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Impact of Transporters in Oral Absorption: A Case Study of *in Vitro* and *in Vivo* Organic Anion Absorption

Luise K. Gram,[†,‡] Gerda Marie Rist,[‡] and Bente Steffansen*[,†]

Molecular Biopharmaceutics, Department of Pharmaceutics and Analytical Chemistry, Faculty of Pharmaceutical Sciences, University of Copenhagen, Denmark, and Department of DMPK and Safety, LEO Pharma, Ballerup, Denmark

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Abstract: A key determinant for oral bioavailability of a drug candidate is the intestinal epithelial permeation of the drug candidate. This intestinal permeation may be affected by interactions on membrane transporters expressed in the intestinal epithelial cells. The purpose of the present study was to investigate whether transporters were involved in the intestinal absorption of an organic anion A275 and to compare the impact of interactions related to transporters in the Caco-2 cell model versus the *in vivo* rat model of intestinal absorption. In both models, it was investigated whether intestinal permeation of A275 was concentration dependent and affected by inhibitors or competitive organic anions. Interactions related to transporters in intestinal permeation was clearly demonstrated in the Caco-2 cell model but was not directly evident for *in vivo* rat absorption. However, an observed biphasic in vivo absorption and a large intervariability between rats might mask a dose-dependent absorption of A275. To avoid these suggested interactions, a dose of at least 10 mg/kg, which saturates the intestinal transporters involved in A275 absorption, should be administered, but at doses below that the risk of such drug interactions should be taken into account.

Keywords: Organic anions; intestinal absorption; transporter-related drug interactions; *in vivo*−*in vitro* correlation

Introduction

Intestinal epithelial permeation of drug candidates may be affected by membrane transporters expressed in the intestinal epithelial cells such as Caco-2. Consequently, intestinal epithelial permeability may be dose dependent and be influenced by interactions related to the involved transporters.[1] Interactions related to intestinal transporters have, for example, been observed to influence nateglinide absorption.[7]

Preclinical investigations of a lead series of aminobenzophenones, which are p38 MAP kinase inhibitors, showed for the organic anion A275 (compound no. 275[11]) vectorial permeability across Caco-2 cells. Consequently there is a risk for this drug candidate to interact on intestinal transporters. A275 is present as a benzoate anion (pK_a 3.9) at intestinal fluid pH as well as at physiological pH but may, according to Lipinski parameters (i.e., MW 402, log *P* 2.3 at pH 7.4, hydrogen bond donors and acceptors, respectively, <5 and 10), be estimated to exhibit good bioavailability. Estrone-3-sulfate is a common substrate for organic anion transporters[3−6] and its permeability in Caco-2 cells is suggested to be influenced by OATP2B1 (organic anion transporting polypeptide 2B1), BCRP (breast cancer resistance protein), and OSTα/β (organic solute transporter α/β).[2] Glipizide

* To whom correspondence should be addressed. Mailing address: Faculty of Pharmaceutical Sciences (PHARMA), University of Copenhagen, Universitetsparken 2, Dk-2100 Copenhagen, Denmark. Tel: +45 3533 6221. Fax: +45 3533 6030. E-mail: bds@farma.ku.dk.

† University of Copenhagen.

‡ LEO Pharma.

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competitively inhibits estrone-3-sulfate exsorptive permeability across Caco-2 cells, and this inhibition is suggested to be due to interactions on the basolateral OSTα/β transporter, whereas it does not interact with estrone-3-sulfate on the apical transporters BCRP and OATP2B1. Pregnenolone sulfate is a substrate for OATP2B1, fumitremorgin C is a specific inhibitor for BCRP, and estradiol-17β glucoronide is a substrate/inhibitor for Pgp, MRP2/4, and BCRP.[2] Consequently estrone-3-sulfate is suggested to be used to screen compounds interacting on anion transporters such as OATP2B1, BCRP, and OSTα/β, whereas glipizide, pregnenolone sulfate, fumitremorgin C, and estradiol-17β glucoronide may be used to further elucidate which of these transporters are involved.[2] Drug interactions related to transporter-mediated permeability across enterocytes observed *in vitro* are however not always confirmed *in vivo*.[8−10]

The purpose of the present study was to investigate whether transporters such as BCRP, OATP2B1, and OSTα/β are involved in the intestinal absorption of the organic anion A275. Furthermore, the purpose was to compare the impact of interactions related to such transporters in an *in vitro* model versus an *in vivo* model of intestinal absorption. The strategy was to study whether A275 permeability into and across Caco-2 cells is dose dependent and influenced by other substrates/inhibitors due to interactions on transporters. Additionally, the impact of intestinal transporters on the *in vivo* oral absorption in rats was studied. Thus, by investigating whether oral absorption of A275 in rats was dose dependent and affected by coadministration of estrrone-3-sulfate and glipizide, the risk of *in vivo* drug interactions related to transporters was addressed.

Experimental Section

Materials. [^{14}C]A275 (56.9 mCi/mmol, 99% radiochemical purity) and unlabeled A275 were manufactured at LEO Pharma (Ballerup, DK). [^{3}H]Mannitol (17 Ci/mmol) and [^{3}H]estrone-3-sulfate (57.3 Ci/mmol) were obtained from Perkin-Elmer Life Science (Boston, MA, USA). Glipizide and estrone-3-sulfate sodium were purchased from Sigma-Aldrich (Brøndby, Denmark). All other reagents were standard grade or better.

In Vitro Permeability in Caco-2 cells. Caco-2 cells were obtained from the American Type Culture Collection (Manassas, VA) and routinely cultured as described previously.[2] The cells were used in passages 25 through 35 for all experiments, which were performed on days 25−28 after seeding on Transwell polycarbonate membrane inserts (1.13 cm^2 growth areas, pore size 0.4 μm). The cell monolayer integrity was accepted if the transepithelial electrical resistance (TEER) exceeded 200 $\Omega \cdot$cm^2. All *in vitro* experiments were performed at 37 °C with orbital rotation (80 rpm). Hanks balanced salt solution (HBSS), containing 5% bovine serum albumin and buffered with *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.4, was used as medium. A275 concentrations above 200 μM could not be investigated due to limited solubility of A275 in the medium used for *in vitro* experiments (solubility aqua 62 μM at pH 7.4; FaSSIF 75 μM at pH 6.5).

Apparent permeability (P_{APP}) and corresponding end-point accumulative permeability (P_{EPA}) of A275 were studied, in both absorptive (apical to basolateral) and exsorptive (basolateral to apical) directions, and initial linear uptake permeability (P_{UP}) was studied at both the apical and basolateral membranes. HBSS supplemented with [^{14}C]A275, the paracellular marker [^{3}H]mannitol, and possible competitive organic anions were added at the donor compartment, and HBSS was added in the opposite compartment. To avoid pH partition-driven permeation, the HBSS was buffered at pH 7.4 in both compartments.[12,13] The amounts of transepithelially permeated [^{14}C]A275 were followed as a function of time by sampling 10% of the receiver chamber volume and immediately replenished with the same volume of buffer. Experiments were stopped by aspirating donor and receiver media, adding ice-cold HBSS, and subsequently rinsing the cells three times in the cold HBSS.

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Samples and cells were analyzed for [^{14}C]A275 and [^{3}H]mannitol in Ultima Gold (Perkin-Elmer, Boston, MA) by liquid scintillation counting (Packard Tri-carb 2100TR, Meriden, CT). [^{3}H]Mannitol was used to determine paracellular diffusion and hence cell integrity in the apparent permeability studies. In uptake and end-point accumulative permeability studies, [^{3}H]mannitol was used to determine extracellular space. Each data point was obtained from the mean of three wells ($n = 3$), and the experiments were repeated in one to three independent cell passages ($N = 1-3$).

Data Analysis. The amount of intracellularly appearing A275 was linearly related to time for 5 min regardless of which donor compartment was used, and initial linear uptake permeability in Caco-2 cells (P_{UP}) was calculated according to eq 1. The amount of apparent permeability (P_{APP}) of A275 was linearly related to time for 1 h, regardless of A275 concentration or whether competitive organic anions were applied. P_{APP} of A275 was calculated according to eq 2. The paracellular permeability was followed during the experiments by apparent mannitol permeability. The apparent mannitol permeability across the Caco-2 cell monolayers was not affected by the presence of A275, estrone-3-sulfate, or glipizide, and only Caco-2 cell monolayers, exhibiting an apparent mannitol permeability of $(3.68 \pm 0.45) \times 10^{-6}$ cm/s, were accepted in the data analysis. At the end of the apparent permeability experiment (60 min), the intracellular end-point accumulative permeability (P_{EPA}) was calculated according to eq 1, as the intracellular accumulated amount of A275 at 1 h related to initial A275 concentration.

$$P_{UP} = P_{EPA} = \frac{Q}{AC_0} \quad (1)$$

$$P_{APP} = \frac{dM/dt}{AC_0} \quad (2)$$

Q is the intracellularly appearing A275 corrected for A275 present in the extracellular space, C_0 is the initial donor concentration, and A is the area of the Caco-2 cell monolayer. The rate of A275 appearance at the receiver compartment is given by dM/dt.

Statistic Analysis and Fitting. Statistical analysis was performed by one-way ANOVA followed by Dunnett's test for comparing with control values using GraphPad Prism (version 4.00, GraphPad Software, San Diego, CA). The dose dependency of A275 permeation was fitted to a sigmoid dose−response curve according to eq 3. The fits were performed as nonlinear regression using GraphPad Prism, where P_X represents either P_{APP} or P_{UP}. The A275 concentration is represented by C, and $P_{X,C}$ is the permeation of A275 at concentration C. $P_{X,min}$ and $P_{X,max}$ are the minimum and maximum possible permeation of A275. EC_{50} represents the A275 concentration causing 50% of maximum permeation increase. K_M is assumed to equal EC_{50} according to Cheng and Prusoff.[14]

$$P_{X,C} = P_{X,min} + \frac{P_{X,max} - P_{X,min}}{1 + 10^{\log EC_{50} - \log C}} \quad (3)$$

In Vivo Absorption in Rats. Sprague−Dawley male rats (210−275 g) were obtained from Taconic Europe (Ry, Denmark). The rats were handled according to the rules for animal ethics and welfare at LEO Pharma. LEO Pharma holds permission to perform animal experiments according to Danish legislation and OECD guidelines on GLP (2005/561-1064). The rats were fasted overnight but had water ad libitum. [^{14}C]A275/A275 was dissolved at the indicated concentrations in the vehicle consisting of ethanol, propylene glycol, and water (1:4:5). Furthermore, 0.9 mM of glipizide or estrone-3-sulfate was added when indicated, and pH was adjusted to 7.4−7.8. To begin the experiments, the rats were dosed orally by gavage (5 mL/kg) or intravenously by tail vein injection (2.5 mL/kg). Blood samples (0.3 mL) were taken from vena sublingualis frequently during the first 8 h, and additional blood samples were taken at 24−48 h. Plasma and blood cells were separated by centrifuging at 4000 rpm at 10 °C for 10 min through a layer of potassium EDTA gel (BD Vacutainer SST). Duplicate samples of 50 μL of plasma were then transferred to scintillation vials containing Pico-Fluor 40 (Perkin-Elmer, Boston, MA), and [^{14}C]A275 was quantified by liquid scintillation counting (Packard Tri-carb 2900TR, Meriden, CT).

Data Analysis. Intravenous Administration. Plasma concentrations of total A275 at each time point were calculated from specific radioactivity. Since A275 is metabolically stable and excreted unchanged in mice and human DMPK studies, and the hepatic extraction ratio is 0.14 in rat hepatocytes (data not shown), the total radioactive concentration is assumed to correspond to A275 concentration. All A275 plasma concentration profiles of iv-administered rats were individually fitted to a pharmacokinetic two-compartment iv-bolus model using WinNonLin (version 5.2 with IVIVC toolkit, Pharsight Corporation, Mountain View, CA), and macroconstants were calculated from the microconstants.

Oral Administration. At each time point, plasma concentrations of total A275 were calculated from specific radioactivitiy. The macroconstants obtained from iv studies were used for deconvolution by WinNonLin using two exponential terms in unit impulse response. For oral administered rats, the deconvolutions were conducted individually on the A275 plasma concentration profiles, using the macroconstants obtained from the corresponding iv-administered rats. The cumulative absorption versus time profiles were obtained for individual rats by deconvolutions, and at each time point, mean values were calculated (Abs). The absorption rate constant (k_a) was calculated from linear regression as the linear slope of log(amount absorbed) vs time in the time interval 0.25−1.25 h. The absorbed fraction (F_a) of A275 was calculated at 4 and 8 h after dosing as the fraction of amount absorbed 20 h after dosing.

Statistic Analysis. Statistical analysis using GraphPad Prism was performed by one-way ANOVA followed by Dunnett's test when comparing to control values or Bon-

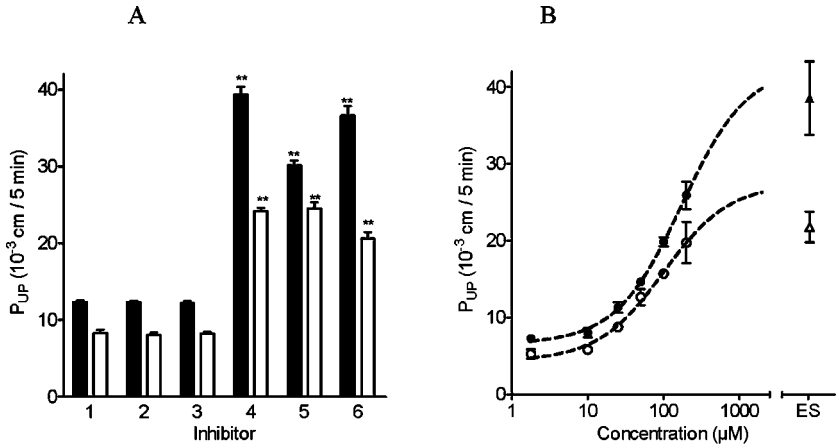

Figure 1. Initial uptake permeability (P_{UP}) of A275 in Caco-2 cells. (A) Apical (filled bars) and basolateral (open bars) P_{UP} (5 min) of 7 μM [^{14}C]A275 in the presence of competitive organic anions, (1) control, (2) fumitremorgin C (0.5 μM), (3) estradiol-17β-glucoronide (100 μM), (4) estrone-3-sulfate (4 mM), (5) glipizide (1 mM), or (6) pregnenolone sulfate (600 μM). Data represent mean $\pm$ SEM ($N = 3$); ** indicates significantly different from control ($p < 0.01$). (B) Apical (filled) and basolateral (open) P_{UP} of 2 μM [^{14}C]A275 in the presence of increasing concentration of nonradiolabeled A275 (2−200 μM, circles) or in the presence of 4 mM estrone-3-sulfate (triangles). Data was fit to eq 1 and represent mean $\pm$ SEM ($N = 3$).

Table 1. Kinetic Parameters of A275 Uptake Permeability (2−200 μM) and Absorptive Apparent Permeability in Caco-2 Cells

	K_M (μM)	$P_{X,max}$
apical uptake permeability	174	$(43 \pm 6) \times 10^{-3}$ cm/(5 min)
basolateral uptake permeability	100	$(28 \pm 5) \times 10^{-3}$ cm/(5 min)
absorptive apparent permeability	163	$(99 \pm 3) \times 10^{-6}$ cm/s

ferroni's multiple comparison test when comparing to dose dependencies.

Results

In Vitro Permeability in Caco-2 Cells. The initial linear uptake permeability at 5 min (P_{UP}) increased significantly, more than 3-fold at both membranes, in the presence of estrone-3-sulfate, glipizide, or pregnenolone sulfate (Figure 1A, $p < 0.01$). At both membranes, P_{UP} increased in a dose-dependent manner in the presence of increasing concentrations of A275 (Figure 1B).

When P_{UP} versus concentration was added to eq 3, the corresponding K_M values were determined to be 174 and 100 μM at the apical and basolateral membranes, respectively (Table 1).

The absorptive apparent permeability (P_{APP}) of A275 was approximately half the exsorptive P_{APP} (Figure 2A, $p < 0.01$). Similarly to P_{UP}, the P_{APP} in both directions was significantly increased in the presence of estrone-3-sulfate or glipizide and at higher A275 concentrations (Figure 2A, $p < 0.01$). The absorptive P_{APP} was increased up to 400%, while the exsorptive P_{APP} was increased to 300%. However, the exsorptive P_{APP} remained higher than the absorptive, although the difference was less pronounced in the presence of estrone-3-sulfate or at higher A275 concentrations. The absorptive P_{APP} increased in the presence of 1 mM glipizide but was not affected at glipizide concentrations below that (Figure 2B). Absorptive P_{APP} was increased in a dose-dependent manner, and K_M was calculated as 163 μM by eq 3 (Figure 2B, Table 1).

The intracellular end-point accumulative permeability (P_{EPA}) of A275 in Caco-2 cells was sensitive to A275 concentration and to presence of competitive organic anions. In both directions, P_{EPA} was significantly increased in the presence of estrone-3-sulfate (Figure 3A, $p < 0.01$). Absorptive P_{EPA} was decreased by glipizide in concentrations of 10−300 μM (Figure 3B), but P_{EPA} was not affected by 1 mM glipizide (Figure 3A,B). In contrast, absorptive P_{EPA} was increased in an A275 concentration-dependent manner (Figure 3B), and exsorptive P_{EPA} was also increased at higher A275 concentrations (Figure 3A, $p < 0.01$).

In Vivo Absorption in Rats. *Intravenous Administration.* The plasma concentration profiles after iv administration (see example in Figure 4) fit a two-comparment distribution. The microconstants for the two-compartment model and volume of distribution for the central compartment were independent of the dose (9 μM to 2 mM) and coadministration of competitive organic anions (oral or iv, $p > 0.05$), and thus the macroconstants were also independent.

Oral Administration. Following oral administration, greater intervariability between rats was observed than for iv-administered rats. Due to a double peak in the plasma concentration profiles (see example in Figure 4), a two-compartment absorption and distribution model could not be applied to the data. The macroconstants from plasma concentration profiles of orally dosed rats were used for individual deconvolution to obtain the cumulative absorption profiles shown in Figure 5. The relative deviation of interindividual absorption ranged from 10% to 39% during

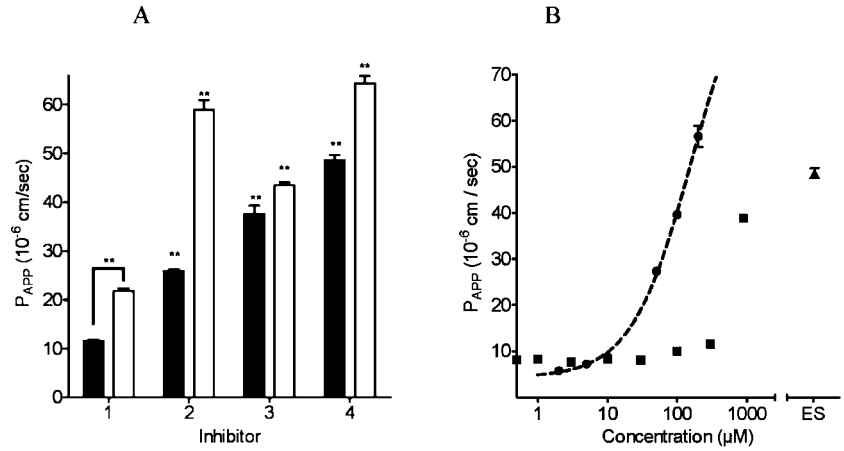

Figure 2. Apparent permeability (P_{APP}) of A275 across Caco-2 cell. (A) Absorptive (filled bars) and exsorptive (open bars) P_{APP} (0−1 h) of 2 μM [^{14}C]A275 in the presence of (1) control, (2) glipizide (1 mM), (3) A275, nonradiolabeled (450 μM), or (4) estrone-3-sulfate (4 mM). Data represent mean ± SE ($N = 1$, $n = 3$); ** indicates significantly different from control ($p < 0.01$). (B) P_{APP} of 2 μM [^{14}C]A275 in the presence of increasing concentrations of nonradiolabeled A275 (2−200 μM, ●), increasing concentrations of glipizide (1−900 μM, ■), or 4 mM estrone-3-sulfate (▲). Data was fit to eq 2 and represents mean ± SE ($N = 1$, $n = 3$).

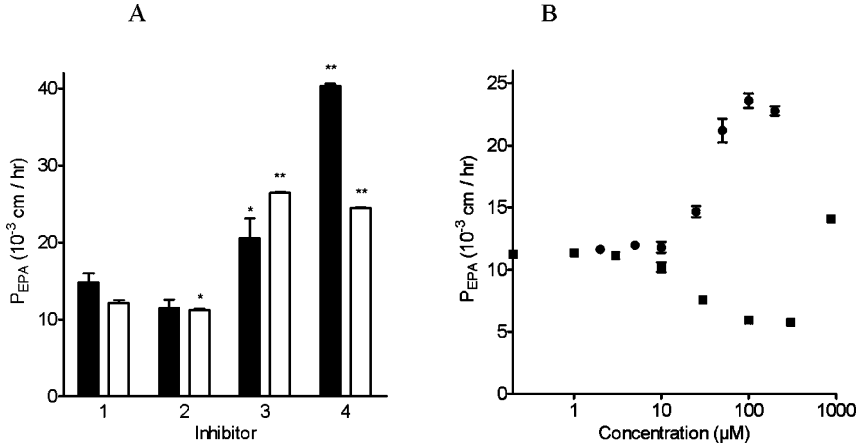

Figure 3. End-point accumulative permeability (P_{EPA}) of A275 in Caco-2 cells. (A) Absorptive (filled bars) and exsorptive (open bars) P_{EPA} of 2 μM [^{14}C]A275 in the presence of (1) control, (2) glipizide (1 mM), (3) A275 (450 μM nonradiolabeled), or (4) estrone-3-sulfate (4 mM). Data represent mean ± SE ($N = 1$, $n = 3$); * indicates significantly different from control at $p < 0.05$; ** indicates significantly different from control at $p < 0.01$. (B) Absorptive P_{EPA} of 2 μM [^{14}C]A275 in the presence of increasing concentrations of nonradiolabeled A275 (2−200 μM, ●) or increasing concentrations of glipizide (1−900 μM, ■). Data represent mean ± SE ($N = 1$, $n = 3$).

the first 4 h. Absorption profiles were characterized by a biphasic absorption pattern in individual rats. The first phase lasted for approximately 4 h followed by a second absorption phase lasting until approx 8 h after dosing (Figure 5).

The initial absorption rate constants (k_a) are given in Table 2. When a high dose of A275 (1 mM, $p < 0.01$) was administered, k_a decreased significantly, whereas k_a was unaffected by lower doses of A275 (Table 2, Figure 5A). However, no consistent dose-dependent effects on fraction absorbed (F_a) were observed in the first (4 h) or second (8 h) phase of absorption (Table 2), and the biphasic absorption pattern was not consistently increased or decreased with increasing doses (Figure 5A). Oral coadministration of glipizide increased k_a significantly ($p < 0.05$), but k_a was not affected by intravenously administered glipizide. The initial absorption rate was not affected by intravenous or oral coadministration of estrone-3-sulfate (Table 2). However, estrone-3-sulfate (oral or iv) clearly decreased F_a of A275 in the first phase (4 h, $p < 0.05$), and estrone-3-sulfate prolonged the absorption of A275 (8 h, $p < 0.01$), (Table 2, Figure 5B). To assess whether this effect of estrone-3-sulfate exerts in the intestinal lumen or the portal blood, plasma concentrations of estrone-3-sulfate after oral and iv administration were analyzed. Plasma concentration of estrone-3-sulfate after iv administration declined rapidly and reached a steady state after 2 h at ~0.2 μg/mL. However, oral absorption of estrone-3-sulfate was slow, and the steady state of ~0.2 μg/mL was not reached until 8 h after oral administration (data not shown).

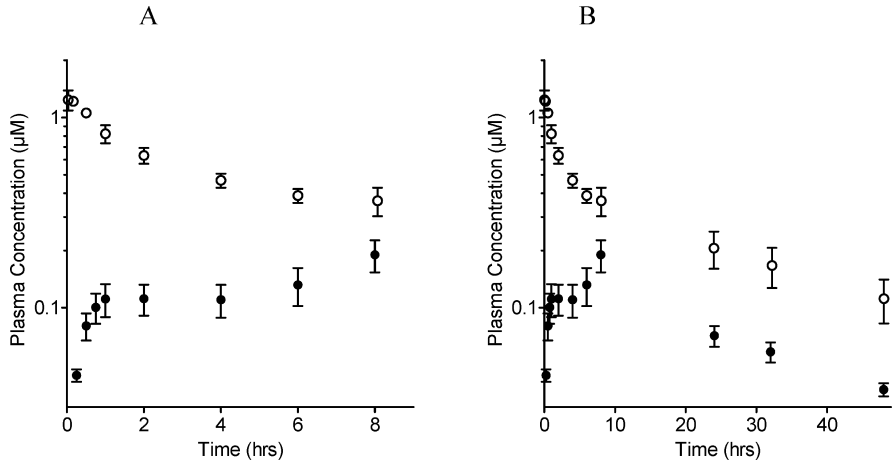

Figure 4. Rat plasma concentration of [^{14}C]A275 following oral administration of 5 mL/kg of 6 μM (●) or iv administration of 2.5 mL/kg of 20 μM (○) of [^{14}C]A275: (A) 0−8 h ($N = 6$); (B) 0−48 h ($N = 6$, $N = 3$ for iv data 8−48 h). All data represent means ± SEM.

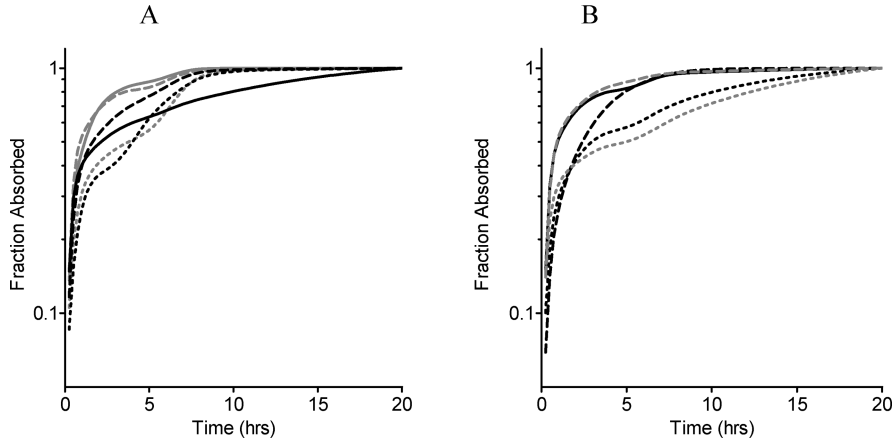

Figure 5. Rat absorption of A275: (A) Absorbed fraction of [^{14}C]A275 in rats following oral administration of 5 mL/kg of 6 μM (•••, gray), 9 μM (−−−, gray), 33 μM (—, gray), 135 μM (•••, black), 0.5 mM (−−−, black) or 1.0 mM (—, black) A275. Data represent means of absorption in three to six rats. (B) Absorbed fraction of 9 μM [^{14}C]A275 after oral administration in rats without coadministration (—) or contemporary administration of 0.9 mM glipizide (−−−) or 0.9 mM estrone-3-sulfate (•••). Oral contemporary administrations were 5 mL/kg (black), and iv contemporary administrations were 2.5 mL/kg iv (gray). Data represent means of absorption in four to six rats.

Discussion

In Vitro Permeability in Caco-2 Cells. The results consistently imply that A275 is a substrate for efflux transporters in both apical and basolateral membranes of Caco-2 cells. This is based on the following observations: (1) The apical initial uptake permeability, P_{UP} (Figure 1), was dose dependent and increased in the presence of the competitive organic anions estrone-3-sulfate, glipizide, and pregnenolone sulfate. However such apical efflux transporter is different from BCRP, Pgp, and MDR2/4 since fumitremorgin C and estradiol-17β glucoronide do not affect the apical P_{UP} of A275. The basolateral P_{UP} is also dose dependent and increased in presence of estrone-3-sulfate, glipizide, and pregnelone sulfate, which may be explained by A275 being a substrate for basolateral efflux transporters, for example, OSTα/β or MRPs.[1,2] (2) The apparent permeability, P_{APP} (Figure 2), in both directions was enhanced by the presence of the competitive organic anions estrone-3-sulfate and glipizide, and absorptive P_{APP} increased in a concentration-dependent manner while the paracellular permeation of mannitol was not affected. (3) Increased end-point accumulative permeability, P_{EPA} (Figure 3), in the presence of estrone-3-sulfate was observed, and absorptive P_{EPA} increased with A275 concentration. The decreased A275 absorptive P_{EPA} in presence of moderate glipizide concentrations (10−300 μM) may be explained if glipizide inhibits reuptake by a basolateral uptake transporter, for example, OSTα/β or OATP3/4A1 (Figure 3B).[1,2]

In Vivo Absorption in Rats. A double peak in the plasma profile during the absorption phase of A275 following oral administration to rats was observed, and deconvolution confirmed A275 absorption to be biphasic. The biphasic absorption may be due to A275 being metabolized in the gut, A275 being exsorbed across the intestinal epithelium by transporters, or the presence of discontinuous A275 absorption sites separated by nonabsorbing sites throughout

Table 2. Characteristic Parameters of A275 Absorption in Rats Following Oral Administration

dose and administration	k_a,[a] h^{-1}	F_a(4 h),[a] fraction	F_a(8 h),[a] fraction
A275 9 μM oral			
ctrl	0.50 ± 0.03	0.80 ± 0.05	0.95 ± 0.02
+ oral glipizide	0.63 ± 0.03[b]	0.71 ± 0.07	0.96 ± 0.02
+ oral estrone-3-sulfate	0.49 ± 0.02	0.55 ± 0.05[b]	0.72 ± 0.06[c]
A275 9 μM oral			
ctrl	0.51 ± 0.03	0.81 ± 0.04	0.97 ± 0.02
+ iv glipizide	0.53 ± 0.03	0.84 ± 0.05	0.96 ± 0.02
+ iv estrone-3-sulfate	0.42 ± 0.05	0.48 ± 0.12[b]	0.63 ± 0.08[c]
A275 oral			
6 μM	0.48 ± 0.02	0.51 ± 0.05	0.92 ± 0.03
12 μM	0.47 ± 0.03	0.81 ± 0.04	0.97 ± 0.02
33 μM	0.47 ± 0.02	0.85 ± 0.07	0.99 ± 0.00
135 μM	0.51 ± 0.02	0.50 ± 0.09	0.91 ± 0.04
0.5 mM	0.47 ± 0.02	0.71 ± 0.12	0.96 ± 0.03
1.0 mM	0.35 ± 0.02[d]	0.60 ± 0.16	0.75 ± 0.15

[a] k_a = absorption rate constant calculated in 0.25−1.25 h. F_a = fraction absorbed of total fraction absorbed after 20 h. Data represent mean ± SE. [b] Significantly different from ctrl at the $p < 0.05$ level. [c] Significantly different from ctrl at the $p < 0.01$. [d] Significantly different from all others in group ($p < 0.05$).

the intestine, as has been suggested for morphine-6-glucuronide absorption.[15−17] Biphasic absorption could also be due to delayed gastric emptying, as suggested for alprazolam and ranitidine, or due to A275 precipitation in GI tract.[18,19] In the present study, the rats were fasted and A275 was dissolved before dosing. Consequently, gastric emptying may not explain the observed biphasic absorption of A275. The biphasic absorption of A275 was observed even at doses corresponding to concentrations below its aquatic solubility, that is, 62 μM. Consequently, A275 precipitation in the GI tract may not explain its biphasic absorption profiles. Finally, A275 is not metabolized in human or mouse plasma and its extraction ratio is low in rat hepatocytes (internal LEO Pharma report). Therefore, metabolism may not explain its biphasic absorption profiles. Thus the observed biphasic absorption of A275 may be due to transporters involved in absorption or enterohepatic recycling of A275 or due to the presence of intestinal absorption windows for A275. Biphasic absorption has also been observed for glibenclamide, which is found to be a substrate of intestinal transporters.[20−23] Thus, the biphasic absorption may indicate A275 being affected by intestinal transporters as is suggested for glibenclamide. However, since the absorption persisted for 8 h, the results indicate enterohepatic recycling to be the cause of biphasic absorption, because residence time in the rat small intestine is only 2−3 h.[24] No consistent dose-dependent effect on absorption rate (k_a) or absorbed fraction (F_a) in the first or second phase of absorption was observed (Table 2, Figure 5A). However, the large intervariability between rats might mask a dose-dependent absorption of A275, as was observed *in vitro*. Glipizide affected the absorption rate of A275 when coadministered (Table 2, Figure 5B). This could be because glipizide, at high intestinal concentrations (1 mM), inhibits exsorptive directed transporter(s), as was the case *in vitro* (Figure 2B).

The effect of estrone-3-sulfate on A275 absorption was a markedly prolonged absorption phase, that is, inhibition of absorption (Table 2, Figure 5B). A higher estrone-3-sulfate plasma concentration after iv administration than that after oral administration was observed during the first 8 h after

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administration. A275 absorption was affected more following iv administration than following oral coadministration of estrone-3-sulfate. Consequently, it is suggested that estrone-3-sulfate affects A275 absorption by a transporter that is located at the serosal side and transports in the absorptive direction, that is, a basolateral efflux transporter.

Relevance of Transporters Involved in A275 Absorption. The *in vitro* estimated K_M values in this study were based on a human model, whereas *in vivo* absorption was evaluated in rats. The functionality and presence of transporters can vary among species, and especially transporters in the OATP family are associated with species differences.[25] Though the apical efflux transporter P-gp has been shown to impact the absorption of its substrates in both mice and Caco-2 cells,[26] the *in vitro* estimated K_M values of human intestinal transporters should be applied to rat intestinal transporters with caution, since the transporters involved in A275 absorption were not identified in this study. Regardless of species, drug interactions on A275 absorption by competitive organic anions occurred in both the human *in vitro* and the rat *in vivo* studies, indicating that transporters were involved in the absorption of A275 in both species. Although the rate of absorption can vary pronounced, the extent of absorption is often shown to be similar between species, including humans. Thus the correlation between percentages of dose absorbed in rat and human are generally shown to be reliable and quantitative.[27] The ratio between percent absorbed in human and rat is found very close to 1, including substances where transporters may be involved in the absorption process.[28] When oral bioavailability does not correlate between species metabolic differences may also play a major role.[29]

Rat gastrointestinal fluid volume is ~20 mL/kg when rats are fasted.[30] As a consequence, the volume is increased 5 times compared with the dose volume (5 mL/kg), and A275 concentration, available for absorption, is diluted approximately 5 times. The rat intestinal fluid concentration, as we have tested it, is thus ~10 μM when interactions with competitive organic anions are studied, and the intestinal fluid A275 concentrations have been tested in the interval ~2−200 μM. According to the K_M of ~174 μM found *in vitro* for the efflux transporter affecting apical uptake and absorptive permeability (Table 1), the concentrations applied in the *in vivo* studies should be designed to recognize whether transporters are affecting A275 absorption. From a pharmacological point of view, the doses applied in this study may seem low. However, limited solubility of the drug candidate could result in intestinal fluid concentrations in the tested range, and therefore a K_M value of ~50 μM or higher at the apical membrane is of pharmacological relevance. Thus, other organic anions that are substrates for the same transporter(s) are capable of affecting the absorption of A275.

The *in vitro* studies revealed basolateral efflux and uptake transporters for A275. The possibility of the intestine having impact as an eliminating organ by clearing drug substances from systemic circulation, that is, whether an intestinal basolateral uptake transporter is of importance for the ADME properties is a matter of some debate, and often hepatobiliary clearance is the major elimination route for drugs excreted in feces.[31−34] In the case of A275, the efflux transporters at the basolateral membrane seemed to be dominant at the tested concentrations up to ~200 μM *in vitro* (Figures 1B and 2B). The possible basolateral uptake transporter is thus not of importance as a clearance mechanism of A275, since the highest acquired plasma concentration in these studies was 80 μM. Furthermore, A275 is >99% bound to plasma proteins, which reduces the availability of A275 for intestinal clearance. In contrast to this, drug interactions involving the basolateral efflux transporter(s) contributing to absorption are found both *in vitro* and *in vivo* when A275 and estrone-3-sulfate arecoadministered (Figure 5B). Thus basolateral transporters can be sites of drug interactions, and so plasma

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concentration of an interacting organic anion is important for the absorption process of the drug candidate.

The large intervariability between subjects in the *in vivo* absorption of A275 may be the result of transporters being involved in the absorption process. In drug discovery settings, the therapeutic range, that is, the range constituted by plasma concentration resulting in pharmacological effects to the plasma concentrations resulting in toxicological effects, needs to be wide whenever intestinal transporters are involved in the absorption of anionic drug candidates. This is partly because of the large intervariability between subjects in absorption and partly because of the risk of interactions with other organic anions. However, if the pharmacologically relevant doses are high so that the transporters involved are saturated, the interactions will not be of any importance. A dose resulting in an intestinal fluid concentration above 1 mM A275 is required for saturating the transporters and thus overcoming these interactions. Such a dose would be at least 10 mg/kg. Therefore the risk of interactions and interindividual variability should be taken into account if therapeutic doses are below 10 mg/kg.

This study aimed at investigating whether transporters affect the absorption of A275. Efflux transporters were found at both the apical and basolateral membranes in the *in vitro* intestinal model, thus affecting A275 permeation of the Caco-2 cell monolayer. The *in vivo* absorption of A275 in rats was biphasic, which may be due to transporters being involved in the process, and a basolateral efflux transporter in rat enterocytes is suggested to be involved in the A275 absorption. Thus, interactions related to transporters in intestinal absorption was clearly demonstrated *in vitro* but was not directly evident *in vivo*. To saturate the intestinal transporters involved in A275 absorption and hereby avoid interactions and high interindividual variation, a dose of at least 10 mg/kg should be administered, but at doses below that the risk of drug interactions should be taken into account.

Abbreviations Used

P_{APP}, apparent permeability across Caco-2 cell monolayer; P_{UP}, apparent uptake permeability in Caco-2 cell monolayer; P_{EPA}, end-point accumulative permeability in Caco-2 cell monolayer; k_a, absorption rate constant in rats (0.25−1.25 h); F_a, amount absorbed as a fraction of absorbed amount 20 h after dosing; FaSSIF, fasted state simulated intestinal fluid.

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