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# P-GLYCOPROTEIN INCREASES PORTAL BIOAVAILABILITY OF LOPERAMIDE IN MOUSE BY REDUCING FIRST-PASS INTESTINAL METABOLISM

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TITLE RUNNING: Enhancement of the Bioavailability of Loperamide by P-gp

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**Nonstandard abbreviations:** ABT = 1α-aminobenzotriazole, AP = Apical, AQ = Absorptive quotient, AUC = Area under the curve, $AUC_{Portal}$ = Area under the curve of portal concentration, $AUC_{Systemic}$ = Area under the curve of systemic concentration, BCS = Biopharmaceutical Classification System, BL = Basolateral, Caco-2 = Human epithelial colorectal adenocarcinoma, $C_D$ = Dose concentration, CYP = Cytochrome P450, CYP3A = Cytochrome P450 3A, DMSO = Dimethyl sulfoxide, ER = Extraction ratio, EtOH = Ethanol, $F_G$ = Portal bioavailability, IV = Intravenous, J = Mass of drug transported over time, KBR = Krebs Bicarbonate Ringer, $M_{Absorbed}$ = Mass of parent drug absorbed from intestine, $M_{Apical}$ = Mass of total metabolite in apical compartment, $M_{Basolateral}$ = Mass of total metabolite in basolateral compartment, $M_{intacellular}$ = Mass of total metabolite within cellular compartment, $M_{Portal}$ = Mass of parent drug in the portal circulation, MRT = mean residence time, NaCl = Sodium chloride, NaOH = Sodium hydroxide, $P_{app}$ = Apparent permeability, $P_{app,AB}$ = Apparent permeability from apical to basolateral compartment, P-gp = P-glycoprotein, $Parent_{Basolateral}$ = Parent drug in basolateral compartment, PK = Pharmacokinetic, $P_{PD}$ = Passive permeability, $Q_{Portal}$ = Portal blood flow, S = Dose concentration, SD = Standard deviation, t = Time.

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**ABSTRACT**

P-glycoprotein (P-gp) and CYP3A/Cyp3a[1] in the intestine can attenuate absorption of orally administered drugs. While some suggest that P-gp enhances intestinal metabolism by CYP3A/Cyp3a during absorption of a dual substrate, others suggest that P-gp reduces the metabolism in the intestine when substrates are at sub-saturating concentrations. Hence, to elucidate the cellular mechanisms that can address these divergent reports, we studied intestinal absorption of the dual substrate, loperamide, in portal vein-cannulated P-gp competent and P-gp deficient mice. These studies showed that at low doses of loperamide, which produced intestinal concentrations near the apparent $K_m$ for oxidative metabolism, the bioavailability across the intestine ($F_G$) was 6-fold greater in the P-gp competent mice than in P-gp deficient mice. The higher $F_G$ of loperamide in the presence of P-gp was attributed to lower loperamide intestinal metabolism. However, at high doses of loperamide, the sparing of first-pass metabolism by P-gp was balanced against the attenuation of absorption by apical efflux, resulting in no net effect on $F_G$. *In vitro* studies with intestinal tissue from P-gp competent and deficient mice confirmed that P-gp reduced the metabolic rate of loperamide during absorptive flux at concentrations near $K_m$ but had little effect on metabolism at higher (saturating) concentrations. Further, studies in which Cyp3a was chemically inactivated by aminobenzotriazole (ABT) in P-gp competent and deficient mice, showed that P-gp and Cyp3a individually attenuated $F_G$ by 8-fold and 70-fold, respectively. These results confirmed that P-gp effectively protects loperamide at low doses from intestinal first-pass metabolism during intestinal absorption.

## INTRODUCTION

The intestinal epithelium is a highly complex barrier with several physical and biochemical features, including CYP3A[1] (oxidative metabolism) and P-glycoprotein (P-gp) (apical (AP) efflux), which limit oral absorption of drugs (Benet, 1996; Paine et al., 1996). These two proteins influence the systemic bioavailability of widely diverse drugs (Castanon-Gonzalez et al., 1995), such as the anti-HIV drug saquinavir (Fitzsimmons and Collins, 1997; Sinko et al., 2004) or the immuno-suppressive agent, cyclosporine (Gomez et al., 1995; Benet, 1996; Gan et al., 1996). The role of CYP3A in drug interactions is well established (Liu et al., 2007; Zhou, 2008)[2]; and recently transporters have also emerged as important players in drug interactions (Giacomini et al., 2010; Proctor, 2010). In the recent FDA guidance on transporter-mediated drug-drug interactions, P-gp is featured prominently (Huang et al., 2007; Zhang et al., 2009; FDA, 2012). P-gp and CYP3A have broad substrate specificity, and the two proteins share many substrates. In the intestinal epithelium, P-gp/CYP3A dual substrates first interact with P-gp before reaching the intracellular membranes of the endoplasmic reticulum where they are metabolized by CYP3A. It is known that P-gp and CYP3A interact in affecting the cellular disposition of their substrates. However, opinions differ on whether P-gp enhances or attenuates CYP3A-mediated metabolism of shared substrates during intestinal absorption. Several *in vitro*

[1]The nomenclature CYP3A is used when reference to cytochrome P450 3A is in general terms (includes reference to the human enzyme), whereas Cyp3a is used when rodent enzyme is specifically mentioned.

[2]also see http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM292362.pdf

studies, conducted to elucidate the effect of the interplay between CYP3A and P-gp on the intestinal absorption of dual substrates using Caco-2 cell monolayers (Gan et al., 1996), (Cummins et al., 2004), suggested that P-gp increased CYP3A4-mediated metabolism of the dual substrates during their absorptive flux across the cell monolayers. Studies with the cysteine protease inhibitor K77 in an *in situ* single-pass rat intestinal perfusion model demonstrated that inhibition of intestinal P-gp efflux with elacridar (GF120918) caused reduction of the extraction ratio of K77, but had no effect on the Cyp3a probe substrate, midazolam (Cummins et al., 2003). It was proposed that P-gp-mediated AP efflux increased intestinal metabolism of the dual substrate during absorption by prolonging the exposure of the drug to Cyp3a through repeated "cycles" of absorption and efflux. Accordingly, P-gp would work synergistically with CYP3A to attenuate the bioavailability of shared substrates for both proteins (Gan et al., 1996; Cummins et al., 2002; Cummins et al., 2003; Benet et al., 2004). However, Tam et al. (2003) and (Knight et al., 2006), based on theoretical considerations, suggested that P-gp would decrease the rate of CYP3A-mediated metabolism under non-saturating conditions by reducing the intracellular concentration of the substrate. Thus, the conclusion that P-gp enhances the metabolism of dual P-gp/CYP3A substrates during absorption based on experimental results from Caco-2 cell model (Gan et al., 1996; Hochman et al., 2000; Cummins et al., 2001; Johnson et al., 2001; Cummins et al., 2002; Li et al., 2002; Cummins et al., 2003; Benet et al., 2004; Cummins et al., 2004) or single pass rat intestinal perfusion model (Cummins et al., 2003) is contradicted by an opposite conclusion reached based on pharmacokinetic (PK) modeling studies and theoretical considerations (Tam et al., 2003; Badhan et al., 2009).

This study addresses the contradictory conclusions about the effect of P-gp on CYP3A-mediated metabolism of dual substrates by assessing this interaction in an *in vivo* mouse model

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coupled with a matched mouse intestinal tissue model. The effect of P-gp on Cyp3a-mediated metabolism in the intestine is examined by comparing the fraction of loperamide (Cyp3a/P-gp probe substrate) dose that appears intact in the portal circulation ($F_G$) of P-gp competent and P-gp deficient mice, and the results are further elucidated by measuring permeability and metabolism kinetics in intestinal tissue from these mice. If the hypothesis that P-gp contributes to an increase in Cyp3A-mediated metabolism of dual substrates is valid, then portal bioavailability of loperamide ($F_G$) should be lower in P-gp competent mice than in P-gp deficient mice. Loperamide was chosen as a probe compound because P-gp attenuates its absorption effectively (this study) and it is subject to extensive first-pass metabolism (Wolf 2011), including by intestinal CYP3A4. We report an unexpected result in that at certain doses of loperamide (low with respect to Cyp3a saturation), the $F_G$ is higher in P-gp competent mice than in P-gp deficient mice, suggesting that P-gp contributes to a decrease in intestinal first-pass metabolism, and that this effect is large enough to compensate for the increased absorption in the P-gp deficient mice due to absence of P-gp. Further, we show that the effect of P-gp on loperamide intestinal metabolism is dose-dependent.

## MATERIALS AND METHODS

### Materials

Loperamide, terfenadine, testosterone, 6β-hydroxytestosterone, troleandomycin, sodium hydroxide, sodium bicarbonate, and Krebs Bicarbonate Ringer (KBR) were purchased from Sigma Aldrich (St. Louis, MO, USA). The metabolites of loperamide (monodemethyl- and didemethyl-loperamide) were generously provided by Janssen Pharmaceutica (Beerse, Belgium). 1-Aminobenzotriazole (ABT) was purchased from Santa Cruz Biochemicals (Santa Cruz, CA, USA). [$^{14}$C]-mannitol (53 μCi/μmol) and [$^{3}$H]-digoxin (23.5 Ci/mmol) were purchased from

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Moravek Biochemicals (La Brea, CA, USA) and were determined to be ≥ 96% pure by the manufacturer. Acetonitrile, 95% ethanol (EtOH), methanol, ethyl acetate, and dimethyl sulfoxide (DMSO) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Ketamine HCl and 0.9% NaCl saline were purchased from Veterinary Medical Supply (Zebulon, NC, USA), and xylazine HCl was purchased from Webster Veterinary (Sterling, MA, USA). 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-/- (P-gp deficient) mice were purchased from Charles River Laboratories (Wilmington, MA, USA). The animals were housed according to requirements and approved protocols of the Association for Assessment and Accreditation of Laboratory Animal Care and the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee. The animal housing facility was under the supervision, care, and husbandry of the University of North Carolina at Chapel Hill's Division of Laboratory Animal Medicine, in compliance with The Animal Welfare Act public laws 89-544 and 91-579. All animals were maintained under a normal 12-hour day/night schedule with lights on at 8:00 am.

**Diffusion Chamber Studies**

P-gp competent and P-gp deficient mice were fasted overnight and then anesthetized with an intraperitoneal (IP) injection of ketamine (140 mg/kg) and xylazine (15 mg/kg). A 10 cm segment of jejunum was gently dissected and immediately flushed with 10 mL of ice-cold KBR with 50% complete EDTA-free protease inhibitor (Roche, Basel, Switzerland). The harvested intestinal segment was separated into four 2 cm segments, mounted between two halves of a diffusion chamber insert and held in place by eight small pins as quickly as possible. The inserts

were then mounted between two side-by-side diffusion chambers (Physiologic Instruments, San Diego, CA, USA). KBR (3 mL) at 37°C was added to each chamber and bubbled with oxygen/carbon dioxide (95:5) gas to maintain tissue viability (Johnson et al., 2002). The intestinal tissues were preincubated (AP and BL) for 30 min at 37°C in 3 mL KBR buffer solution or for Cyp3a inhibition studies, with KBR buffer containing 100 μM of the Cyp3a inhibitor troleandomycin or 1mM of the general Cyp inhibitor ABT.

After the equilibration period, transepithelial electrical resistance (TEER) was measured to test the epithelial integrity of the intestinal tissue. Intestinal tissues with TEER ≥ 250 $\Omega$*$cm^2$ were used for experiments with no difference observed between P-gp competent and P-gp deficient intestinal tissue. The KBR buffer from the BL chamber was removed and replaced with fresh buffer (3 mL), while the buffer in the AP chamber was replaced with 3 mL of an appropriate concentration of loperamide (0.75, 1, 7.5, 10, 25, 75, 100, or 125 μM) or testosterone (0.1, 0.25, 0.5, and 1.0 mM) in KBR buffer containing 0.1% EtOH. The intestinal tissues were incubated for 90 min at 37°C after which KBR buffer from the AP and BL chambers was collected. The intestinal tissues were washed ten times in ice-cold KBR buffer, homogenized for 30 sec with a sonic dismembrator in 200 μL of 50:50 KBR and 2N NaOH, and vortexed with 0.5 mL ethyl acetate for 1 min. The homogenates were centrifuged at 9,000 g at 4°C for 10 min, and the supernatants collected. The homogenized tissues were further washed with 0.5 mL ethyl acetate, vortexed, centrifuged, and the supernatants collected two additional times. The supernatants were combined, evaporated under nitrogen gas, and reconstituted in KBR. All samples were stored at -20°C until analysis using liquid chromatography-mass spectrometry (LC/MS/MS). [$^{14}$C]-mannitol and [$^{3}$H]-digoxin were measured using liquid scintillation counting (1600 TR Liquid Scintillation Analyzer, Packard Instrument Company, Downers Grove, IL, USA).

## *In Vivo* Studies

### Portal Vein Cannulation of Mouse

P-gp competent and P-gp deficient mice were fasted overnight and anesthetized as previously described. The depth of anesthesia was monitored throughout the surgical procedure and experiment by the toe pinch reflex and if appropriate, a 35 mg/kg dose of ketamine was administered intramuscularly in the hind limb to maintain the anesthetic plane. A 2 cm abdominal midline incision was made and the intestine was gently pushed to the side exposing the portal vein. A saline filled silastic catheter (0.025" OD x 0.012" ID) (Braintree Scientific, Braintree, MA), with a 28 ga. needle tip (Becton, Dickinson Biosciences, Franklin Lakes, NJ USA) attached to the end, was inserted into the portal vein and secured to the surrounding tissue with a micro-serrefines vascular clamp (FST, Foster City, CA). The animal's body temperature was monitored throughout the experiment by placement of a rectal thermometer (Fisher Scientific, Pittsburgh, PA, USA) and maintained at 37°C on surgical board (VWR, Radnor, PA), which was maintained at 37°C with a heating pad (Jarden Corporation, New York, NY, USA). Doses of 0.23, 0.47, and 0.95 mg/kg of loperamide were administered by oral gavage in 300 μL of 0.9% NaCl with 1% EtOH, which produce approximate intestinal concentration of 50, 100, or 200 μM, respectively, to the portal vein cannulated P-gp competent (n=6) or P-gp deficient mice (n=6). For Cyp inhibition studies, ABT was dosed (50 mg/kg oral dose in 100 μL = ~130 mM ABT), as adapted from studies by Balani et al. (2004), three hours prior to initiation of the study. An additional 50 mg/kg ABT was co-administered with a 0.23 mg/kg dose of loperamide in 300 μL of 0.9% NaCl with 1% EtOH at the initiation of the study. Portal blood samples (20 μL) were withdrawn through the portal cannula over four hours (at 10, 30, 60, 120, 180, and 240 min). Each blood sample was replaced with an equal volume of 10 U heparinized 0.9% NaCl

saline. The blood samples were placed in heparinized microcentrifuge tubes (Fisher Scientific, Pittsburgh, PA, USA) and stored on ice until the completion of the experiment. Following centrifugation at 9,000 g at 4°C for 10 min, plasma was collected, and the samples were stored at -80°C.

The oral absorption of loperamide in portal cannulated mice was studied over four hours, which is several times longer than the standard 30-60 min small intestinal transit time that is observed in mice (Hsu, 1982; Suckow et al., 2001). However it must be noted that for these studies it was necessary to maintain cannulated mice under anesthesia using a mixture of ketamine and xylazine. The maximum delay in small intestinal transit from the administration of anesthesia is typically around two hours; specifically, the administration of xylazine results in up to a 2-fold delay in small intestinal transit time (Hsu, 1982). It is therefore expected that the four-hour study period would encompass the entire transit time of loperamide through the mouse small intestine.

**Jugular Cannulation of Mouse**

P-gp competent and P-gp deficient mice were fasted overnight and then anesthetized, as described above, and implanted with an acute indwelling jugular catheter. A 1 cm incision was made in the neck and the jugular vein gently exposed by blunt dissection. A silastic jugular catheter (0.05" OD x 0.01" ID) (Braintree Scientific, Braintree, MA) was inserted into the right jugular vein and advanced to the junction of the vena cava and the right atrium and then sutured to tissue surrounding the vein. The catheter was then tunneled subcutaneously to the back where the cannula was exteriorized between the scapulae. The animal's body temperature was monitored throughout the entirety of the surgical procedure and experiment, as described above. Doses of 0.23, 0.47, and 0.95 mg/kg of loperamide, were administered by oral gavage in 300 μL

of 0.9% NaCl saline with 1% EtOH to jugular vein cannulated P-gp competent (n=6) or P-gp deficient mice (n=6). Cyp inhibition in mice was achieved as described above. Systemic blood samples (20 μL) were withdrawn through the jugular cannula over four hours (at 10, 30, 60, 120, 180, and 240 min) and prepared as described previously and then stored at -80°C.

**Loperamide Blood to Plasma Ratio**

Fresh blood was collected from P-gp competent mice in heparinized centrifuge tubes (Fisher Scientific, Pittsburgh, PA, USA), and 1 μΛ of blood was then spiked with loperaμide to obtain a final concentration of 5 ng/mL. The blood was then gently shaken at 37°C for 30 min, after which an aliquot of 100 μL whole blood was collected in quadruplicates. Plasma samples were obtained by centrifuging 100 μL aliquots of whole blood at 9000 g at 4°C for 10 min. Whole blood and plasma samples (50 μL) were processed by precipitation with 250 μL of ice-cold acetonitrile containing a 5 ng/mL terfenadine internal standard, and analyzed by LC-MS/MS.

**Analytical Procedure**

**Liquid Chromatography-Mass Spectrometry (LC-MS/MS)**

Each portal and systemic plasma sample (10 μL) was prepared for analysis by precipitation with the addition of 50 μL of ice-cold acetonitrile, containing terfenadine (10 ng/mL) as the internal standard. For diffusion chamber studies, 50 μL of each experimental buffer sample was mixed with 50 μL of ice-cold acetonitrile, containing a 10 ng/mL terfenadine internal standard. All samples were then vortexed for 1 min and centrifuged at 9000 g at 4°C for 10 min. Loperamide (4-[4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl]-N,N-dimethyl-2, 2-diphenylbutanamide) and its two metabolites (Figure 1), monodemethyl-loperamide and didemethyl-loperamide, were quantified in samples obtained from the receiver and donor compartments of the diffusion apparatus, the intestinal tissue, with LC-MS/MS. For in vivo

studies, only loperamide was quantified from each portal and jugular plasma sample using LC-MS/MS. Metabolites of loperamide were not quantified from portal or jugular plasma samples because concentrations were below the limit of quantification. The LC/MS/MS system comprised 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-quadropole mass spectrometer (Applied Biosystems, Foster City, CA). The mobile phases consisted of (A) 0.1% formic acid in water and (B) 0.1% formic acid in methanol. The chromatographic separation of analytes was performed with a linear gradient of 5-95% A in B at a flow rate of 0.8 mL/min over four min and a sample injection volume of 5 μL. The analytical column was an Aquasil C18, 50 x 2.1 mm, with a 5 μm particle size (Thermo-Scientific, Waltham, MA, USA). The samples were ionized using TuroboIonSpray ion source and the positive ions were monitored at the following Q1/Q3 transitions (m/z): 477/266 for loperamide, 463/252 for monodemethyl-loperamide, and 449/238 for didemethyl-loperamide, and 472/436 for terfenadine. Calibration standard curves that ranged from 0.05-100 ng/mL were linear ($R^2 >$ 0.99) with the coefficient of variation (CV) <10% and were analyzed at the beginning and end of the run for each experimental group for sample quantification. The data were acquired and processed using Analyst 1.4.1 (Applied Biosystems, Foster City, CA, USA).

**Analysis of Cyp3a-mediated metabolism of Testosterone by High Performance Liquid Chromatography (HPLC)**

To examine the testosterone metabolism during its absorptive flux across mouse intestinal tissue, samples were prepared by mixing 40 μL of each experimental buffer sample with 60 μL of methanol that contained corticosterone (25 ng/mL) internal standard. All samples were vortexed for 1 min, centrifuged at 9000 g at 4°C for 10 min and then transferred to glass

micro-inserts (National Scientific, Rockwood, TN, USA) placed in autosampler vials (National Scientific, Rockwood, TN, USA). Testosterone and its Cyp3a specific metabolite, 6β-hydroxytestosterone, was quantified in triplicate as previously described (Knight, 2007), using a HP1100 HPLC (Agilent Technologies, Santa Clara, CA) with UV detection at 242 nm for testosterone and 260 nm for corticosterone. The mobile phases consisted of (A) water and (B) methanol. The analytical column was a Zorbax Eclipse XDB-C8, 150 x 4.6 mm, with 5 µm particle size (Agilent Technologies, Santa Clara, CA). The samples were injected in triplicate with an injection volume of 50 µL and chromatographic separation was performed with a linear gradient of 40-75% A in B at a flow rate of 1.0 mL/min over 10 min. The standard curves of testosterone ranged from 0.2-200 µg/mL were linear ($R^2 > 0.99$) with a CV of <5% and were analyzed at the beginning and end of each run.

**Data Analyses**

For the metabolic studies, a Michaelis-Menten model (Equation 1) was fit to the data.

$$V = \frac{V_{max} \times [S]}{K_m + [S]} \qquad \textbf{Equation 1}$$

V represents the rate of metabolism (formation of total monodemethyl-loperamide and didemethyl-loperamide in intestinal tissue and the apical and basolateral, compartments) achieved over 90 min, $V_{max}$ is the maximum rate of metabolism, $K_m$ is the loperamide concentration at which half of the maximum metabolic rate is achieved, and S is the loperamide dose concentration. The Michaelis-Menten model was fit to the data using GraphPad Prism® 4.0 (GraphPad Software Inc., La Jolla, CA, USA) with the goodness of fit confirmed by $R^2 > 0.90$ and a replicates test.

The apparent permeability ($P_{app}$) of loperamide across the mouse intestinal tissue was calculated according to Equation 2,

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$$P_{app,AB} = \frac{J}{C_D \times A} \qquad \textbf{Equation 2}$$

where J represents the mass of drug transported, integrated over time (dQ/dt), A is the area of the intestinal tissue, and $C_D$ is the initial concentration of drug in the donor compartment, with studies conducted under sink conditions (Artursson and Karlsson, 1991).

The efficiency of P-gp in attenuating the passive permeability during intestinal absorption was quantitatively expressed as the absorptive quotient (AQ) (Equation 3).

$$AQ = \frac{P_{PD} - P_{app,AB}}{P_{PD}} \qquad \textbf{Equation 3}$$

The AQ was determined experimentally by measuring (i) apparent permeability (AP to BL) in the presence of P-gp mediated efflux ($P_{app,AB}$) and (ii) passive permeability in the absence of P-gp ($P_{PD}$) (P-gp inhibited with GF120918 or as in the current study with intestinal tissue from P-gp deficient mice) and by calculating the decrease in permeability caused by P-gp in relation to the $P_{PD}$ (Troutman and Thakker, 2003a; Troutman and Thakker, 2003b). The loperamide permeability measurements were conducted with the intestinal tissue that was treated with the Cyp3a inhibitor troleandomycin (100 μM) to prevent loperamide from being metabolized during the measurement. Thus, for a compound with an AQ of 0.9, P-gp efflux attenuates the $P_{PD}$ during absorptive flux by 90%.

The $F_G$ was quantified as previously described by Hoffman et. al (1995) as briefly described below.

$$M_{Absorbed} = M_{Portal} - M_{Systemic} \qquad \textbf{Equation 4}$$

$M_{Portal}$ is the mass of parent drug calculated from measured concentrations in the portal blood over time, $M_{Absorbed}$ is the total mass of the parent drug absorbed across the intestine over time,

and $M_{Systemic}$ is the mass of the parent drug returned to the portal vein from the systemic circulation over time.

The total drug in the portal vein that was absorbed directly from the intestine (Equation 5) or from the systemic circulation (Equation 6) was quantified from the portal blood flow ($Q_{Portal}$) in mouse of 1.45 mL/min (Davies and Morris, 1993), the area under the portal blood concentration-time curve to time (t) ($AUC_{Portal\ (0\text{-}t\ hr.)}$), and the mean area under the systemic blood concentration-time curve to t ($AUC_{Systemic\ (0\text{-}t\ hr.)}$) following the oral administration of the dose. The blood concentration of loperamide in the portal and systemic was calculated by conversion of portal and systemic plasma concentration (Uchimura et al., 2010) using the blood (4.8 ± 1.1 ng/mL) to plasma (6.7 ± 0.71 ng/mL) ratio of loperamide (0.71 ± 0.06).

$$M_{Portal} = Q_{Portal} \times AUC_{Portal\ (0\text{-}4hr)} \qquad \textbf{Equation 5}$$

and

$$M_{Systemic} = Q_{Portal} \times AUC_{Systemic\ (0\text{-}4hr)} \qquad \textbf{Equation 6}$$

Expressing $M_{Absorbed}$ (Equation 4) relative to the mass of the dose allows for a direct quantification of the fraction of dose absorbed into the portal circulation ($F_G$), as expressed in Equation 7.

$$F_G = \frac{M_{Absorbed}}{Dose} \qquad \textbf{Equation 7}$$

**Statistical Analysis**

The overall difference in measured values (e.g. metabolic rate, $K_m$, $P_{app}$, and $F_G$) between the mouse strains or treatments was determined using a two-way analysis of variance (ANOVA) followed by a Bonferroni post-test, unless specifically noted, using GraphPad Prism® statistical software 4.0 for Mac (GraphPad Software Inc., La Jolla, CA, USA). All values are expressed as

the mean ± standard deviation (SD). The criterion for a significant difference in values was considered as $p < 0.05$.

**RESULTS**

**Metabolism of Loperamide during Absorptive Flux across Intestinal Tissue from P-gp Competent or P-gp Deficient Mice**. The rate of loperamide metabolism during absorptive flux, i.e. AP to BL compartment, across intestinal tissue from P-gp competent and P-gp deficient mice is shown as a function of the initial concentration in the donor compartment (Figure 2). The rate of metabolism was greater in the absence of P-gp than in its presence throughout the concentration range, causing a leftward shift of the metabolic rate *versus* concentration profile for intestinal tissue from P-gp deficient mice relative to that from P-gp competent mice (Figure 2). The difference in the metabolic rate due to P-gp was greatest at concentrations near the apparent $K_m$ for Cyp3a-mediated metabolism. At high concentrations, as loperamide metabolism appeared to approach saturation, this difference gradually decreased. Intestinal tissue from P-gp competent and P-gp deficient mice showed no difference in metabolic activity toward testosterone (data not shown), a Cyp3a probe substrate (Kenworthy et al., 1999) that is not subject to AP efflux by P-gp, thus providing evidence that Cyp3a activity was not affected in the P-gp deficient mice. When the Michaelis-Menten model (Equation 1) was fit to the metabolic rate *versus* concentration data, the analysis showed that the apparent $K_m$ for Cyp3a-mediated metabolism of loperamide was approximately 5-fold lower ($p < 0.001$) in P-gp deficient (4.5 ± 2.4 μM) *versus* P-gp competent (22 ± 4.8 μM) intestinal tissue (Table 1), but there was no difference in the $V_{max}$. Intestinal tissue from P-gp competent (37 ±8.0 pmol/min/cm$^2$) and P-gp deficient (36 ± 4.0 pmol/min/cm$^2$) mice showed no difference in metabolic activity toward testosterone (Figure 3A), a Cyp3a probe substrate (Kenworthy et al., 1999) that is not subject to

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AP efflux by P-gp. Treatment of mouse intestinal tissue from P-gp competent or P-gp deficient mice with 100 μM troleandomycin completely abolished oxidative metabolism of testosterone $0.5 \pm 0.5$ pmol/min/cm$^2$).

**Intestinal Absorption of Loperamide into the Portal Circulation of P-gp Comptent and P-gp Deficient Mice.** The oral absorption of loperamide was studied over four hours in portal vein cannulated P-gp deficient and P-gp competent mice. The rationale was that if P-gp influences the absorption of loperamide by a combination of effects on first-pass metabolism and permeability, this should be evaluated in the portal circulation before loperamide is further metabolized in the liver or excreted in bile. The loperamide portal plasma concentration *versus* time profiles following oral doses of 0.23, 0.47, and 0.94 mg/kg are shown in Figure 3. The low dose (0.23 mg/kg) was selected to achieve intraluminal concentration of ~50 μM, which is close to the apparent loperamide $K_m$ for Cyp3a-mediated metabolism during absorptive flux across intestinal epithelium (Figure 2). The goal was to select a concentration that was in the linear metabolism range and yet provide sufficiently high plasma concentrations in the portal circulation to generate pharmacokinetic profile via serial 20 μl portal blood sampling over four hours. For this dose, the exposure of intact loperamide into the portal circulation was much higher in the P-gp competent mice compared to P-gp deficient mice as evidenced by significantly higher ($p < 0.01$) portal $AUC_{0\text{-}240\ min}$ in P-gp competent mice than in P-gp deficient mice (Figure 3 and Table 2). This result is consistent with the observation in Figure 2 that P-gp reduces metabolism of loperamide during its absorptive flux across the intestinal epithelium, and suggests that the attenuation of loperamide absorption by P-gp is offset by reduced first-pass intestinal metabolism in P-gp competent mice, causing overall increased absorption of intact loperamide into the portal circulation. At higher doses (0.47 mg/kg and 0.94 mg/kg), selected to

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achieve intraluminal concentrations that are significantly above loperamide $K_m$ for Cyp3a and near saturating for Cyp3a, the portal vein plasma concentrations of loperamide were comparable in P-gp competent mice and P-gp deficient mice (Figure 3 and Table 2). Accordingly, the $F_G$ of loperamide was 6-fold greater ($p < 0.001$) in P-gp competent mice (2.6 ± 0.70 %) than in P-gp deficient mice (0.40 ± 0.09 %) (Figure 4) at the lowest dose, whereas the $F_G$ in P-gp competent and P-gp deficient mice was comparable at the middle (1.7 ± 1.0 % and 1.1 ± 0.22 %, respectively) and highest (16 ± 11 % and 17 ± 12 %, respectively) dose (Figure 4). Comparison of the $F_G$ values obtained with the middle dose with those at the lowest dose show that the $F_G$ value dropped by nearly 1/3$^{rd}$ for P-gp competent mice but increased by nearly 3-fold P-gp deficient mice. These results suggest the intestinal metabolism was nearly saturated at the middle dose and that P-pg is not as effective at sparing the first-pass metabolism at 0.47 mg/kg dose as it is at 0.23 mg/kg dose. Nearly 10-fold higher loperamide $F_G$ in P-gp competent mice and 15-fold higher $F_G$ in P-gp deficient mice when the dose was increased from 0.47 mg/kg (50 µM) to 0.94 mg/kg (100 µM) would suggest saturation of both intestinal Cyp3a and P-gp at 0.94 mg/kg dose.

**Individual and Combined Barrier Effects of P-gp-mediated Apical Efflux and Intestinal First-pass Metabolism on Loperamide Absorption into Portal Circulation.** The results from the loperamide transport and metabolism studies in the intestinal tissue suggest that higher portal loperamide concentrations and higher $F_G$ in P-gp competent *versus* P-gp deficient mice are likely due to sparing of loperamide from intestinal first-pass metabolism by P-gp during oral absorption of the drug. The overlapping and opposing effect of P-gp to cause efflux and to spare first-pass intestinal metabolism in the oral absorption of loperamide, was investigated by comparing the $F_G$ at a 0.23 mg/kg dose in four groups of mice: P-gp competent mice (Figure 4, 0.23 mg/kg dose),

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P-gp deficient mice (Figure 4, 0.23 mg/kg dose),, and mice in each of these groups that were treated with the mechanism-based Cyp inhibitor, ABT, at a dose that completely abolishes Cyp metabolic activity in mice (Balani et al., 2004). The complete inhibition of Cyp3a activity by ABT (1 mM) in P-gp competent and deficient mouse intestine was confirmed by the observation that metabolic conversion of testosterone was absent (i.e., no testosterone metabolite detected) during absorptive flux across mouse intestinal tissue, while robust metabolism of testosterone occurred in intestinal tissue from untreated animals (P-gp competent = 37 ± 8.4 pmol/min/cm$^2$ and P-gp deficient = 36 ± 4 pmol/min/cm$^2$). Further, mice that were treated with ABT orally had a greater than 40-fold increase in the oral absorption of testosterone (0.4 mg/kg dose) into the portal circulation compared to P-gp competent mice ($AUC_{0\text{-}120\ min}$ = 2.6 ± 0.35 μg*min/mL) treated with saline ($AUC_{0\text{-}120\ min}$ = 0.06 ± 0.03 μg*min/mL). There was no difference in passive permeability of [$^{14}$C]-mannitol (4.6 ± 3.0, 3.9 ± 2.1) and [$^{3}$H]-digoxin (17.9 ± 8.8, 14.7 ± 6.6) across intestinal tissue in the presence of ABT, respectively, showing that ABT does not cause unintended changes in the permeability of the intestinal epithelium.

When the $F_G$ of loperamide was compared in P-gp competent (3.6 ± 3.8 %) and P-gp deficient (28 ± 20 %) mice in which Cyp3a was completely inactivated, it was observed that P-gp attenuated the intestinal absorption of loperamide by 8-fold ($p < 0.001$) (Figure 5). Although variability in $F_G$ was high in P-gp competent group (likely due to multiple treatment (ABT followed by loperamide) of portal vein canulated animals coupled with low $F_G$), the large attenuation of loperamide absorption by P-gp was clearly demonstrated. A comparison of loperamide $F_G$ in P-gp deficient mice with functional Cyp3a or with inactivated Cyp3a revealed that first-pass metabolism attenuated absorption of intact loperamide by approximately 70-fold

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($p < 0.001$) (Figure 5). Interestingly, the attenuation of $F_G$ by Cyp3a was eliminated by functional P-gp in wild-type mice (Figure 5).

**The Effect of Interplay between Intestinal P-gp and Cyp3a on Systemic Availability of Loperamide in Mouse.** In contrast to the increase in $F_G$ caused by P-gp due to its interactions with Cyp3a in the intestine, systemic plasma concentrations were similar in P-gp competent and P-gp deficient mice after an oral loperamide dose of 0.23 mg/kg (Figure 6 and Table 2). The systemic exposure of loperamide following a 0.47 mg/kg oral dose was approximately 10-fold greater ($p < 0.001$) in P-gp deficient mice *versus* P-gp competent mice (Figure 6 and Table 2). These observations suggest that hepatic first-pass metabolism may mask or reverse P-gp's effect on the intestinal first-pass metabolism. These results are consistent with the report by Sadeque et al. (2000) that inhibition of P-gp by quinidine caused an increase in systemic concentration of loperamide over a part of the plasma concentration – time profile (Sadeque et al., 2000).

## DISCUSSION

The present study systematically evaluate the effect of P-gp on (i) first-pass metabolism and (ii) $F_G$ of a P-gp/Cyp3a dual substrate using portal vein cannulated P-gp competent and P-gp deficient mice, and a matched *in vitro* model of mouse intestinal transport and metabolism. The use of both in vivo and in vitro models is necessary because the interactions between the two proteins are expected to be complex due differences in the expression of the two proteins along the length of the intestinal tract (Paine et al., 1997; Tamura et al., 2002; Tamura et al., 2003; Iida et al., 2005) and other physiologic factors. The functional activity of intestinal Cyp3a in P-gp deficient and P-gp competent mice was equivalent as determined by quantification of first-pass metabolism of the Cyp3a probe substrate testosterone. Testosterone is not a substrate for efflux by P-gp (Chan et al., 2004), and therefore it would truly reflect Cyp3a differences if such

differences existed. It was recognized that to elucidate the effect of P-gp on intestinal first-pass metabolism of a compound, systemic sampling would introduce artifacts due to hepatic first-pass metabolism and/or biliary excretion. Therefore, an acute indwelling catheter was inserted into the portal and jugular veins of mice so that the test compound absorbed across the intestinal epithelium could be sampled in the bloodstream before it entered the liver. The kinetic parameters for transport and metabolism were generated from the *in vitro* studies with intestinal tissue of the same strains of mice, which enabled proper design and interpretation of the *in vivo* experiments

Loperamide, a μ-opioid receptor agonist that is used in the treatment of diarrhea, was chosen as a probe compound for this study because it has a low oral bioavailability (Heykants et al., 1974; Miyazaki et al., 1979) that is attributed to efficient P-gp mediated efflux (Schinkel et al., 1996; Acharya et al., 2006) and extensive CYP3A-mediated first-pass metabolism (Kim et al., 2004). In fact, a quantitative measure of efficiency of P-gp (AQ, Equation 3, (Troutman and Thakker, 2003b)) toward loperamide revealed that P-gp attenuated its absorptive flux by ~60% in Caco-2 cells (AQ = 0.60 ± 0.09) (Knight, 2007) and ~ 90% in mouse intestinal tissue (Table 1).

The *in vivo* studies produced a surprising result that the portal plasma concentrations of loperamide (0.23 mg/kg, 50 μM) were several fold higher (Figure 3A), and accordingly, $F_G$ was as much as 6-fold higher in mice with normal P-gp expression in their intestine than in P-gp deficient mice (Figure 4). Since intestinal absorption of loperamide is expected to be attenuated by P-gp, a greater amount of loperamide in the portal blood of mice with normal intestinal P-gp suggests that a lower intestinal first-pass metabolism in these mice (compared to P-gp deficient) compensated for P-gp's effect to reduce absorption. These findings are consistent with the

results obtained in the *in vitro* study presented here, which showed that the metabolism of loperamide was significantly lower during absorptive flux across intestinal tissue from P-gp competent *versus* P-gp deficient mice (Figure 2).

The only other *in vivo* study in which P-gp's effect on intestinal first-pass metabolism was directly measured involved the use of a rat single-pass intestinal perfusion model, in which the extent of the P-gp/Cyp3a dual substrate K77 metabolized was found to be lower when P-gp was inhibited (Cummins et al., 2003). Thus the results in the present study contradict the results reported in Cummins et al. (2003) and the hypothesis proposed by Benet et al. that P-gp efflux would enhance intestinal first-pass metabolism by increasing the exposure of parent drug to metabolizing enzymes due to cycling between enterocytes and the luminal space as it travels down the length of the intestine (Cummins et al., 2002; Cummins et al., 2003; Benet et al., 2004; Cummins et al., 2004). We believe that the contradiction can be explained in part by reexamining the results reported by Cummings et al. (2003). The extent of metabolism of the dual P-gp/CYP3A substrate, K77, was estimated and expressed in that study as extraction ratio, which was calculated as a ratio of total metatbolites found in the perfusate and blood to total metabolites plus the absorbed substrate (measured in the blood) (see Knight et al., 2006 for detailed analysis and interpretation of this approach). As Knight et al. (2006) demonstrated in their analysis, the use of ER, as proposed by Cummins and Benet (Cummins et al., 2002; Cummins et al., 2003; Benet et al., 2004; Cummins et al., 2004), does not accurately estimate the extent of metabolism in the presence or absence of P-gp. This is because P-gp directly influences the amount of parent drug that crosses the intestinal barrier, and by definition affects the ER, irrespective of its effect on metabolism (Knight et al., 2006), thus distorting the estimates of the P-gp effect on metabolism. Further, a part of the differences between the two studies may

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be attributed to the differential effect of P-gp on metabolism of a dual substrate depending on the extent of CYP3A saturation achieved by the substrate.

At higher doses (0.47 mg/kg, ~100 μM; 0.94 mg/kg, ~200 μM), the portal plasma concentrations and $F_G$ of loperamide in P-gp competent and P-gp deficient mice were nearly equal (Figure 3, 4). This suggested that P-gp-mediated sparing of the first-pass intestinal metabolism of loperamide was not as large at higher doses as at the 0.23 mg/kg dose, presumably due to saturation of Cyp3a-mediated metabolism, as would be predicted from the *in vitro* study (Figure 2). Interestingly, the loperamide concentration-time profiles in the portal circulation generated for the 0.23 mg/kg and 0.47 mg/kg doses in P-gp competent mice were nearly identical (Figures 3A and 3B). This suggests that doubling the dose from 0.23 to 0.47 mg/kg in P-gp competent mice doubles the first-pass metabolism rate and as a result, the net intact loperamide that reaches the portal circulation remained unchanged. In contrast, the portal concentrations increased ~4-fold in P-gp deficient mice when the dose is doubled from 0.23 to 0.47 mg/kg (Figure 4), suggesting that intestinal first-pass metabolism was saturated, at least partially, between these two doses. The increase in portal concentration with increase in dose from 0.47 and 0.94 mg/kg was even more disproportionate (~15-fold). This is likely due to saturation of both P-gp and intestinal first-pass metabolism in P-gp competent mice (Figure 3B and 3C).

The chemical inhibition of Cyp-metabolic activity achieved by treating P-gp competent and P-gp deficient mice with the mechanism-based Cyp inhibitor, ABT, provided a valuable *in vivo* model to dissect the individual role of P-gp and Cyp3a in attenuating intestinal absorption of loperamide without interactions with each other. In the absence of Cyp activity, the $F_G$ (0.23 mg/kg oral dose) of loperamide was reduced by > 8-fold in P-gp competent mice compared to P-gp deficient mice (Figure 5, bars on the right side), demonstrating that intestinal absorption of

loperamide is strongly influenced by P-gp. This result is consistent with high loperamide AQ (P-gp's efficiency to reduce absorptive flux) of 0.9 in mouse intestinal tissue. The major role of Cyp-mediated metabolism in attenuating loperamide $F_G$ is evidenced by the result that in P-gp deficient mice, the $F_G$ increased by ~70-fold when Cyp was inhibited (Figure 5). These results show that P-gp and Cyp3a are both individually effective in attenuating absorption of loperamide across intestinal epithelium with Cyp3a being so effective that it nearly completely prevents absorption of intact loperamide. However, P-gp reduces the rather formidable barrier effect of Cyp3a, spares loperamide from efficient first-pass metabolism, and increases $F_G$ (Figure 5). Thus, in P-gp competent mice, Cyp3a has little impact on $F_G$ (Figure 5).

The effect of P-gp and Cyp3a interaction on oral absorption of loperamide into the systemic circulation was studied using jugular vein cannulated mice that were competent or deficient of P-gp expression. For the 0.23 mg/kg dose, the systemic exposure of loperamide was similar in P-gp competent and P-gp deficient mice (Figure 6), with no apparent effect of P-gp on loperamide systemic bioavailability. This result suggests that hepatic metabolism and biliary excretion of loperamide partially reverses the effect of intestinal P-gp to increase $F_G$; this trend continued for the higher dose of 0.47 mg/kg, so that the systemic exposure to loperamide was significantly greater in P-gp deficient mice *versus* P-gp competent mice despite similar $F_G$ (Figure 6).

In conclusion, we have demonstrated for the first time that for a compound with high intestinal first-pass metabolism and high P-gp-mediated attenuation of absorptive flux (e.g. loperamide), P-gp can increase $F_G$ by sparing first-pass metabolism, and that the interactions of P-gp and Cyp3a are dose-dependent. However, it is recognized that the effect of P-gp on first-pass metabolism of other dual substrates with different kinetic behavior with respect to P-gp and

CYP3A (either due to intrinsic differences in the interactions with the two proteins or due to different permeability) may be different. The present study makes a strong case for a more systematic evaluation of interactions between P-gp and CYP3A as well as other transporter-enzyme combinations.

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## AUTHORSHIP CONTRIBUTIONS

Participated in research design: Dufek, M.B, Knight, B.M., and Thakker, D.R.

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Wrote or contributed to the writing of the manuscript: Dufek, M.B, Knight, B.M., and Thakker, D.R.

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**FOOTNOTES**

Title Page:

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[1]The nomenclature CYP3A is used when reference to cytochrome P450 3A is in general terms (includes reference to the human enzyme), whereas Cyp3a is used when rodent enzyme is specifically mentioned.

[2]also see http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM292362.pdf

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**LEDGEND FOR FIGURES**

**Figure 1. The chemical structure of loperamide and its major metabolites (monodesmethyl-loperamide and didesmethyl-loperamide), which are produced by CYP3A-mediated metabolism.**

**Figure 2. The rate of metabolism during absorptive flux of loperamide across P-gp competent (solid circle) or P-gp deficient (open circle) mouse intestinal tissues that were mounted in side-by-side diffusion chambers.** Solid and dotted lines represent the fit of the Michaelis-Menten model of enzyme kinetics to the data from P-gp competent (solid line) ($V_{max}$ = 31 ± 2.1 pmol/min/cm$^2$, $K_m$ = 22 ± 4.8 μM) and P-gp deficient (dotted line) ($V_{max}$ = 30 ± 3.2 pmol/min/cm$^2$, $K_m$ = 4.5 ± 2.4 μM) mouse intestinal tissue. Values are expressed as the mean ± SD of n=3 mice with four intestinal tissues per mouse. Statistical significance of difference groups was determined by two-way ANOVA with Bonferroni multiple comparison test. * = $p < 0.05$ or ** = $p < 0.01$ for the comparison between intestinal tissue from P-gp competent or P-gp deficient mice.

**Figure 3. The plasma concentration of loperamide in the portal circulation of P-gp competent (solid circle) or P-gp deficient (open circle) mice treated with 0.23, (A), 0.47 (B) or 0.95 (C) mg/kg oral dose.** The portal $AUC_{0\text{-}240\ min}$ values obtained at these doses are 266 ± 77, 269 ± 140, and 4900 ± 3000 ng*min/mL, respectively, for P-gp competent mice and 90 ± 14, 420 ± 54, and 5700 ± 2900 ng*min/mL, respectively, for P-gp deficient mice. Values are

expressed as the mean ± SD of n=6 mice. Statistical significance was determined using a two-tailed t-test analysis of the $AUC_{0\text{-}240\ min}$ of the portal loperamide plasma concentration profiles.

**Figure 4. The $F_G$ of loperamide in P-gp competent or deficient mice.** The $F_G$ of loperamide was quantitatively derived as outlined in the Methods Section (equations 4-7) after conversion of portal and systemic loperamide plasma concentration to whole blood concentrations using the mean ratio, 71% (range: 66% – 81%), of the whole blood to plasma concentration of loperamide measured at 10 nM in mouse. The $F_G$ of loperamide in P-gp competent (open bars) or P-gp deficient (closed bars) mice at doses of loperamide that produce intestinal concentrations similar to and higher relative to the apparent $K_m$ (22 μM) for Cyp3a-mediated metabolism. The $F_G$ for P-gp competent and P-gp deficient mice, respectively, was 2.6 ± 0.70 and 0.40 ± 0.09 % for the 0.23 mg/kg dose; 1.7 ± 1.0 and 1.1 ± 0.22 % for the 0.47 mg/kg dose; and 16 ± 11 and 17 ± 12 % for the 0.94 mg/kg dose. Values are expressed as the mean + SD of n=6 mice.

$F_G$ = portal bioavailablity. *** = $p < 0.001$ for the 0.23 mg/kg loperamide dose for the comparison of P-gp competent and P-gp deficient mice, or ## = $p < 0.01$ represents the comparison of the 0.23 mg/kg to the 0.47 mg/kg dose of P-gp deficient mice determined by a two-way ANOVA with Bonferroni post hoc test.

**Figure 5. The effect of P-gp and Cyp3a on $F_G$ of loperamide.** The $F_G$ of loperamide (0.23 mg/kg) in P-gp competent (open bars) or P-gp deficient (closed bars) mice in the presence (+Cyp, same data as Figure 4, 0.23 mg/kg dose) or absence (-Cyp) of Cyp-mediated metabolism. (-Cyp) mice were created by treatment with the pan-Cyp inactivator ABT (50 mg/kg, 2x). Values are expressed as the mean ± SD of n=6 mice.

$F_G$ = portal bioavailablity. * = $p < 0.05$ for –Cyp group or *** = $p < 0.001$ for +Cyp group represents the comparison of P-gp competent and P-gp deficient mice, ### = $p < 0.001$ represents the comparison +Cyp and –Cyp of P-gp deficient mice.

**Figure 6. The systemic exposure of loperamide upon oral administration to P-gp competent or P-gp deficient mice.** The systemic plasma concentrations [(A) 0.23mg/kg and (B) 0.47 mg/kg] were measured over 240 min in P-gp competent (solid circle) or P-gp deficient (open circle) mice that had jugular vein cannula implanted. Values are expressed as the mean + SD of n=6 mice.

Statistical significance was determined using a two-tailed t-test analysis of the $AUC_{0\text{-}240\ min}$ of the systemic loperamide plasma concentration profiles.

**TABLE**

**Table 1. The kinetic parameters of loperamide metabolism and permeability in intestinal tissue from P-gp competent or P-gp deficient mice.**

| | $V_{max}$ | $K_m$ | $P_{app\ (-Cyp)}$ (20 μM) |
|---|---|---|---|
| **P-gp Competent** | 31 ± 1 | 22 ± 4.8 *** | 0.61 ± 0.047 *** |
| **P-gp Deficient** | 30 ± 3.2 | 4.5 ± 2.4 | 5.5 ± 1.1 |

The kinetic parameters of loperamide metabolism, $V_{max}$ and $K_{m,}$ were derived by measuring loperamide metabolites during its absorptive flux across intestinal tissue from P-gp competent or P-gp deficient mice (as described in the Methods Section). The permeability values are reported as $P_{app\ (-Cyp)}$ and were determined in the presence of troleandomycin (100 μM) to inhibit oxidative metabolism of loperamide by Cyp3a. In the absence of troleandomycin, In the absence of troleandomycin, approximately 7% and 14% of loperamide (20 μM) was metabolized over the 90 min period by the intestinal tissue from P-gp competent and P-gp deficient mice, respectively during flux measurement (Figure 2). Values are expressed as the mean ±SD of n=3 mice with four intestinal tissues from each mouse. AQ for loperamide in mouse intestinal tissue is thus [0.61/(0.61+5.5)] = 0.1.

$V_{max}$ = pmol metabolite/min/$cm^2$, $K_m$ = μM, and $P_{app}$ = nm/sec. *** = $p < 0.001$ significance between P-gp competent and P-gp deficient mice, as determined using a two-tailed Wilcoxon t-test.

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**Table 2. Portal and Systemic Exposure of Loperamide in P-gp competent or P-gp deficient mice.**

| | AUC (ng*min/mL) | | |
|---|---|---|---|
| **Dose** | **0.23 mg/kg** | **0.47 mg/kg** | **0.96 mg/kg** |
| **Portal Exposure** | | | |
| P-gp Competent | 270 ± 77 | 270 ± 140 | 4900 ± 3000 |
| P-gp Deficient | 90 ± 14 | 420 ± 54 | 5700 ± 2900 |
| **Systemic Exposure** | | | |
| P-gp Competent | 56 ± 16 | 31 ± 11 | - |
| P-gp Deficient | 62 ± 23 | 260 ± 120 | - |

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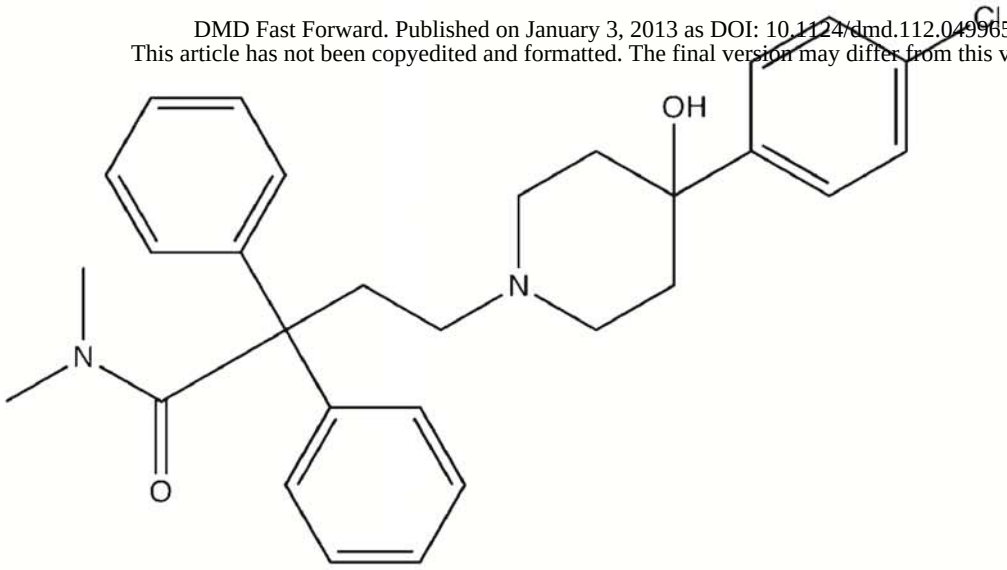
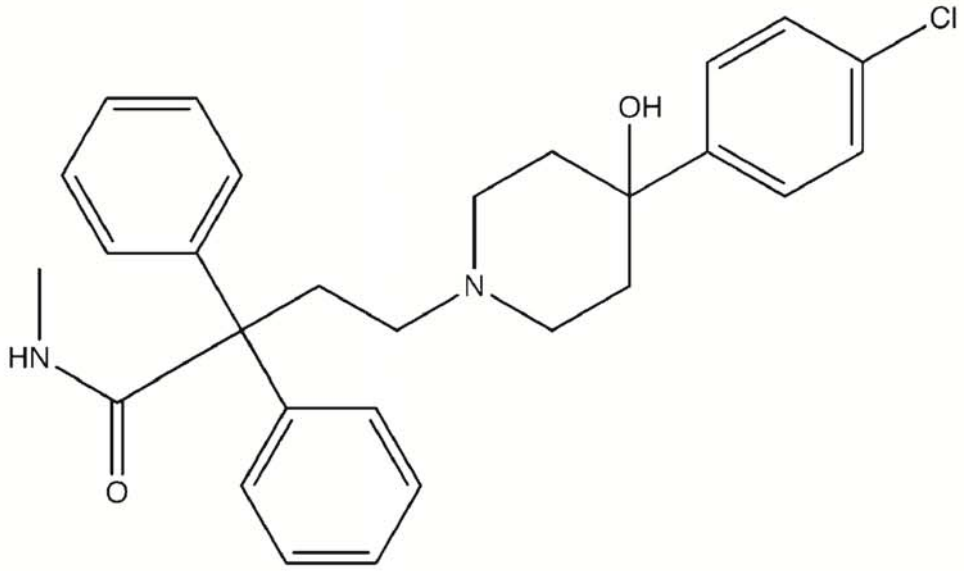
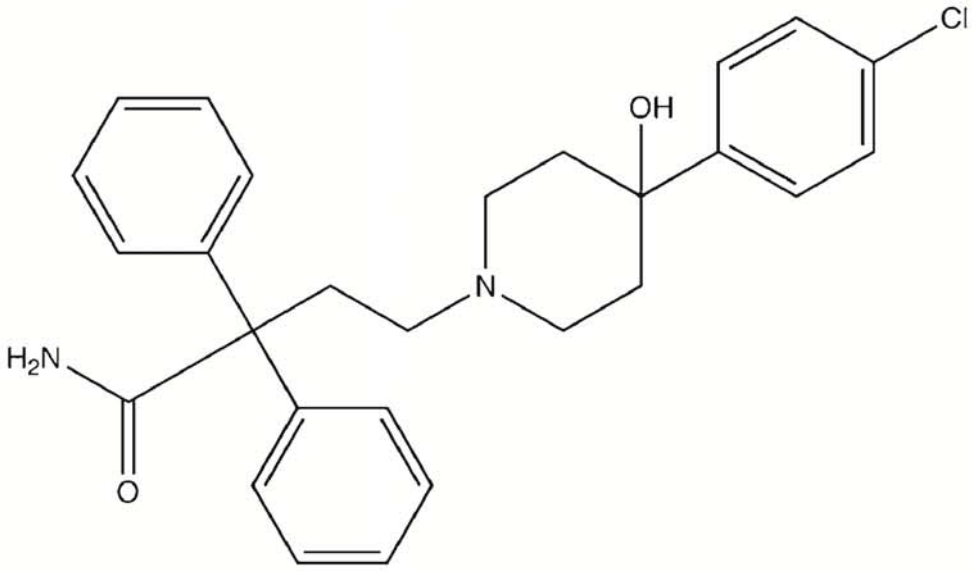



Loperamide




Monodemethyl-loperamide




Didemethyl-loperamide

Figure 1

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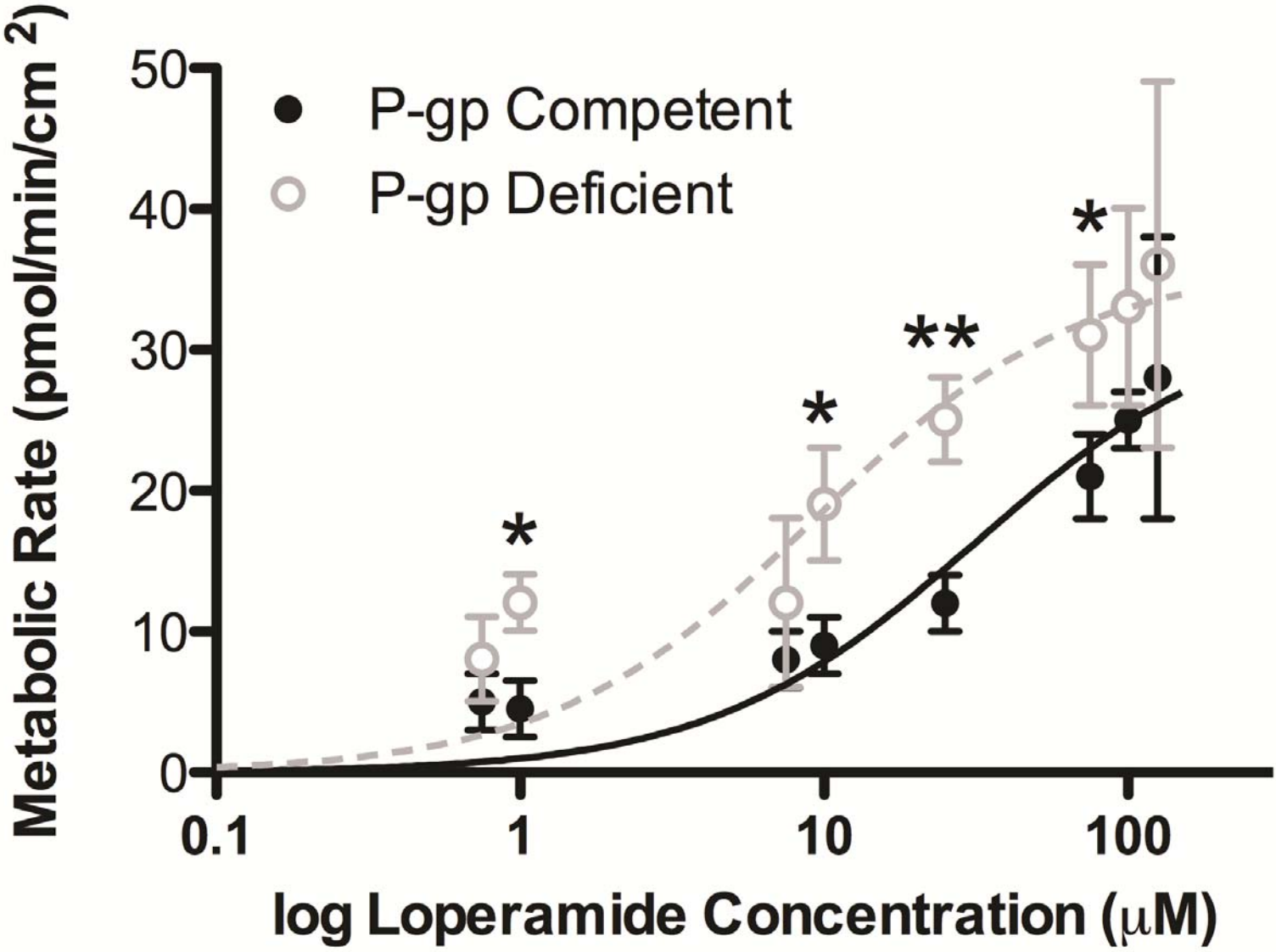


Figure 2

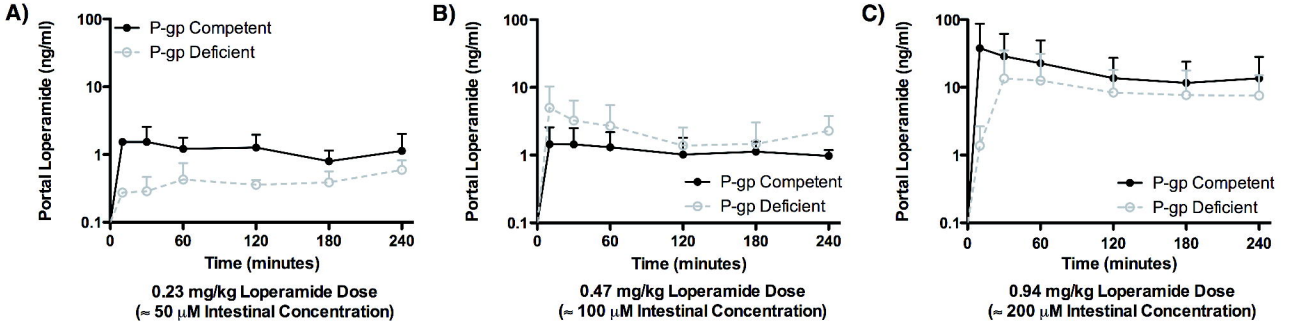
A)
Portal Loperamide (ng/ml)
100
10
1
0.1
P-gp Competent
P-gp Deficient
0
60
120
180
240
Time (minutes)
0.23 mg/kg Loperamide Dose
(≈ 50 μM Intestinal Concentration)
B)
Portal Loperamide (ng/ml)
100
10
1
0.1
P-gp Competent
P-gp Deficient
0
60
120
180
240
Time (minutes)
0.47 mg/kg Loperamide Dose
(≈ 100 μM Intestinal Concentration)
C)
Portal Loperamide (ng/ml)
100
10
1
0.1
P-gp Competent
P-gp Deficient
0
60
120
180
240
Time (minutes)
0.94 mg/kg Loperamide Dose
(≈ 200 μM Intestinal Concentration)

Figure 3

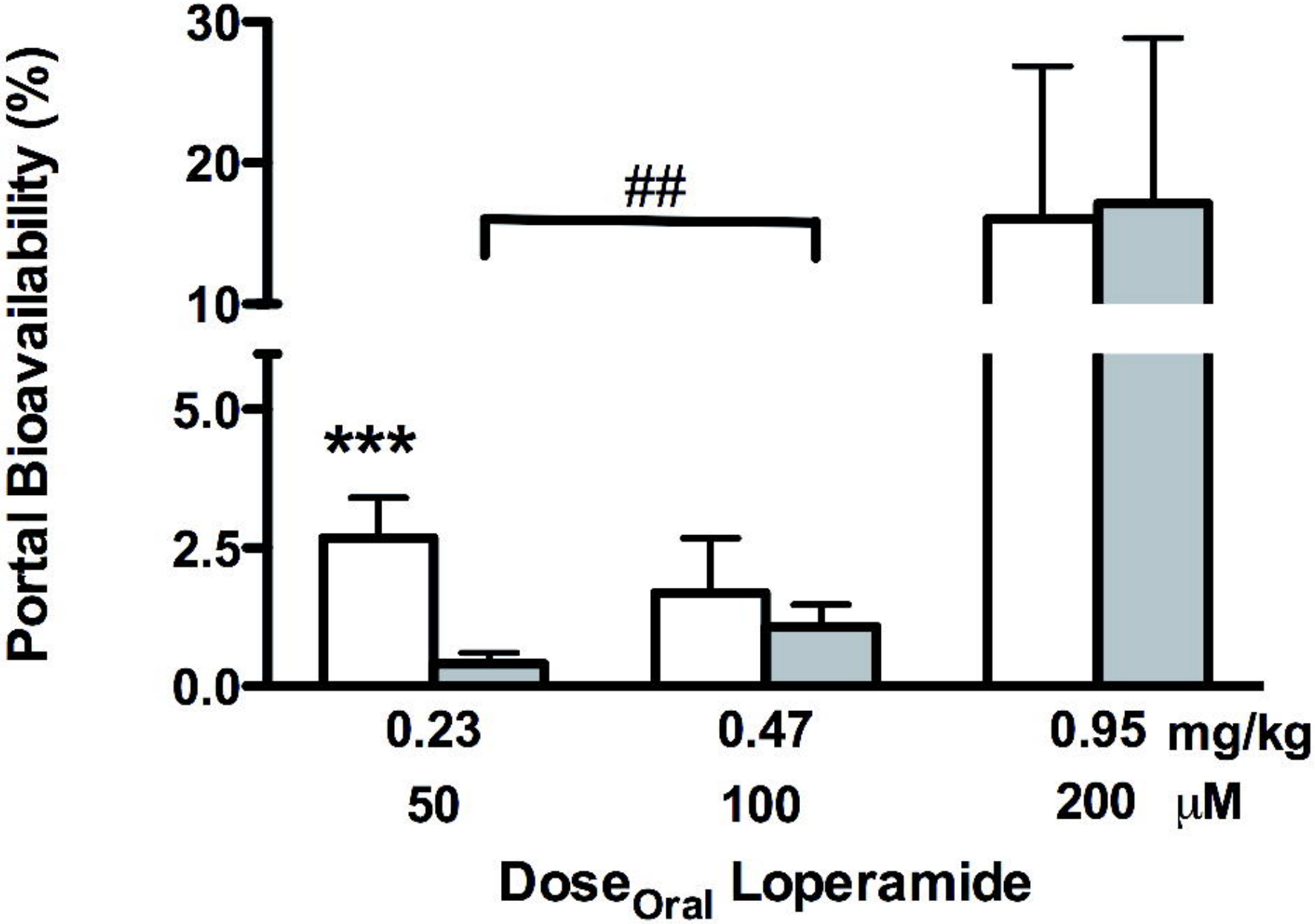
Portal Bioavailability (%)
30
20
10
5.0
2.5
0.0
##
***
0.23
0.47
0.95 mg/kg
50
100
200 μM
Dose Oral Loperamide

Figure 4

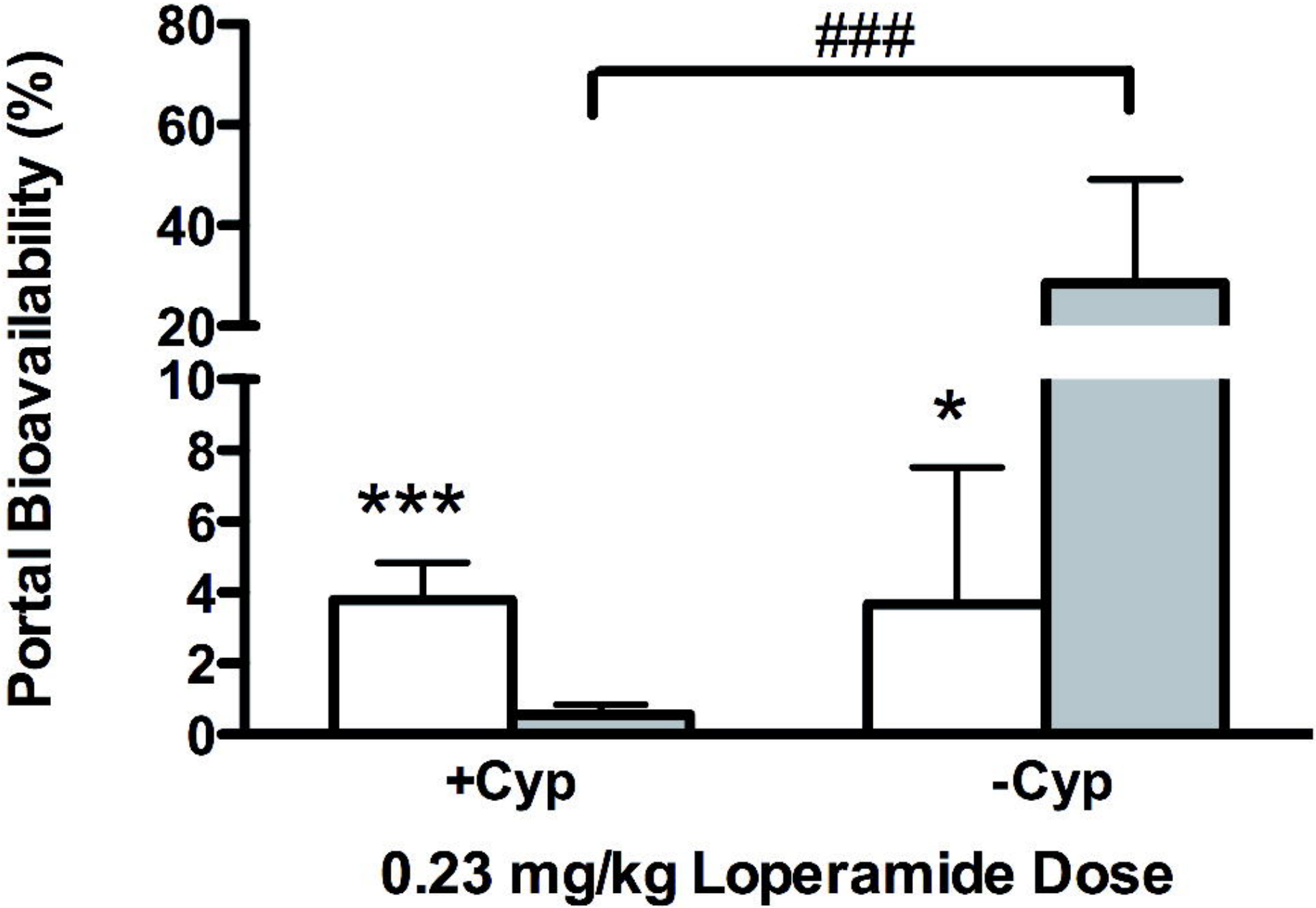
Portal Bioavailability (%)
80
60
40
20
10
8
6
4
2
0
###
***
*
+Cyp
-Cyp
0.23 mg/kg Loperamide Dose

Figure 5

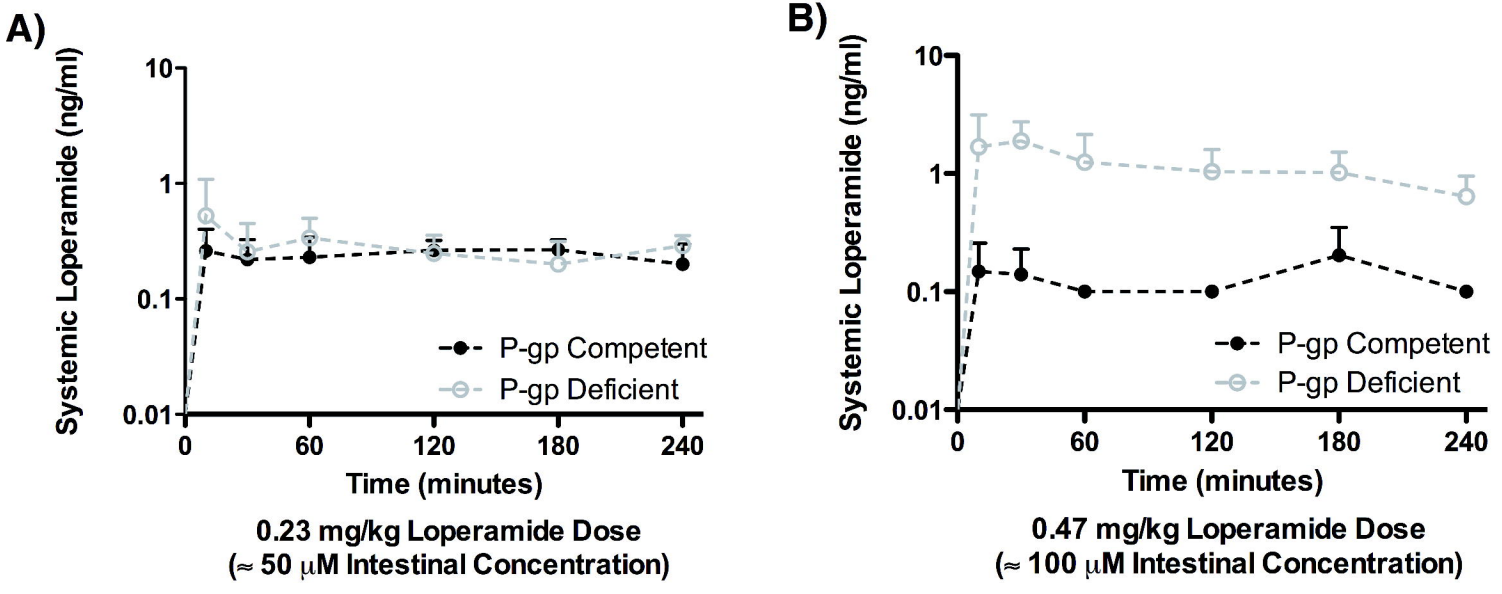


Figure 6