA) column was eluted at 0.75 ml/min with the mobile phases A (35 mM formic acid and 15 mM ammonium formate in deionized water) and B (80:20 mixture of acetonitrile and deionized water). The analyte was injected at a 3 μl volume, and chromatographic separation started with 100% mobile phase A (0.6 minutes), followed by a linear gradient from 0% to 95% mobile phase B (0.6–4 minutes), a wash at 95% mobile phase B for 1 minute, and then a re-equilibration at 100% mobile phase A for 1 minute. The samples were ionized using an Atmospheric Pressure Chemical Ionization source and the positive ions of amprenavir were monitored at transitions of 477/266. Amprenavir calibration standard curves that ranged from 0.015 to 1000 nM were linear throughout the range, and were analyzed at the beginning and end of the run for sample quantification. The positive ions of ritonavir (internal standard) were monitored in the AP and BL compartments and the intestinal tissue) after 90 minutes, $V_{max}$ is the maximum rate, $K_m$ is the concentration of amprenavir at which half maximal metabolic velocity is achieved, and $S$ the concentration in the donor compartment. The kinetic parameters $V_{max}$ and $K_m$ determined for P-gp competent or P-gp deficient mouse intestinal tissue were used to estimate the in vitro intrinsic clearance ($CL_{int}$) ($\mu$/min per cm$^2$) for the formation of the M2–M5 metabolites of amprenavir using Eq. 2.

$$CL_{int} = \frac{V_{max}}{K_m}$$

The apparent permeability ($P_{app}$) across the mouse intestinal tissue for permeability studies conducted under sink condition was calculated according to Eq. 3.

$$P_{app} = \frac{J}{C_D \times A}$$

where $P_{app}$ represents the permeability across the intestinal tissue in the presence of P-gp, $J$ (dQ/dt) represents the mass of drug flux across the intestinal tissue over time, $A$ is the area of the intestinal tissue membrane, and $C_D$ is the initial concentration of drug in the donor compartment (Artursson and Karlsson, 1991).

The efficiency of P-gp in attenuating the rate of flux during intestinal absorption is defined as AQ (Eq. 4).

$$AQ = \frac{P_{PD} - P_{app,AB}}{P_{PD}}$$

This parameter can be obtained by measuring the apparent permeability (AP to BL) across P-gp competent intestinal tissue ($P_{app,AB}$) and the passive permeability ($P_{PD}$) across intestinal tissue from P-gp deficient mice (the intestinal tissue was treated with 100 μM of the Cyp3a inhibitor troleandomycin to prevent metabolism of amprenavir during measurement of permeability values).
and then calculating the decrease in the absorptive permeability caused by P-gp efflux as a fraction of $P_{PD}$ (Troutman and Thakker, 2003). Thus, for a compound with an $AQ$ of 0.7, P-gp efflux attenuates the $P_{PD}$ during absorptive flux by 70%.

The $F_G$ was quantified as previously described by Hoffman et al. (1995) and Dufek et al. (2013).

**Statistical Analysis.** Two-way analysis of variance followed by a Bonferroni multiple comparisons post test was used to assess the difference between the experimental groups being compared using GraphPad Prism 6 statistical software. All values are expressed as the mean ± S.D. The criterion for a significant difference in values was considered as $P < 0.05$.

**Results**

**Metabolism of Amprenavir During Absorptive Flux Across Intestinal Tissue from P-gp competent and P-gp deficient Mice.** The rate of amprenavir metabolism during the absorptive flux of amprenavir across the intestinal tissue from P-gp competent and P-gp deficient mice was determined by quantifying the M2–M5 metabolites of amprenavir (Fig. 1) formed over the course of the transport experiment. M1 was not used for quantification of the metabolic parameters because it was below the limit of quantitation. M3 and M4 were the major metabolites of amprenavir formed in mouse intestinal tissue, followed by, in decreasing order, M5, M2, and M1. Although CYP2D6 and CYP2C9 contribute to the metabolism of amprenavir, this drug is predominantly metabolized by CYP3A4 (Polk et al., 1999; Tréluyer et al., 2003). Ritonavir, a potent inhibitor of CYP3A, increased the oral bioavailability of amprenavir and fosamprenavir by approximately 2-fold, suggesting a role of the CYP3A4-mediated first-pass metabolism in attenuating the oral bioavailability of these drugs (Sadler et al., 2001; Wire et al., 2006). The role of Cyp3a in the mouse intestinal metabolism of amprenavir was demonstrated by the treatment of the intestinal tissue with the CYP3A/Cyp3a-specific mechanism-based inactivator, troleandomycin, which completely abolished the metabolism of amprenavir. The metabolites of amprenavir were preferentially distributed in the AP compartment and intestinal tissue from P-gp competent mice and in the intestinal tissue from P-gp deficient animals. Fig. 2 depicts the rate of amprenavir metabolism during its absorptive flux across intestinal tissue from P-gp competent and P-gp deficient mice plotted against the initial donor compartment concentrations. The metabolic rate was slightly higher for the intestinal tissue from P-gp deficient mice than that from P-gp competent mice, resulting in a slight leftward shift of the metabolic rate versus concentration profile for P-gp deficient mice (Fig. 2). However, there was no difference in the apparent $K_m$ for metabolism in the intestinal tissue from P-gp competent mice ($53 ± 12 \mu M$) compared with P-gp deficient mice ($50 ± 11 \mu M$) (Table 1). These $K_m$ values are comparable with the $K_m$ values determined for the formation of the two major metabolites, M2 (19 $\mu M$) and M3 (32 $\mu M$), by human liver microsomes (Tréluyer et al., 2003). There was no difference in the predicted $V_{max}$ for metabolism in intestinal tissue from P-gp competent or P-gp deficient mice (390 ± 29 pmol/min per cm²) or P-gp deficient (8.9 ± 1.1 $\mu l/min per cm²$) (Table 1).

**Effect of P-gp mediated Efflux on the Absorptive Permeability of Amprenavir Across Mouse Intestinal Tissue.** The efficiency of the intestinal tissue from P-gp deficient mice (53 ± 12 $\mu M$) compared to that from P-gp competent mice was 4.3 ± 1.9 $\mu M$ (Table 1). The lower efficiency of P-gp in attenuating the absorptive flux of amprenavir (AQ = 0.69) compared with that of loperamide (AQ = 0.90) (Dufek et al., 2013) may explain a more modest effect of P-gp in sparing the intestinal metabolism of amprenavir (Fig. 2). Dufek et al. (2013) showed that the in vitro metabolism of loperamide in mouse intestinal tissue from P-gp competent and P-gp deficient mice predicted the effect of P-gp on the intestinal first-pass metabolism of loperamide, and in turn on its $F_G$, in vivo in the same species. Hence, we anticipated that P-gp would only provide moderate protection of amprenavir from intestinal first-pass metabolism in vivo, which was borne out in the studies described below.

**Interaction Between P-gp Efflux and P450-mediated Metabolism of Amprenavir during Intestinal Absorption of Intact Amprenavir in Portal Vein Cannulated Mice.** A 0.42 mg/kg dose of amprenavir, administered via oral gavage in a 0.3 ml volume, which would generate an intestinal concentration of approximately 100 $\mu M$, produced similar plasma concentrations and exposures in the portal circulation of P-gp competent mice [area under the curve 0–240 min (AUC$_{0-240}$) min] = 2.8 ± 0.5 $\mu g$·min/ml and 3.7 ± 2.1 $\mu g$·min/ml for P-gp competent and P-gp deficient mice, respectively; $P > 0.05$ (Fig. 3A). Accordingly, there was no significant difference ($P > 0.05$) in the $F_G$ between P-gp competent (1.8% ± 0.48%) and P-gp deficient mice (2.9% ± 0.87%), although there was a trend toward a higher $F_G$ in P-gp deficient mice (Fig. 4). At the higher dose of 2.1 mg/kg, which is expected to saturate intestinal Cyp3a (Fig. 2), there was no difference in the plasma concentrations of amprenavir in the portal circulation between P-gp competent and P-gp deficient mice (AUC$_{0-240}$ min = 29 ± 30 $\mu g$·min/ml and 25 ± 18 $\mu g$·min/ml, respectively; $P > 0.05$).
Although there was a similar trend in the P-gp deficient group, it did not reach statistical significance due to higher variability in the measurements.

The role of P-gp in modulating the intestinal first-pass metabolism of amprenavir (0.42 mg/kg oral dose) was further investigated by inactivating P450 with ABT (Balani et al., 2002, 2004; Dufek et al., 2013) and determining the effect of P-gp on the portal concentrations/exposure as well as the Fc in the presence and absence of P450 activity in mice. Complete inactivation of P450 in mice treated with ABT was confirmed in a previous study (Dufek et al., 2013) by the complete absence of metabolic conversion of testosterone into the portal circulation of ABT-treated mice compared with saline-treated mice. When P450 was inactivated by treatment of

respectively) (Fig. 3C). Similarly, the Fc of amprenavir at the 2.1 mg/kg dose was comparable in P-gp competent mice (3.4% ± 1.8%) and P-gp deficient mice (4.5% ± 1.6%) (Fig. 4). The area under the curve (AUC), Cmax, and Fc values of amprenavir in P-gp competent and P-gp deficient mice are summarized in Table 2. Comparisons of the AUC0–240 min between the two dose groups (Fig. 3, A and C; Table 2) revealed a 7- to 10-fold increase in the intestinal absorption of amprenavir into the portal circulation in P-gp competent and P-gp deficient mice when the dose was increased by 5-fold, presumably due to saturation of the intestinal first-pass metabolism at the higher dose. A nearly 2-fold increase (P < 0.01) in the Fc between the 0.42 mg/kg and 2.1 mg/kg oral doses for the P-gp competent mouse group (Fig. 4; Table 2) further confirmed saturation of the first-pass metabolism. Although there was a similar trend in the P-gp deficient group, it did not reach statistical significance due to higher variability in the measurements.

The role of P-gp in modulating the intestinal first-pass metabolism of amprenavir in portal vein cannulated mice. The plasma concentration versus time profiles for amprenavir in the portal circulation were generated by analyzing the serial blood samples that were withdrawn from an implanted portal vein cannula in P-gp competent (square) or P-gp deficient mice (circle) over 4 hours. The plots show the log portal vein amprenavir concentrations as a function of time after (A) a 0.42 mg/kg dose of amprenavir + P450, (B) a 0.42 mg/kg dose of amprenavir - P450, (C) a 2.1 mg/kg dose of amprenavir + P450, and (D) a 2.1 mg/kg dose of amprenavir - P450.

**TABLE 1**

The amprenavir permeability and kinetic parameters for metabolism during its absorptive translocation across intestinal tissue from P-gp competent and P-gp deficient mice.

<table>
<thead>
<tr>
<th>Mice</th>
<th>P_app (CYP3A)</th>
<th>(V_{\max})</th>
<th>(K_m)</th>
<th>(Cl_{int})</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-gp competent</td>
<td>440 ± 6.6</td>
<td>390 ± 26</td>
<td>53 ± 12</td>
<td>7.3 ± 0.78</td>
</tr>
<tr>
<td>P-gp deficient</td>
<td>12 ± 7.3</td>
<td>440 ± 29</td>
<td>50 ± 11</td>
<td>8.9 ± 1.1</td>
</tr>
</tbody>
</table>

Values are expressed as the mean ± S.D. of three mice with four intestinal tissues from each mouse.

The apparent permeability (P_app) of [\(^3\)H]-amprenavir and the kinetic parameters for metabolism of amprenavir were measured during its absorptive flux from the AP to the BL side of the intestinal tissue from P-gp competent or P-gp deficient mice that was mounted in an Ussing-type diffusion chamber. Treatment of mouse intestinal tissue from P-gp competent or P-gp deficient mice with 100 \(\mu\)M troleandomycin completely abolished the oxidative metabolism of testosterone (Dufek et al., 2013). The P_app values were determined after inactivation of Cyp3a by pretreatment of the intestinal tissue with troleandomycin [P_app(-Cyp3a)]. The P_app(-Cyp3a) value across intestinal tissue from P-gp deficient mice represents the passive permeability of amprenavir (10 \(\mu\)M) since there is no influence of P-gp-mediated efflux.

**Fig. 3.** The role of P-gp and intestinal first-pass metabolism in the oral absorption of amprenavir in portal vein cannulated mice. The plasma concentration versus time profiles for amprenavir in the portal circulation were generated by analyzing the serial blood samples that were withdrawn from an implanted portal vein cannula in P-gp competent (square) or P-gp deficient mice (circle) over 4 hours. The plots show the log portal vein amprenavir concentrations as a function of time after (A) a 0.42 mg/kg dose of amprenavir (AUC0–240 min = 2.8 ± 0.5 \(\mu\)g-min/ml and 3.7 ± 2.1 \(\mu\)g-min/ml for P-gp competent and P-gp deficient mice, respectively) and (C) a 2.1 mg/kg dose (AUC0–240 min = 29 ± 30 \(\mu\)g-min/ml and 25 ± 18 \(\mu\)g-min/ml for P-gp competent and P-gp deficient mice, respectively), which produce intestinal concentrations of approximately 100 and 500 \(\mu\)M, respectively. Similar plots of the amprenavir portal concentrations as a function of time were also generated in mice whose P450 was inactivated by treatment with ABT (see Materials and Methods section) after (B) a 0.42 mg/kg dose (AUC0–240 min = 34 ± 12 \(\mu\)g-min/ml and 43 ± 16 \(\mu\)g-min/ml for P-gp competent and P-gp deficient mice, respectively) and a (D) 2.1 mg/kg dose (AUC0–240 min = 28 ± 10 \(\mu\)g-min/ml and 28 ± 8 \(\mu\)g-min/ml for P-gp competent and P-gp deficient mice, respectively). Values are expressed as the mean ± S.D. of five mice, with the significance determined using a two-tailed t test of the AUC.
the mice with ABT, there was no difference in the portal exposure of amprenavir in the 2.1 mg/kg dose group for both P-gp competent and P-gp deficient mice (AUC0–240 min = 28 ± 10 µg·min/ml and 28 ± 8 µg·min/ml, respectively) (Fig. 3, C and D; Table 2). For the 0.42 mg/kg dose, there was an approximately 10-fold increase (P < 0.001) in the portal concentrations/exposure when P450 in mice was inactivated (Fig. 3, A and B; Table 2). The increase in the portal exposure of amprenavir at this dose was slightly higher (P > 0.05) in P-gp deficient mice compared with P-gp competent mice (AUC0–240 min = 43 ± 16 µg·min/ml and 34 ± 12 µg·min/ml, respectively) (Fig. 3B).

After the 0.42 mg/kg dose of amprenavir, there was an approximately 10-fold increase in the Fg (P < 0.001) associated with inactivation of P450 activity (Fig. 4). In contrast to P450, P-gp reduced the Fg of amprenavir by only 2-fold (28% ± 3.7% versus 13% ± 7.3% for P-gp deficient and P-gp competent mice, respectively) (P < 0.05) in the P450-null group (Fig. 4). These results demonstrate that P450-mediated metabolism in the intestine reduces amprenavir intestinal absorption and the Fg by nearly 10-fold, whereas P-gp has a modest effect compared with P450 in attenuating the intestinal absorption of amprenavir.

**Interaction Between P-gp Efflux and P450-mediated Metabolism of Amprenavir during the Absorption of Amprenavir into the Systemic Circulation of Intact Cannulated Mice.** The systemic exposure of amprenavir was equivalent in P-gp competent and P-gp deficient mice after a 0.42 mg/kg dose of amprenavir (AUC0–240 min = 1.1 ± 0.68 µg·min/ml and 0.89 ± 0.30 µg·min/ml, respectively) (Fig. 5). For the same dose, inactivation of P450 resulted in a >10-fold increase in the amnivarin systemic concentrations in both strains of mice (AUC0–240 min = 16 ± 11 µg·min/ml and 13 ± 6 µg·min/ml for P-gp competent and P-gp deficient mice, respectively) as was the case for portal concentrations, showing no additional impact due to inactivation of hepatic P450. There was an approximately 2-fold decrease in the AUCs of amprenavir between the portal and systemic circulation (Figs. 3A and 5A), which is indicative of a small contribution of first-pass hepatic clearance in addition to the intestinal clearance in determining the systemic exposure of amprenavir.

**Discussion**

The studies with intestinal tissue from P-gp competent and P-gp deficient mice show that the oxidative metabolism of amprenavir in mouse intestinal tissue during absorptive flux was only modestly reduced (Km, Vmax, CLint) by P-gp (Fig. 2). This is in contrast to loperamide, which was metabolized at a significantly slower rate and exhibited an approximately 5-fold increase in the apparent Km during its absorptive flux across the intestinal tissue from P-gp competent compared with P-gp deficient mice (Dufek et al., 2013). There was no difference in the oxidative metabolism of the Cyp3a probe substrate testosterone in intestinal tissue from P-gp competent and P-gp deficient mice (Dufek et al., 2013), thus showing that Cyp3a activity

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**TABLE 2**

<table>
<thead>
<tr>
<th>Mice</th>
<th>0.42 mg/kg Amprenavir</th>
<th>2.1 mg/kg Amprenavir</th>
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<tbody>
<tr>
<td></td>
<td>AUC0–240 min</td>
<td>Fg</td>
</tr>
<tr>
<td></td>
<td>µg/ml·min</td>
<td>µg/ml</td>
</tr>
<tr>
<td>Portal sampling</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-gp competent, +P450</td>
<td>2.8 ± 0.5</td>
<td>1.8 ± 0.48</td>
</tr>
<tr>
<td>P-gp deficient, +P450</td>
<td>3.7 ± 2.1</td>
<td>2.9 ± 0.87</td>
</tr>
<tr>
<td>P-gp competent, −P450</td>
<td>34 ± 12</td>
<td>13 ± 7.3</td>
</tr>
<tr>
<td>P-gp deficient, −P450</td>
<td>43 ± 16</td>
<td>28 ± 3.7</td>
</tr>
<tr>
<td>Systemic sampling</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-gp competent, +P450</td>
<td>1.1 ± 0.68</td>
<td>ND</td>
</tr>
<tr>
<td>P-gp deficient, +P450</td>
<td>0.89 ± 0.30</td>
<td>ND</td>
</tr>
<tr>
<td>P-gp competent, −P450</td>
<td>16 ± 11</td>
<td>ND</td>
</tr>
<tr>
<td>P-gp deficient, −P450</td>
<td>13 ± 6</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Values are expressed as the mean ± S.D. of five mice.*
was not altered in the P-gp deficient mice. AP efflux of amprenavir is predominantly mediated by P-gp (Polli et al., 1999; van der Sandt et al., 2001) and amprenavir is not known to be a substrate for other transporters; thus, any compensatory change in the expression of other transporters or metabolizing enzymes in P-gp deficient mice is not expected to influence the intestinal metabolism or absorption of amprenavir. The lesser effect of P-gp on the metabolism of amprenavir in mouse intestinal tissue is likely due to the lower efficiency of P-gp in attenuating the absorptive flux of amprenavir (AQ = 0.69) than of loperamide (AQ = 0.90). The effect of P-gp in reducing the absorptive permeability of amprenavir in the mouse is higher than what is described in Caco-2 cell monolayers, where the absorptive flux of amprenavir was reduced by approximately 50% (Polli et al., 1999), but the fact remains that P-gp is less efficient in attenuating the absorptive flux of amprenavir than of loperamide. Furthermore, amprenavir is subject to an approximately 10-fold higher rate of oxidative metabolism compared with loperamide in mice (Dufek et al., 2013), which may also contribute to the smaller effect of P-gp–mediated efflux on the intestinal metabolism of amprenavir.

The interaction of P-gp and Cyp3a during intestinal absorption was investigated by measuring portal absorption over 4 hours, which is expected to encompass the entire transit time of amprenavir through the mouse small intestine, as discussed previously (Hsu, 1982; Suckow et al., 2001; Dufek et al., 2013). The low dose (0.42 mg/kg) of amprenavir was selected to achieve intestinal lumen concentrations of approximately 100 μM, which was a subsaturating concentration with respect to intestinal metabolisms (Fig. 2). The portal concentrations of amprenavir (Fig. 3A) and the F_G (Fig. 4) were similar in P-gp competent and P-gp deficient mice for the 0.42 mg/kg dose. Thus, it appears that any attenuation of amprenavir intestinal absorption due to P-gp–mediated efflux was negated by sparing of intestinal first-pass metabolism by P-gp, thus resulting in no net effect of P-gp on the F_G of amprenavir (Fig. 4). Alternatively, P-gp has no influence on either the intestinal first-pass metabolism or absorption of amprenavir relative to the impact of its high first-pass intestinal metabolism in mouse and the resultant effect on F_G. The high dose of 2.1 mg/kg was chosen to achieve an intestinal concentration of approximately 500 μM, which is approximately 10-fold higher than the apparent K_m for oxidative metabolism of amprenavir (Table 1), and would be expected to saturate intestinal metabolism (Fig. 2). For the high dose, as with the lower dose, the portal concentrations of amprenavir and the F_G were not affected by P-gp (Figs. 3C and 4).

To determine the effect of P-gp–mediated efflux, independent of the effect of P450-mediated intestinal first-pass metabolism, the above study was repeated in mice whose P450 was chemically inactivated by treatment with ABT. The results showed that at the 0.42 mg/kg dose, P450 attenuated the intestinal absorption by approximately 10-fold irrespective of whether P-gp was functional (Fig. 3, A and B). Interestingly, P-gp attenuated the intestinal absorption of amprenavir slightly in the absence of P450 (Fig. 3B), although the decrease was not statistically significant (P > 0.05), and P-gp had no such effect in the presence of P450 (Fig. 3A). Thus, it is clear that P450 is a much greater barrier than P-gp to intestinal absorption of amprenavir. At the higher dose (2.1 mg/kg, approximately 500 μM intestinal concentration), the effect of P450 on the intestinal absorption of amprenavir was much smaller (Fig. 3, C and D), presumably due to saturation of the metabolizing enzymes. Surprisingly, when Cyp3a was inhibited, there was no difference (P > 0.05) in the exposure to amprenavir between the 0.42 and 2.1 mg/kg doses (Fig. 3, B and D). This suggests that amprenavir may have reached solubility limit at the high dose and the absorption may have become dissolution rate-limited. The small effect of P-gp on the intestinal absorption of amprenavir, observed at the 0.42 mg/kg dose in the absence of P450 activity, was not observed at the 2.1 mg/kg dose (Fig. 3D).

Interestingly, P-gp also did not seem to have an effect on systemic concentrations/exposure of amprenavir, regardless of whether P450 was active or not (Fig. 5, A and B). In contrast, P450 reduced systemic concentrations/exposure by approximately 10-fold as was the case for the effect of P450 on portal concentrations/exposure. Comparison of the portal (Fig. 3, A and C) and systemic (Fig. 5) concentrations of amprenavir reveal that the hepatic first-pass clearance contributes to an approximately 2-fold reduction of the amprenavir exposure.

In summary, we conclude that the magnitude of the P-gp effect on intestinal first-pass metabolism is dependent on both the efficiency of efflux by P-gp and the activity of intestinal metabolism. Thus, for a drug with moderate efflux by P-gp and high intestinal first-pass metabolism, the intestinal first-pass metabolism, and not P-gp–mediated efflux, plays a dominant role in attenuating the absorption of the drug as was seen in this study on the effect of P-gp on intestinal first-pass metabolism of amprenavir by Cyp3a and its oral absorption in mice. This behavior is very different from that of loperamide, a drug that is subject to highly efficient P-gp–mediated efflux, whose intestinal first-pass metabolism was significantly spared by P-gp, and consequently both P-gp and intestinal metabolism significantly affected its intestinal absorption in mice (Dufek et al., 2013). Additional dual substrates with varying AQ values but similar intestinal P450 metabolism (and vice versa) need to be studied to provide a better understanding of the impact of varying efficiency for efflux by P-gp on metabolism. In addition to these experimental approaches, modeling can also provide better insights regarding the impact of the
relative roles of the two barriers on their interactions in the intestinal epithelium and overall effect on oral absorption of dual substrates.

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Authorship Contributions

Participated in research design: Dufek, Bridges, Thakker.
Conducted experiments: Dufek.
Performed data analysis: Dufek, Bridges.
Wrote or contributed to the writing of the manuscript: Dufek, Thakker.

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