Evaluation and prediction of potential drug-drug interactions of
linagliptin using in vitro cell culture methods

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Number of words in Discussion: 1215
**ABBREVIATIONS:**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
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<tr>
<td>AtoB</td>
<td>Apical-to-basal</td>
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<tr>
<td>BtoA</td>
<td>Basal-to-apical</td>
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<td>BCRP</td>
<td>breast cancer resistance protein</td>
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<tr>
<td>CCK8</td>
<td>cholecystokinin-8</td>
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<tr>
<td>DDI</td>
<td>drug-drug interaction</td>
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<td>DPP-4</td>
<td>dipeptidyl peptidase-4</td>
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<tr>
<td>E$_{217}$$\beta$G</td>
<td>estradiol 17$\beta$-D-glucoronide</td>
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<tr>
<td>E-sul</td>
<td>estrone 3-sulfate</td>
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<td>ITC</td>
<td>international transporter consortium</td>
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<td>MPP$^+$</td>
<td>1-methyl-4-phenylpyridinium</td>
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<td>OAT</td>
<td>organic anion transporter</td>
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<td>OATP</td>
<td>organic anion transporting polypeptide</td>
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<td>OCT</td>
<td>organic cation transporter</td>
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<td>organic cation/carnitine transporter</td>
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<td>PAH</td>
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<td>apparent permeability coefficient</td>
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<td>P-gp</td>
<td>P-glycoprotein</td>
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<td>S.D.</td>
<td>standard deviation</td>
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<tr>
<td>SLC</td>
<td>solute carrier</td>
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<td>TEA</td>
<td>tetraethylammonium</td>
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**Abstract**

Linagliptin is a highly potent dipeptidyl peptidase-4 (DPP-4) inhibitor approved for the treatment of type 2 diabetes. Unlike other DPP-4 inhibitors, linagliptin is cleared primarily via the bile and gut. We used a panel of stably- and transiently-transfected cell lines to elucidate the carrier-mediated transport processes that are involved in linagliptin disposition in vivo and to assess the potential for drug-drug interactions (DDI). Our results demonstrate that linagliptin is a substrate of organic cation transporter 2 (OCT2) and P-glycoprotein (P-gp) but not of organic anion transporting-polypeptide 1B1 and 1B3, organic anion transporter 1, 3 and 4, OCT1, carnitine/organic cation transporter 1 and 2, suggesting that OCT2 and P-gp play a role in the disposition of linagliptin in vivo. Linagliptin inhibits transcellular transport of digoxin by P-gp with an apparent IC$_{50}$ = 66.1 μM, but did not inhibit activity of multidrug resistance associated protein 2 and breast cancer resistance protein represented by transport of probe substrate into membrane vesicles from respective transporter-expressing cells. In addition, inhibitory effect of linagliptin on major solute carrier transporter isoforms was investigated. Linagliptin showed inhibitory potency to only OCT1 and OCT2 out of all major solute carrier transporter isoforms examined and those inhibition potencies using three different in vitro probe substrates were in a substrate-specific manner. Considering the low therapeutic plasma concentration of linagliptin, our data clearly suggest a very low risk for transporter-mediated DDIs on comedications in clinical practice.
Introduction

Inhibitors of dipeptidyl peptidase-4 (DPP-4) help improve glucose homeostasis in patients with diabetes by blocking the degradation of incretins, hormones secreted in response to nutrient intake and which in turn increase insulin secretion (Drucker and Nauck, 2006; Nauck, 2011). Linagliptin, a very potent inhibitor of DPP-4, has a high therapeutic index (Scheen, 2010; Forst et al., 2011) and has recently been approved in several countries, including US, Japan and EU for treatment of type 2 diabetes (Thomas et al., 2008; Deacon and Holst, 2010; Forst et al., 2011). Unlike other DPP-4 inhibitors, such as sitagliptin, which are cleared from the body via the kidneys (Herman et al., 2005), linagliptin is cleared primarily via the bile and subordinately via gut which represents an alternative clearance pathway in case of liver failure (Heise et al., 2009; Blech et al., 2010; Fuchs et al., 2012).

In recent years, research on carrier-mediated drug transport processes has helped elucidate their central role in drug disposition (Dresser et al., 2001; Litman et al., 2001; Kusuhara and Sugiyama, 2002). This knowledge has provided a better understanding of factors that affect drug bioavailability, distribution, and elimination, and thereby determination of the correct clinically effective and safe drug dose (Ayrton and Morgan, 2001; Fricker and Miller, 2002; Goh et al., 2002; Mizuno et al., 2003; Giacomini et al., 2010). There are two major classes of carrier-mediated drug transporters: solute carrier (SLC) transporters which generally facilitate drug entry into cells and ATP binding cassette (ABC) transporters which generally facilitate drug exit from cells (Mizuno et al., 2003). The differential distribution of these transporters in intestine, liver, and kidney, all tissues involved in drug absorption, metabolism, and excretion, and their
impact on the pharmacokinetics and bioavailability of drugs and potential drug-drug interactions (DDI) has been well studied (Choo et al., 2000; Dresser et al., 2001; Kusuhara and Sugiyama, 2002; Hirano et al., 2004; Shimizu et al., 2005). The availability of cloned transporter genes (Tanigawara et al., 1992; Muller et al., 1994; Tamai et al., 1997; Tamai et al., 1998) has caused a major impact on drug discovery and development processes (Mizuno et al., 2003) by facilitating drug evaluation using in vitro cell culture systems to predict in vivo drug metabolism and disposition (Goh et al., 2002).

In this manuscript, we use various in vitro cell culture systems to further characterize linagliptin interaction with transporters based on the recommendations from the International transporter consortium (ITC) and availability of experimental systems. Specifically, we set out to determine whether linagliptin behaves as a substrate or an inhibitor of selected SLC and ABC transporters and to assess potential in vivo DDIs.

Materials and Methods

Chemicals. Aminohippuric acid (PAH), probenecid, and taurocholate were obtained from Wako, Japan. Carnitine, cholecystokinin-8 (CCK8), cimetidine, cyclosporine A, digoxin, estrone 3-sulfate (E-sul), estradiol 17β-D-glucoronide (E217βG), 1-methyl-4-phenylpyridinium (MPP+), prazosin, and quinidine were obtained from Sigma-Aldrich, USA. The multidrug resistance-associated protein (MRP) inhibitor MK571 was obtained from Alexis Biochemicals, Switzerland. [3H(G)]Digoxin, [3H]PAH, [3H]E-sul, [3H]E217βG, and [3H]CCK8 were obtained from PerkinElmer, USA. [3H]Ergothioneine was obtained from Moravek Biochemicals, Inc., USA.
DMD #48470

[3H]Benzylpenicillin, [3H]MPP+, [14C]metformin, and [ethyl 1-14C]-tetraethylammonium bromide (TEA) were obtained from American Radiolabeled Chemicals, Inc., USA. [3H]Carnitine was obtained from GE Healthcare, UK. Linagliptin and [14C]linagliptin were synthesized by Boehringer Ingelheim Pharma GmbH, Germany. All other chemicals were of the highest reagent grade available from commercial sources.

**Plasmids.** Organic cation transporter (OCT) 1 and OCT2, and organic anion transporter (OAT) 1 and OAT3 cDNAs were amplified from Marathon-ready human kidney cDNA (BD Biosciences) by polymerase chain reaction (PCR). Briefly, oligonucleotide primers specific to the 5’ and 3’ coding regions of the OAT1 sequence (forward primer: 5’-TCC TCC AGC GAA GGA CAG CAG GC-3’ and reverse primer: 5’-CCT CTT CCT CCT TGT GTG GG-3’), the OAT3 sequence (forward primer: 5’-TAC TAC AGC AGC TGC CGG CCC C-3’ and reverse primer: 5’-AGG GAG AAC AAG GGC AGG GAT GGC-3’), the OCT1 sequence (forward primer: 5’-CGG GAT CCA TGC CCA CCG TGG ATG ACA TTC TGG-3’ and reverse primer: 5’-GCT CTA GAC TCC ATC TTC ATC CCT CCA ACA CG-3’) and the OCT2 sequence (forward primer: 5’ CGG GAT CCA CTC CCC TCT TTG ACT TCT GGC C 3’ and reverse primer: 5’-GCT CTA GAT CTA CTT TTG GTC TTG CTG CCA TCA-3’) were synthesized (Sigma Genosys, Japan) and used in a standard PCR reaction with KOD plus DNA polymerase (Takara, Japan) according to manufacturer’s instructions. The PCR products amplified were cloned into the TOPObluntII expression vector (Invitrogen, Carlsbad, CA) according to manufacturer’s instructions. cDNA fragments of OAT1, OAT3, OCT1 and OCT2 were obtained by a restriction enzyme digestion by NotI
and KpnI for OAT1 and OAT3, and by BamHI and Xbal for OCT1 and OCT2. The fragments were collected and ligated into the NotI-KpnI site (OAT1 and OAT3) or BamHI-Xbal site (OCT1 and OCT2) of pcDNA3.1Zeo(+) mammalian expression vector. cDNAs were sequenced and the amino acid sequence of each transporter isoform was confirmed to be identical to that of gene accession number AB009698 for OAT1 and NM_004254 for OAT3, NM_003057 for OCT1 and NM_003058 for OCT2, respectively. cDNA of organic anion transporting-polypeptide (OATP)1B1 and OATP1B3 subcloned into expression vector pcDNA3.1 were obtained from Prof. Sugiyama, University of Tokyo, Tokyo, Japan (Hirano et al., 2004). OAT4 cDNA in oocyte expression vector was obtained from Prof. Tamai, Kanazawa University, Kanazawa, Japan (Iwanaga et al., 2005) and the cDNA was subcloned to a mammalian expression vector pcDNA3.1(-) (Invitrogen, Carlsbad, CA). The transport activity of each cell line was confirmed by examining the uptake of probe substrates.

**Cell Culture.** HEK293 cells were purchased from Health Science Research Resource Bank, Japan. HEK293 cells stably transfected with either vector, organic cation/carnitine transporter (OCTN)1 or OCTN2 were obtained from GenoMembrane, Inc., Japan. HEK293 cells were maintained in DMEM culture medium (low-glucose Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, USA), 100 U/mL penicillin, 100 g/mL streptomycin, and 0.25 g/mL amphotericin B) at 37°C, 5% CO₂, and 95% relative humidity. Parental LLC-PK1 cells were obtained from Health Science Research Resources Bank, Japan, and maintained in M199 culture medium supplemented with 3% FBS at 37°C, 5% CO₂ and 95% relative humidity. P-glycoprotein (P-gp)–expressing LLC-PK1 cells (LLC-GA5-
COL150) were purchased from Riken Cell Bank, Japan, (Tanigawara et al., 1992; Ueda et al., 1992) and maintained in M199 culture medium supplemented with 10% FBS and 150 ng/mL colchicine at 37°C, 5% CO₂ and 95% relative humidity. Parental MDCKII cells and MDCKII cells expressing human MRP2 were obtained from Dr. Piet Borst, The Netherlands Cancer Institute, The Netherlands (Evers et al., 1998; Tang et al., 2002). Breast cancer resistance protein (BCRP)–expressing cells were established by transfection of human BCRP cDNA into MDCKII cells (Ishiguro et al., 2008). Parental and transporter-expressing MDCKII cells were maintained in the same medium and conditions as HEK293 cells.

**Transport Experiments.**

**Cellular Uptake.** For expression of OAT, OATP, and OCT transporter isoforms, parental HEK293 cells were seeded onto lysine-coated 24-well plates at a density of 0.75 x 10⁵ cells/well. On the next day cells were exposed to serum-free Opti-MEM culture medium containing plasmid (0.2 µg) and FuGENE6 (0.6 µL). Approximately 24 h after transfection, the plasmid-FuGENE6 mixture was removed, cells rinsed in DMEM culture medium supplemented with 5 mM sodium butyrate, and incubated in culture medium for 24 h more to allow for plasmid transporter gene expression. For OCTN1 and OCTN2, HEK293 cells stably expressing OCTN1 or OCTN2, or stably transfected with empty vector were seeded onto lysine-coated 24-well plates at a density of 0.75 x 10⁵ cells/well and were cultured for two days.

Both stably- or transiently-expressing cells were then rinsed twice and preincubated in transport buffer (modified Krebs-Henseleit buffer supplemented with 25 mM HEPES, 1.5 mM calcium chloride) at 37°C for 15–60 min. One percent bovine
serum albumin was additionally supplemented only for substrate assay using linagliptin as substrate. The transport buffer was removed and replaced with fresh transport buffer containing radiolabeled drug and varying concentrations of inhibitor specific for each transporter (Table 1). Initial drug concentration was determined from a 50 μL sample at the initiation of the incubation (t0). The concentration of the solvent in which the drug was initially dissolved did not exceed 0.5% by volume. The incubation was stopped by aspirating the buffer and replacing it with 0.5 mL of ice-cold transport buffer. The cells were rinsed three times with 0.5 mL of ice-cold transport buffer. After the final rinse, the buffer was aspirated and the cell protein extracted with 250 μL of 1 N NaOH for 1 h at 37°C. The reaction was neutralized with 250 μL of 1 N HCl. Radioactivity in the cells was determined by transferring 400 μL aliquots from each well to scintillation vials containing 3 mL of scintillation cocktail (Hionic Fluor) and counting for 3 min in a liquid scintillation analyzer (TRI-CARB 3100TR and 3110TR, Packard, USA). The protein concentration for cells was measured using the Lowry method (Lowry et al., 1951).

**Vesicular uptake experiments.** Membrane vesicles were prepared from MRP2-expressing MDCKII cells, BCRP-expressing MDCKII cells and parental MDCKII cells using a slightly modified hypotonic method (Muller et al., 1994). Briefly, cells were harvested, washed twice with ice-cold phosphate-buffered saline pH 7.4, and collected by centrifugation at 50 g for 5 min at 4°C. The pellet (1-2 mL) was diluted 40-fold with a hypotonic buffer (1 mM Tris-0.1 mM EDTA pH 7.4) and the suspension was stirred gently for 90 min on ice in the presence of protease inhibitors (2 mM PMSF, 0.5 μg/mL leupeptin, 0.1 μg/mL pepstatin, and 0.5 μg/mL aprotinin). The cell lysate was centrifuged at 100,000 g for 30 min at 4°C, and the resulting pellet was suspended in 10
mL of isotonic Tris-sucrose buffer (1 mM Tris- 0.25 M sucrose pH 7.4) and homogenized with a Dounce-B homogenizer (glass/glass, tight pestle, 30 strokes). The crude membrane fraction was layered on top of a 38% sucrose solution and centrifuged in a Beckman SW41 rotor at 280,000 g for 1 h at 4°C. The turbid layer at the interface was collected, diluted to 20 mL with isotonic Tris-sucrose buffer, and centrifuged at 100,000 g for 30 min at 4°C. The resulting pellet was suspended in 400 μL of isotonic Tris-sucrose buffer. Vesicles were formed by passing the suspension 30 times through a 27-gauge needle with a syringe. The membrane vesicles were frozen in liquid nitrogen and stored at –80°C until use in transport assays. The protein concentration for vesicles was measured using the Lowry method (Lowry et al., 1951). Vesicle protein concentration was adjusted to 1 μg/μL prior to the experiment.

Transport medium consisting of 7 μL of membrane vesicle transport buffer (10 mM magnesium chloride in Tris-sucrose buffer, pH 7.4), 4 μL of creatinine phosphokinase (0.5 mg/mL membrane vesicle transport buffer), 2 μL of 50 mM ATP and 2 μL radiolabelled ligand (containing 0.03 μCi in the final volume; 0.033 μM of E217βG for MRP2, and 0.035 μM of E-sul for BCRP) was preincubated in a 1.5 mL Eppendorf tube at 37°C for 3 minutes. To evaluate uptake in the absence of ATP, 2 μL of 50 mM AMP was substituted for ATP. The transport reaction was started by rapidly mixing 5 μL of membrane vesicles (1μg/μL protein) to 15 μL pre-incubated transport medium. After up to 2 min for BCRP and 5 min for MRP2 at 37°C, the reaction was stopped by the addition of 1 mL of ice-cold membrane vesicle stop buffer (100 mM sodium chloride in Tris-sucrose buffer, pH 7.4). Aliquots (900 μL) from each reaction were filtered through a 0.45 μm membrane filter HAWP02500 and washed twice with 5 mL of ice-cold
membrane vesicle stop buffer under vacuum. Each membrane filter and 50 μL of the residual reaction mixture were transferred to scintillation vials containing 2 mL of scintillation cocktail (Hionic Fluor) and the radioactivity measured as described earlier.

**Transcellular Transport.** Parental and P-gp–expressing LLC-PK1 cells were seeded onto 12-well transwell filter inserts at a density of 2.0 x 10^5 cells/filter and 2.5 x 10^5 cells/filter, respectively. The inserts were placed into 12-well tissue culture plates with 0.5 mL culture medium in the apical compartment and 1.5 mL culture medium in the basal compartment. The cells were cultured until they were confluent (6–7 days) with one change of medium.

Approximately 1 h before the start of the experiment, culture medium in both the apical and basal chambers was replaced with equal volume of serum-free M199 medium. Apical-to-basal (AtoB) transport experiments were initiated by replacing the medium in the apical compartment with equal volume of serum-free M199 medium containing radiolabeled substrate with or without inhibitor and the medium in the basal compartment with equal volume of serum-free M199 medium with or without inhibitor (Table 1). The media were reversed for basal-to-apical (BtoA) transport experiments. The cells were incubated at 37ºC and 200 μL samples taken at designated times from the receiver compartment. Initial substrate concentration was determined from a 10 μL sample from the donor compartment at the initiation of the experiment (t0). The concentration of the solvent in which the drug was initially dissolved did not exceed 0.5% by volume. The samples were transferred to scintillation vials containing 2 mL of scintillation cocktail (Hionic Fluor) and the radioactivity measured as described earlier.

**Calculations.**
Cellular Uptake. Cellular uptake was normalized to the amount of radioactivity in the medium and protein concentration in each well and calculated as given in the equation below:

\[
\text{Uptake} = \frac{C_{\text{cell}}}{C_{\text{medium}} \cdot \text{Protein}}
\]

Uptake \(\mu\text{L/designated time/mg}\)

\(C_{\text{cell}}\) Radioactivity in the cell (dpm/designated time/well)

\(C_{\text{medium}}\) Radioactivity concentration in the medium (dpm/\(\mu\text{L}\))

Protein Amount of protein (mg/well)

Transporter-mediated uptake was calculated by subtracting the uptake in vector-transfected cells from that in transporter-expressing cells.

Vesicular uptake. Vesicular uptake was normalized to the amount of radioactivity in the medium and protein concentration as given in the equation below:

\[
\text{Uptake} = \frac{C_{\text{filter}} - B_{\text{filter}}}{C_{\text{medium}} \cdot \text{Protein}}
\]

Uptake \(\mu\text{L/designated time/mg}\)

\(C_{\text{filter}}\) Radioactivity of the membrane filter in the presence of membrane vesicles (dpm/designated time)

\(B_{\text{filter}}\) Radioactivity of the membrane filter in the absence of membrane vesicles (dpm/designated time)

\(C_{\text{medium}}\) Radioactivity concentration in the medium (dpm/\(\mu\text{L}\))

Protein Protein concentration (mg/\(\mu\text{L}\))
**Net Permeability Coefficient (Papp).** The net permeability coefficient (Papp) was calculated from the initial radiolabelled ligand concentration in the donor compartment and the amount of transport using the equations given below:

\[
Papp = \frac{1}{A \times C_{t0}} \times \frac{V_R \times \Delta C_R}{\Delta t}
\]

- **Papp:** Permeability coefficient (cm/sec)
- **C_{t0}:** Radioactivity in the donor compartment at time 0 (dpm/mL)
- **A:** Area of the filter (cm\(^2\))
- **V_R:** Volume in the receiver compartment (mL)
- **\Delta C_R/\Delta t:** Change in substance concentration over time in the receiver compartment (dpm/mL·sec)

The transport rate (\(V_R \times \Delta C_R/\Delta t\)) was calculated from the linear part of the drug concentration versus time curve in the receiver compartment.

**Efflux Ratio.** The ratio of BtoA to AtoB transport was calculated using the equation given below:

\[
\text{Efflux ratio} = \frac{Papp, BtoA, transfected}{Papp, AtoB, transfected} \times \frac{Papp, BtoA, parent}{Papp, AtoB, parent}
\]

- **Papp, BtoA, transfected**  BtoA transport in transfected cells
- **Papp, AtoB, transfected**  AtoB transport in transfected cells
- **Papp, BtoA, parent**  BtoA transport in parental cells
- **Papp, AtoB, parent**  AtoB transport in parental cells
Kinetic Parameters. The kinetic parameters, $K_m$ value, were calculated using the computer program WinNonlin (ver. 4.1, Pharsight, USA), assuming Michaelis-Menten kinetics for the active transport. The permeability coefficient for passive transport is independent of drug concentration.

$$P_{\text{observed}} = P_{\text{app}}$$
$$P_{\text{max}} = P_{\text{app}} \text{ at } C = \infty$$
$$P_{0} = P_{\text{app}} \text{ at } C = 0$$

$P$: Observed Papp of substrate (cm/sec)

$P_{\text{max}}$: Papp of substrate at $C = \infty$ (cm/sec)

$P_{0}$: Papp of substrate at $C = 0$ (cm/sec)

$C$: Concentration of substrate in donor compartment ($\mu$M)

$K_m$: Michaelis-Menten constant ($\mu$M)

$h$: Slope factor

50% Inhibition of Drug Transport (apparent $IC_{50}$). The apparent $IC_{50}$ was calculated as given in the equation below using a nonlinear least-squares regression analysis assuming Michaelis-Menten kinetics for active transport and a non-saturable process independent of drug concentration for passive transport.

$$E = E_{\text{max}} \left( \frac{E_{\text{max}} - E_{\text{min}}}{IC_{50}^h + I^h} \right)$$

$E$: The observed transporter-mediated uptake of substrate ($\mu$L/designated time/mg) in the presence of an inhibitor

$E_{\text{max}}$: The maximum transporter-mediated uptake of substrate in the absence of an inhibitor ($\mu$L/designated time/mg)
E\textsubscript{min}: The non-saturable uptake of substrate in the presence of an inhibitor (µL/designated time/mg)

I: Inhibitor concentration (µM)

IC\textsubscript{50}: Inhibitor concentration for 50% inhibition of transporter-mediated uptake (µM)

h: The slope factor

**Statistical Analysis.** Data are presented as mean ± standard deviation (S.D.) of at least three experiments. Comparisons between two groups were performed with the use of the unpaired student’s t test. Differences were considered statistically significant when the calculated p value was < 0.05.

**Results**

**Linagliptin Uptake.** The possibility of linagliptin acting as a substrate for one or more transporters was examined using HEK293 cells transfected with various transporters or vector. There was no statistically significant difference in [\textsuperscript{14}C]linagliptin uptake between HEK293 cells transfected with OAT1, OAT3, OAT4, OATP1B1, OATP1B3, OCT1, OCTN1 or OCTN2, or the vector alone (Fig. 1). A statistically significant difference (p < 0.05) in [\textsuperscript{14}C]linagliptin uptake was observed only in HEK293 cells transfected with OCT2 plasmid compared with cells transfected with vector plasmid (Fig. 1E and F). The specificity of this uptake was tested by adding 10 mM of the OCT inhibitor cimetidine to the incubation. Under these conditions, cimetidine blocked [\textsuperscript{14}C]linagliptin uptake by the OCT2 transfected HEK293 cells (Fig. 1F).
Inhibition of OCT1 and OCT2 Transporter Activity by Linagliptin. The ability of linagliptin, over a concentration range from 0.3 to 100 µM, to inhibit the uptake of three known substrates of OCT1 and OCT2 transporters was examined using HEK293 cells expressing the transporter being evaluated (Fig. 2). The three substrates tested were [3H]MPP+ (Fig. 2A and B), [14C]metformin (Fig. 2C and D), and [ethyl 1-14C]-TEA (Fig. 2E and F). In cells expressing OCT1, linagliptin blocked the uptake of both [14C]metformin (Fig. 2C) and [ethyl 1-14C]-TEA (Fig. 2E) by > 50% resulting in apparent IC50s of 41 µM and 45 µM, respectively, but not that of [3H]MPP+ (Fig. 2A). In cells expressing OCT2, linagliptin blocked the uptake of [3H]MPP+ partially (Fig. 2B), [14C]metformin significantly (Fig. 2D; apparent IC50 = 80 µM), but did not block the uptake of [ethyl 1-14C]-TEA (Fig. 2F). For all other transporters evaluated, inhibition by linagliptin at the maximum concentration tested (100 µM) was < 50% (Table 2). A positive control was included for each experiment.

Kinetics of P-gp-Mediated Linagliptin Transport Since asymmetric transport across cell monolayers of a transwell system is a strong indicator for the involvement of ABC transporters, their role in disposition of linagliptin was evaluated in both directions, AtoB and BtoA. Specifically, the kinetics of P-gp-mediated linagliptin transport were evaluated using P-gp-expressing LLC-PK1 cells and [14C]linagliptin at concentrations ranging from 2 µM to 500 µM in the donor compartment. There was a concentration-dependent effect on the net permeability coefficients (Papp) in both directions in P-gp-expressing LLC-PK1 cells (Fig. 3A). The mean AtoB Papp increased with linagliptin concentration from 4.21 x 10^-6 cm/sec at 2 µM to 26.7 x 10^-6 cm/sec at 500 µM, whereas the mean BtoA Papp decreased from 39.4 x 10^-6 cm/sec at 2 µM to 15.7 x 10^-6 cm/sec
at 500 μM (Fig. 3B). These data indicate that while the mean Papp was similar in either direction at 500 μM linagliptin, the highest concentration tested, the BtoA permeability was 9.35 times greater than AtoB permeability at 2 μM linagliptin, the lowest concentration tested. Based on these data, the calculated mean $K_m$ of P-gp-associated transport was 187 μM (Table 3). There was no significant change in Papp in either direction in the parental LLC-PK1 cells regardless of linagliptin concentration in the donor compartment (Fig. 3A). The efflux ratio (BtoA to AtoB) calculated based on the directional Papp data in two types of cell lines showed almost complete saturation at 500 μM linagliptin substrate concentration (Fig. 3C).

**Inhibition of ABC Transporters by Linagliptin.** The ability of linagliptin, over a concentration range of 3 μM to 100 μM, to inhibit the uptake of known substrates specific to various ABC transporters was examined in transcellular transport experiments for P-gp or vesicular uptake experiments for MRP2 and BCRP.

As would be expected, digoxin transport in P-gp–expressing LLC-PK1 cells was asymmetric in the absence of any inhibitors with BtoA $P_{app} = 11.9 \times 10^{-6}$ cm/sec, AtoB $P_{app} = 1.06 \times 10^{-6}$ cm/sec, and an efflux ratio of 14.8. This transport was almost completely inhibited by 3 μM cyclosporine A, a well-known inhibitor of P-gp. Linagliptin also inhibited the vectorial transport of digoxin in P-gp–expressing LLC-PK1 cells in a concentration-dependent manner (Fig. 4A). The maximum inhibition observed for linagliptin at 100 μM was similar to that observed for 3 μM cyclosporine A (Fig. 4B). The calculated apparent IC$_{50}$ for linagliptin inhibition of digoxin BtoA transport was 66.1 μM. Parental LLC-PK1 cells were used as controls and demonstrated symmetric transport of digoxin in the presence or absence of linagliptin or cyclosporine A (Fig. 4A).
In the vesicular uptake experiments, linagliptin did not inhibit MRP2-mediated E$_{217}\beta$G transport (Fig. 5A) and only partially inhibited (24.7%) BCRP-mediated E-sul transport (Fig. 5B). Transport of the two substrates was inhibited completely by 25 $\mu$M MK571 and 50 $\mu$M prazosin, respectively.

**Potential Drug-Drug Interactions of Linagliptin.** The calculated apparent IC$_{50}$ values for linagliptin inhibition of specific substrate transport by P-gp, OCT1, and OCT2 were 66 $\mu$M, 41 $\mu$M, and 80 $\mu$M, respectively (Table 4). Linagliptin did not appear to inhibit substrate transport by OAT3 or OATP1B1 since the calculated apparent IC$_{50}$ for both transports was $>100$ $\mu$M (Table 4). The unbound C$_{\text{max}}$ calculated based on total steady state C$_{\text{max}}$ in humans administered a daily oral dose of 5 mg linagliptin being 11.1 nM (Heise et al., 2009), at least 80% of which is bound to protein (Fuchs et al., 2009b) was 2.2 nM. The potential for DDIs due to interference with P-gp-transported drugs was calculated in accordance with the decision trees published by the ITC (Giacomini et al., 2010). The ratio of unbound C$_{\text{max}}$ (2.2 nM) to the apparent IC$_{50}$ of linagliptin for P-gp-mediated substrate transport (66 $\mu$M) was 0.000033, well below the limit of 0.1 set by the Consortium. Based on accepted calculations (Zhang et al., 2008), the theoretical maximal intestinal concentration of linagliptin in humans administered a daily oral dose of 5 mg of the drug is 42.3 $\mu$M and its ratio to the apparent IC$_{50}$ of linagliptin for P-gp-mediated substrate transport is 0.64, again far below the threshold of 10 set by the ITC, indicating that a clinical DDI via P-gp inhibition by linagliptin is highly unlikely.
Discussion

Drug transporters play a central role in determining drug disposal via hepatobiliary excretion, renal excretion and reabsorption, and intestinal excretion and absorption (Ayrton and Morgan, 2001; Fricker and Miller, 2002; Goh et al., 2002; Mizuno et al., 2003; Giacomini et al., 2010). Transporters are expressed preferentially in different tissues with the liver expressing the SLC transporters OATP1B1, OATP1B3, and OCT1, the kidney expressing the SLC transporters OAT1, OAT3, OAT4, OCT2, OCTN1, and OCTN2, and the intestine expressing OATP2B1, and all three tissues expressing the ABC transporters P-gp, MRP2 and BCRP (Giacomini et al., 2010). Using in vitro systems, we investigated the potential for a spectrum of transporters to affect the disposition of linagliptin and to assess their possible impact on the bioavailability, and consequently the clinical dose, of linagliptin.

Linagliptin is a highly potent inhibitor of DPP-4 (Thomas et al., 2008; Deacon and Holst, 2010) approved for the treatment of type 2 diabetes. Clinical studies indicated that linagliptin was primarily excreted through the biliary route (Heise et al., 2009; Blech et al., 2010). As such, the most physiologically relevant interaction we found was between linagliptin and P-gp, a transporter that is highly expressed on the apical membrane of the cells lining the intestine as well as hepatocytes. Our in vitro cell data demonstrated clearly that linagliptin be transported by P-gp suggesting that inhibitors of P-gp could increase the concentration of free linagliptin in the milieu. These data are consistent with recently published in vivo data from rats (Fuchs et al., 2012) and clinical study data in prescribing information of Tradjenta® (Boehringer Ingelheim Pharmaceuticals, 2011). Linagliptin bioavailability increased in rats administered oral linagliptin in the presence of
zosuquidar, an inhibitor of P-gp. Both our in vitro results and the published in vivo data from rats demonstrated that the interaction between linagliptin and P-gp was dose-dependent. In clinical study, linagliptin AUC after 5 mg single dose was 2-fold higher than that without co-administration with 200 mg ritonavir twice daily, although no dose adjustment is recommended (Boehringer Ingelheim Pharmaceuticals, 2011).

Furthermore, P-gp inducer rifampin decreased linagliptin AUC to 60% after repeated once daily dosing of 600 mg rifampin till steady state, suggesting a potential reduction of efficacy of linagliptin (Neumiller and Setter, 2012).

As linagliptin is a substrate for P-gp-mediated transport, there is a potential for DDIs with other drugs that are also substrates or inhibitors of P-gp activity. Apparent IC$_{50}$ values are generally affected by the concentration of the probe substrate used, and assay conditions close to the expected in vivo condition were selected for assessment of the DDI potential of linagliptin. Oral administration of 0.25 mg digoxin should result in a theoretical intestinal concentration of 1.28 $\mu$M, assuming that the compound is completely dissolved in a volume of 250 mL of intestinal fluid. We therefore used 1 $\mu$M of digoxin as probe substrate concentration for determining the apparent IC$_{50}$ of linagliptin in vitro. We evaluated our findings in the context of the decision trees provided by the ITC (Giacomini et al., 2010) using the two criteria recommended for determining the probability of DDIs, the ratio of unbound C$_{\text{max}}$ of linagliptin to its apparent IC$_{50}$ for P-gp-mediated transport and the ratio of the theoretical maximal intestinal concentration of linagliptin to its apparent IC$_{50}$ for P-gp-mediated transport. The values for both these ratios were well below the threshold set by the ITC indicating that while linagliptin was a substrate for P-gp-mediated transport, it posed a very low
risk for DDIs affecting other P-gp substrates. The validity of this assessment has been confirmed in the clinical DDI study using digoxin as substrate of P-gp and linagliptin as putative P-gp inhibitor at oral dose of 5 mg (Friedrich et al., 2011). These data are similar to those obtained for sitagliptin from in vitro and clinical DDI evaluations (Chu et al., 2007).

The observed in vitro interaction between linagliptin and the SLC transporter OCT2, which primarily is involved in renal uptake, was counterintuitive since linagliptin is eliminated primarily via bile with only 5% of the oral therapeutic dose excreted at steady state via urine in humans (Graefe-Mody et al., 2011). One simple explanation is that this finding is an overestimation by the in vitro transfected cell system used to evaluate these interactions. Another, more intriguing, explanation is that it may reflect a physiological response to high doses of linagliptin, such as used in a clinical phase I study (Hüttner et al., 2008). Under those conditions, it is possible that the plasma and renal DPP-4 proteins that bind linagliptin under normal therapeutic doses may be saturated thereby resulting in increased unbound linagliptin and consequently increased renal clearance higher than demonstrated in mice, both wild-type and DPP-4-knockout, after high oral doses (Fuchs et al., 2009a; Fuchs et al., 2009b; Retlich et al., 2009), or humans administered a single oral dose of linagliptin of up to 600 mg (Hüttner et al., 2008) or an intravenous dose of 10 mg (Retlich et al., 2010). Although linagliptin is likely to be actively secreted through OCT2 only at very high administered doses, such as in the phase I study, it is important that DDI assessment be done at the therapeutic dose of 5 mg. Based on the criteria set by the Consortium for transporter substrates, our data seem to indicate that linagliptin also does not exhibit a risk for DDI due to it being a
substrate for OCT2 because of very minor contribution of renal excretion process to overall disposal of linagliptin from body at therapeutic dose. In contrast to linagliptin, active renal clearance accounts for approximately 70% of sitagliptin disposal in healthy human subjects (Bergman et al., 2007). Therefore, although it has not yet been tested in a clinical situation, sitagliptin exposure may be increased by interactions with other drugs such as ibuprofen, fenofibric acid, and probenecid by blocking the SLC transporter OAT3 (Chu et al., 2007).

Members of the OCT family of transporters appear to have two binding sites (Minuesa et al., 2009). In our experiments, linagliptin demonstrated differential ability to inhibit the transport of three different substrates, MPP⁺, metformin, and TEA, in cells expressing either OCT1 or OCT2. For example, in cells expressing OCT1, linagliptin significantly inhibited metformin and TEA uptake but not that of MPP⁺. On the other hand, in cells expressing OCT2, linagliptin significantly inhibited the uptake of only metformin, but only partially inhibited MPP⁺ uptake and did not block TEA uptake at all. These data are consistent with the multiple binding site hypothesis and suggest that several substrates should be used to comprehensively assess the potential for DDIs of any compound being evaluated. The DDI assessment using the decision tree established by the International Transporter Consortium and our apparent IC₅₀ values of linagliptin using metformin as probe substrate for OCT1 and OCT2 returned a prediction that no clinical DDI is expected between linagliptin and metformin (Boehringer Ingelheim Pharmaceuticals, 2011). Furthermore, the change of metformin exposure due to linagliptin was within the established range of 80% –125% for bioequivalence (Center
suggesting no clinical DDI between these two drugs.

Overall, our results are consistent with the clinical findings for linagliptin pharmacokinetics and excretion. Based on these data, there appears to be a low risk for linagliptin to demonstrate significant DDIs in clinical use.
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Participated in research design: Naoki Ishiguro, Wataru Kishimoto, and Thomas Ebner
Conducted experiments: Hidetada Shimizu, Wataru Kishimoto
Contributed new reagents or analytic tools: Hidetada Shimizu
Performed data analysis: Hidetada Shimizu, Wataru Kishimoto
Wrote or contributed to the writing of the manuscript: Naoki Ishiguro, Wataru Kishimoto, Thomas Ebner, Olaf Schaefer
References


Hirano M, Maeda K, Shitara Y, and Sugiyama Y (2004) Contribution of OATP2 (OATP1B1) and OATP8 (OATP1B3) to the hepatic uptake of pitavastatin in humans. *J Pharmacol Exp Ther* **311:**139-146.


transporters to the hepatic uptake of fexofenadine in humans. *Drug Metab Dispos* **33**:1477-1481.


Footnotes

This study was supported by Boehringer Ingelheim.
Figure Legends

FIG. 1. Time-dependent [14C]linagliptin uptake by cells transfected with OAT1, OAT3 (A); OAT4 (B); OATP1B1, OATP1B3 (C); OCTN1, OCTN2 (D); and OCT1, OCT2, (E and F) compared with those transfected with vector plasmid. Time course experiments are shown in panels A–E and effect of a OCT inhibitor cimetidine on [14C]linagliptin uptake at 5 min is shown in panel F.

FIG. 2. Dose-dependent inhibition of [3H]MPP+ (A and B), [14C]metformin (C and D), or [14C]TEA (E and F) uptake by linagliptin in HEK293 cells transfected with either OCT1 (A, C and E) or OCT2 (B, D, and F).

FIG. 3. Dose-dependent effect of linagliptin on the apparent permeability coefficients (Papp) (A); BtoA and AtoB Papp (B); and efflux ratios (C) of [14C]linagliptin in P-gp–expressing and parental LLC-PK1 cells.

FIG. 4. Dose-dependent effect of linagliptin on the apparent permeability coefficients (Papp) (A) and percentage of control transport of [3H]digoxin (B) in P-gp–expressing and parental LLC-PK1 cells. Cyclosporine A was used as a positive control.

FIG. 5. Dose-dependent effect of linagliptin on the vesicular uptake of radiolabeled substrate in vesicles generated from MDCKII cells expressing MRP2 (A) or BCRP (B).
### TABLE 1

**Substrates and inhibitors of transporters used in various experiments**

<table>
<thead>
<tr>
<th>Transporter</th>
<th>Substrate</th>
<th>Inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>OATs</td>
<td>$^{[14]}$C-Linagliptin (1 μM)</td>
<td>Probenecid (100~1000 μM)</td>
</tr>
<tr>
<td>OATPs</td>
<td>$^{[14]}$C-Linagliptin (1 μM)</td>
<td>Taurocholate (1000 μM)</td>
</tr>
<tr>
<td>OCTs</td>
<td>$^{[14]}$C-Linagliptin (1 μM)</td>
<td>Cimetidine (10 mM)</td>
</tr>
<tr>
<td>OCTNs</td>
<td>$^{[14]}$C-Linagliptin (1 μM)</td>
<td>Quinidine (1500 μM)</td>
</tr>
<tr>
<td>OAT1</td>
<td>$^{[3]}$H-PAH (0.2 μM)</td>
<td>Linagliptin (0.3–100 μM)</td>
</tr>
<tr>
<td>OAT3</td>
<td>$^{[3]}$H-E-sul (0.1 μM)</td>
<td>Linagliptin (0.3–100 μM)</td>
</tr>
<tr>
<td>OAT4</td>
<td>$^{[3]}$H-E-sul (0.1 μM)</td>
<td>Linagliptin (0.3–100 μM)</td>
</tr>
<tr>
<td>OATP1B1</td>
<td>$^{[3]}$H-E$_2$17βG (0.1 μM)</td>
<td>Linagliptin (0.3–100 μM)</td>
</tr>
<tr>
<td>OATP1B3</td>
<td>$^{[3]}$H-CCK8 (0.1 μM)</td>
<td>Linagliptin (0.3–100 μM)</td>
</tr>
<tr>
<td>OCT1</td>
<td>$^{[14]}$C-Metformin (10 μM)</td>
<td>Linagliptin (0.3–100 μM)</td>
</tr>
<tr>
<td>OCT1</td>
<td>[ethyl 1-$^{14}$C]-TEA (10 μM)</td>
<td>Linagliptin (0.3–100 μM)</td>
</tr>
<tr>
<td>OCT1</td>
<td>$^{[3]}$H-MPP$^+$ (1 μM)</td>
<td>Linagliptin (0.3–100 μM)</td>
</tr>
<tr>
<td>OCT2</td>
<td>$^{[14]}$C-Metformin (10 μM)</td>
<td>Linagliptin (0.3–100 μM)</td>
</tr>
<tr>
<td>OCT2</td>
<td>[ethyl 1-$^{14}$C]-TEA (10 μM)</td>
<td>Linagliptin (0.3–100 μM)</td>
</tr>
<tr>
<td>OCT2</td>
<td>$^{[3]}$H-MPP$^+$ (1 μM)</td>
<td>Linagliptin (0.3–100 μM)</td>
</tr>
<tr>
<td>OCTN1</td>
<td>$^{[3]}$H-Ergothioneine (1 μM)</td>
<td>Linagliptin (0.3–100 μM)</td>
</tr>
<tr>
<td>OCTN2</td>
<td>$^{[3]}$H-Carnitine (0.1 μM)</td>
<td>Linagliptin (0.3–100 μM)</td>
</tr>
<tr>
<td>P-gp</td>
<td>$^{[3]}$H(G)Digoxin (0.1 μM)</td>
<td>Linagliptin (0.3–100 μM)</td>
</tr>
<tr>
<td>BCRP</td>
<td>$^{[3]}$H-E-sul (0.035 μM)</td>
<td>Linagliptin (1–100 μM)</td>
</tr>
<tr>
<td>MRP2</td>
<td>$^{[3]}$H-E$_2$17βG (0.033 μM)</td>
<td>Linagliptin (1–100 μM)</td>
</tr>
</tbody>
</table>
### TABLE 2

**Inhibition of substrate uptake for each SLC transporter by 100 µM linagliptin**

<table>
<thead>
<tr>
<th>Transporter</th>
<th>Substrate</th>
<th>Percent Inhibition</th>
<th>Linagliptin</th>
<th>Prototypical Inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>OAT1</td>
<td>[3H]PAH (0.2 µM)</td>
<td>1.07 8.33</td>
<td>96.2</td>
<td>0.57</td>
</tr>
<tr>
<td>OAT3</td>
<td>[3H]E-sul (0.1 µM)</td>
<td>0 (–5.70)</td>
<td>8.71</td>
<td>88.5</td>
</tr>
<tr>
<td>OAT4</td>
<td>[3H]E-sul (0.1 µM)</td>
<td>0 (–26)</td>
<td>7.98</td>
<td>81.4</td>
</tr>
<tr>
<td>OATP1B1</td>
<td>[3H]E217βG (0.1 µM)</td>
<td>35.5 3.93</td>
<td>98.4</td>
<td>0.81</td>
</tr>
<tr>
<td>OATP1B3</td>
<td>[3H]CCK8 (0.1 µM)</td>
<td>3.39 5.78</td>
<td>97.4</td>
<td>2.01</td>
</tr>
<tr>
<td>OCTN1</td>
<td>[3H]Ergothioneine (1 µM)</td>
<td>19.4 3.60</td>
<td>83.9</td>
<td>0.35</td>
</tr>
<tr>
<td>OCTN2</td>
<td>[3H]Carnitine (0.1 µM)</td>
<td>15.5 1.25</td>
<td>89.9</td>
<td>0.93</td>
</tr>
</tbody>
</table>

SD, standard deviation
## TABLE 3

*Kinetic parameters of membrane permeability from transwell experiments on LLC-PK1 cells expressing P-gp transporter*

<table>
<thead>
<tr>
<th>Parameter</th>
<th>BtoA</th>
<th>AtoB</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_m$ (μM)</td>
<td>154</td>
<td>220</td>
<td>187</td>
</tr>
<tr>
<td>$P_c$ (cm/sec)</td>
<td>$27.4 \times 10^{-6}$</td>
<td>$-25.1 \times 10^{-6}$</td>
<td>–</td>
</tr>
<tr>
<td>$P_m$ (cm/sec)</td>
<td>$14.7 \times 10^{-6}$</td>
<td>$27.3 \times 10^{-6}$</td>
<td>$21.0 \times 10^{-6}$</td>
</tr>
<tr>
<td>$h$</td>
<td>2.71</td>
<td>4.40</td>
<td>3.56</td>
</tr>
</tbody>
</table>

$h$, slope factor; $P_c$, transporter-mediated active membrane permeability; $P_m$, non-transporter-related passive membrane permeability
**TABLE 4**

*Likelihood of drug-drug interactions with respect to inhibitor*

<table>
<thead>
<tr>
<th>Transporter</th>
<th>Apparent IC\textsubscript{50}</th>
<th>Calculated Unbound</th>
<th>Unbound C\textsubscript{max}/ apparent IC\textsubscript{50}</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-gp</td>
<td>66 (\mu\text{M})</td>
<td>0.0022 (\mu\text{M}) at 5 mg</td>
<td>0.000033</td>
</tr>
<tr>
<td>OCT1</td>
<td>41 (\mu\text{M})#</td>
<td>steady state (total C\textsubscript{max} is 0.011(\mu\text{M})* of which 20%† is unbound)</td>
<td>0.000050</td>
</tr>
<tr>
<td>OCT2</td>
<td>80 (\mu\text{M})#</td>
<td>0.000028</td>
<td></td>
</tr>
<tr>
<td>OAT3</td>
<td>&gt;100 (\mu\text{M})</td>
<td>0.000022</td>
<td></td>
</tr>
<tr>
<td>OATP1B1</td>
<td>&gt;100 (\mu\text{M})</td>
<td>0.000022</td>
<td></td>
</tr>
</tbody>
</table>

*From Heise et al., 2009; †from Fuchs et al., 2009. #Apparent IC\textsubscript{50} values from in vitro studies using metformin as in vitro probe substrate.*
Figure 1

A

B

C

D

E

F

Linagliptin uptake (L[Ug/d]oz./g/min)

Time (min)

Vector
OAT1
OAT3

Vector
OAT4

Vector
OAT1B1
OAT1B3

Vector
OCTN1
OCTN2

Vector
OCT1
OCT2

None
10 mM Cmedidine

Vector
OCT1
OCT2

Vector
OCT1
OCT2

Linagliptin uptake (L[Ug/d]oz./g/min)

Time (min)
Figure 2

(A) 

(B) 

(C) 

(D) 

(E) 

(F)
Figure 3

A

B

C

DMD Fast Forward. Published on October 16, 2012 as DOI: 10.1124/dmd.112.048470
Figure 4

A

B

Papp (cm/ sec)

% of control transport

[Linagliptin] μM

[Linagliptin] μM

Parent AtoB

Parent BtoA

P-gp AtoB

P-gp BtoA

0

3

10

30

100

CsA

3 μM

0

3

10

30

100

CsA

3 μM

100

80

60

40

20

0

120
Figure 5

A

$[^3]H$E$_217bG$ uptake (mL/5 min/mg)

$[^3]H$E$_217bG$ uptake (mL/5 min/mg)

[Linagliptin] $\mu$M

0  1  10  100  MK-571 25 $\mu$M

Parent
MRP2

B

$[^3]H$E-sul$uptake(\mu L/2 min/mg)$

$[^3]H$E-sul$uptake(\mu L/2 min/mg)$

[Linagliptin] $\mu$M

0  1  10  100  Prazosin 50 $\mu$M

Parent
BCRP