Evaluation and Prediction of Potential Drug-Drug Interactions of Linagliptin Using In Vitro Cell Culture Methods

Naoki Ishiguro, Hidetada Shimizu, Wataru Kishimoto, Thomas Ebner, and Olaf Schaefer

Nippon Boehringer Ingelheim Co., Ltd., Kobe, Japan (N.I., H.S., W.K., O.S.); and Boehringer Ingelheim Pharma GmbH, Biberach, Germany (T.E.)

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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 (DDIs). 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; or organic cation/carnitine 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\textsubscript{50} of 66.1 \mu M, but it did not inhibit activity of multidrug resistance-associated protein 2 and breast cancer resistance protein as represented by transport of probe substrate into membrane vesicles from respective transporter-expressing cells. In addition, the inhibitory effect of linagliptin on major solute carrier transporter isoforms was investigated. Linagliptin showed inhibitory potency against only OCT1 and OCT2 out of all major solute carrier transporter isoforms examined, and those inhibition potencies, evaluated using three different in vitro probe substrates, were substrate-specific. Considering the low therapeutic plasma concentration of linagliptin, our data clearly suggest a very low risk for transporter-mediated DDIs with comedinations 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 the US, Japan, and European Union, 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 the 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 (DDIs) have 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, 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 article, 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 to other DPP-4 inhibitors, linagliptin is cleared primarily via the bile and gut, which represents an alternative clearance pathway in case of liver failure (Heise et al., 2009; Blech et al., 2010; Fuchs et al., 2012).

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In this article, 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

Aminophylline acid (PAH), probenecid, and taurocholate were obtained from Wako Pure Chemical Industries (Osaka, Japan). Carnitine, cholecytokinin-8 (CCK8), cimetidine, cyclosporine A, digoxin, estrone 3-sulfate (E-sul), estradiol 17β-glucuronide (E17βG), 1-methyl-4-phenylpyridinium (MPP+), prazosin, and quinidine were obtained from Sigma-Aldrich, St. Louis, MO. The multidrug resistance-associated protein (MRP) inhibitor MK-571 [6-[(3-[2-(7-chloro-2quinolinyl)ethyl]phenyl][3-[3-dimethylamino-3-oxopropyl]phenyl]prop- anonic acid] was obtained from Alexis Biochemicals (Lazen, Switzerland). [3H]H[G] Digoxin, [3H]PAH, [3H]sul, [3H]E17βG, and [3H]CCK8 were obtained from PerkinElmer Life and Analytical Sciences (Waltham, MA). [3H]Ergothioneine was obtained from Moravek Biochemicals, Inc., (Brea, CA). [3H]Benzylenepicillin, [3H]MPP+, [3H]Menformin, and [ethyl 1-14C]tetraethylammonium bromide (TEA) were obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). [3H]Carnitine was obtained from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). Linagliptin and [14C]linagliptin were synthesized by Boehringer Ingelheim GmbH (Ingelheim, 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, San Jose, CA) by polymerase chain reaction (PCR). Briefly, oligonucleotide primers specific to the 5′- and 3′-coding regions of the OAT1 sequence (forward primer: 5′-TCC CCC AGC GAA GGA CAG CAG GC-3′; reverse primer: 5′-CCT CCT CTT TCT TGT TGG CCA-3′), the OAT3 sequence (forward primer: 5′-TAC TAC AGC AGC TGC CGG CCC GC-3′; reverse primer: 5′-AGG GAG AAC AAG GGC AGG GAT GGC-3′), the OCT1 sequence (forward primer: 5′-CGG GAT CCA TGC CCA CGG TCG ATG ACC TGG G-3′; 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 TGT ACT TCT GGC C-3′; reverse primer: 5′-GCT CTA GAT CTA CTT TTG GTG CTA CGC-3′) were synthesized (Sigma-Genosys, Tokyo, Japan) and used in a standard PCR reaction with KOD plus DNA polymerase (Takara, Shiga, Japan) according to the manufacturer’s instructions. The PCR products amplified were cloned into the TOPObluntII expression vector (Invitrogen, Carlsbad, CA) according to the 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 XbaI for OCT1 and OCT2. The fragments were collected and ligated into the NotI-KpnI site of OAT1 (OAT3) or BamHI-XbaI site (OCT1 and OCT2) of pcDNA3.1 Zeo mammalian expression vector. cDNAs were sequenced, and the amino acid sequence of each transporter isoform was confirmed to be identical to that of GenBank accession numbers AB009698 for OAT1, NM_004254 for OAT3, NM_003057 for OCT1, and NM_003058 for OCT2. cDNAs of organic anion–transporting polypeptide (OATP) 1B1 and OATP1B3 subcloned into expression vector pcDNA3.1 were obtained from Dr. Sugiyama University of Tokyo (Tokyo, Japan) (Hirano et al., 2004). OAT4 cDNA in oocyte expression vector was obtained from Dr. Tamao Kanazawa University (Kanazawa, Japan) (Iwanaga et al., 2005) and the cDNA was subcloned to mammalian expression vector pcDNA3.1(−) (Invitrogen). 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 Resources Bank (Osaka, Japan). HEK293 cells stably transfected with vector, organic cation/carnitine transporter (OCTN) 1, or OCTN2 were obtained from GenoMembrane, Inc. (Tokyo, Japan). HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) culture medium (low-glucose DMEM [Invitrogen] supplemented with 10% fetal bovine serum [FBS] [Invitrogen], 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B) at 37°C, 5% CO2, and 95% relative humidity. Parental LLC-PK1 cells were obtained from Health Science Research Resources Bank and maintained in M199 culture medium supplemented with 3% FBS at 37°C, 5% CO2, and 95% relative humidity. Parental MDCCKII cells and MDCCKII cells expressing human MRPs were obtained from Dr. Piet Borst, The Netherlands Cancer Institute (Amsterdam, The Netherlands) (Everson et al., 1998; Tang et al., 2002). Breast cancer resistance protein (BCRP)-expressing cells were established by transfection of human BCRP cDNA into MDCCKII cells (Ishiguro et al., 2008). Parental and transporter-expressing MDCCKII 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 × 105 cells/well. On the next day cells were exposed to serum-free Opti-MEM 1 culture medium containing plasmid (0.2 μg) and FuGENE 6 (0.6 μl). Approximately 24 hours after transfection, the plasmid-FuGENE 6 mixture was removed and cells were rinsed in culture medium supplemented with 5 mM sodium butyrate and incubated in culture medium for 24 hours 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 × 105 cells/well and were cultured for 2 days.

Both stably and transiently expressing cells were then rinsed twice and preincubated in transport buffer (modified Krebs-Henseleit buffer supplemented with 25 mM Heps and 1.5 mM calcium chloride) at 37°C for 15–60 minutes. Bovine serum albumin (1%) 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 (0). 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.

<table>
<thead>
<tr>
<th>Transporter</th>
<th>Substrate</th>
<th>Inhibitor</th>
</tr>
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<tbody>
<tr>
<td>OATs</td>
<td>[14C]Linagliptin (1 μM)</td>
<td>Probenecid (100–1000 μM)</td>
</tr>
<tr>
<td>OATNs</td>
<td>[3H]Linagliptin (1 μM)</td>
<td>Taurocholate (1000 μM)</td>
</tr>
<tr>
<td>OATTs</td>
<td>[3H]Linagliptin (1 μM)</td>
<td>Cimetidine (10 nM)</td>
</tr>
<tr>
<td>OCTNs</td>
<td>[3H]Linagliptin (1 μM)</td>
<td>Quinidine (1500 μM)</td>
</tr>
<tr>
<td>OCT1</td>
<td>[3H]PAH (0.2 μM)</td>
<td>Linagliptin (0.3–100 μM)</td>
</tr>
<tr>
<td>OCT3</td>
<td>[3H]sul (0.1 μM)</td>
<td>Linagliptin (0.3–100 μM)</td>
</tr>
<tr>
<td>OCT4</td>
<td>[3H]E17βG (0.1 μM)</td>
<td>Linagliptin (0.3–100 μM)</td>
</tr>
<tr>
<td>OCTP1B1</td>
<td>[3H]MPP+ (0.1 μM)</td>
<td>Linagliptin (0.3–100 μM)</td>
</tr>
<tr>
<td>OCTP1B3</td>
<td>[3H]CCK8 (0.1 μM)</td>
<td>Linagliptin (0.3–100 μM)</td>
</tr>
<tr>
<td>OCT1</td>
<td>[14C]Mefoxin (10 μM)</td>
<td>Linagliptin (0.3–100 μM)</td>
</tr>
<tr>
<td>OCT1</td>
<td>[14C]TEA (10 μM)</td>
<td>Linagliptin (0.3–100 μM)</td>
</tr>
<tr>
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</tr>
<tr>
<td>OCT1</td>
<td>[14C]TEA (10 μM)</td>
<td>Linagliptin (0.3–100 μM)</td>
</tr>
<tr>
<td>OCT2</td>
<td>[14C]Mefoxin (10 μM)</td>
<td>Linagliptin (0.3–100 μM)</td>
</tr>
<tr>
<td>OCT2</td>
<td>[14C]TEA (10 μM)</td>
<td>Linagliptin (0.3–100 μM)</td>
</tr>
<tr>
<td>OCTN1</td>
<td>[3H]Ergothioneine (1 μM)</td>
<td>Linagliptin (0.3–100 μM)</td>
</tr>
<tr>
<td>OCTN2</td>
<td>[3H]Carnitine (0.1 μM)</td>
<td>Linagliptin (0.3–100 μM)</td>
</tr>
<tr>
<td>P-gp</td>
<td>[3H]G-Digoxin (0.1 μM)</td>
<td>Linagliptin (0.3–100 μM)</td>
</tr>
<tr>
<td>BCRP</td>
<td>[3H]sul (0.035 μM)</td>
<td>Linagliptin (1–100 μM)</td>
</tr>
<tr>
<td>MRPs</td>
<td>[3H]E17βG (0.035 μM)</td>
<td>Linagliptin (1–100 μM)</td>
</tr>
</tbody>
</table>

BCRP, breast cancer resistance protein; CCK8, cholecytokinin-8; E17βG, estradiol 17β-glucuronide; E-sul, estrone 3-sulfate; MPP+, 1-methyl-4-phenylpyridinium; MRPs, multidrug resistance-associated protein; OAT, organic anion transporter; OATP, organic anion–transporting polypeptide; OCT, organic cation transporter; OCTN, organic cation/carnitine transporter; PAH, aminophylline acid; P-gp, P-glycoprotein; TEA, tetraethylammonium.

In vivo DDIs.
Fig. 1. Time-dependent $[^{14}C]$linagliptin uptake by cells transfected with OAT1 and OAT3 (A), OAT4 (B), OATP1B1 and OATP1B3 (C), OCTN1 and OCTN2 (D), and OCT1 and OCT2 (E) compared with those transfected with vector plasmid. Time course experiments are shown in (A–E), and the effect of an OCT inhibitor, cimetidine, on $[^{14}C]$linagliptin uptake at 5 minutes is shown in (F).
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 hour 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; PerkinElmer Life and Analytical Sciences) and counting for 3 minutes in a liquid scintillation analyzer (Tri-Carb 3100TR and 3110TR; Packard). 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). In brief, cells were harvested, washed twice with ice-cold phosphate-buffered saline at pH 7.4, and collected by centrifugation at 50g for 5 minutes 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 minutes on ice in the presence of protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, 0.1 μg/ml pepstatin, and 0.5 μg/ml aprotinin). The cell lysate was centrifuged at 100,000g for 30 minutes 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 280,000g for 1 hour at 4°C. The turbid membrane fraction was transferred to scintillation vials containing 3 ml of scintillation cocktail and counted for 3 minutes in a liquid scintillation analyzer. The protein concentration for vesicles was measured using the Lowry method (Lowry et al., 1951).

**Fig. 2.** Dose-dependent inhibition of [³H]MPP⁺ (A, B), [¹⁴C]metformin (C and D), and [ethyl 1-¹⁴C]TEA (E and F) uptake by linagliptin in HEK293 cells transfected with either OCT1 (A, C, and E) or OCT2 (B, D, and F).
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 before 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 creatine phosphokinase (0.5 mg/ml membrane vesicle transport buffer), 2 μl of 50 mM ATP, and 2 μl of radiolabeled ligand (containing 0.03 μCi in the final volume; 0.033 μM E217βG for MRPE2 and 0.035 μM 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 (1 μg/μl protein) with 15-μl preincubated transport medium. After up to 2 minutes for BCRP and 5 minutes for MRPE2 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, Millipore, MA) 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 2.0×10⁵ cells/filter, respectively. The inserts were placed into 12-well tissue culture plates with 0.5-ml culture medium in the apical compartment and 2.5 ml of membrane filter in each well tissue culture plates with 0.5-ml culture medium in the apical compartment. The cells were cultured until they were confluent (6–7 days) with one change of medium.

Approximately 1 hour before the start of the experiment, culture medium in both the apical and basal chambers was replaced with equal volumes of serum-free M199 medium. Apical-to-basal (AtoB) transport experiments were initiated by replacing the medium in the apical compartment with an equal volume of serum-free M199 medium containing radiolabeled substrate with or without inhibitor and the medium in the basal compartment with an 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 37°C, and 200-μl samples were 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:

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

where Uptake is μl/designated time/mg; Ccell is radioactivity in the cell (dpm/designated time/well); Cmedium is the radioactivity concentration in the medium (dpm/μl); and Protein is the 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:

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

where Uptake is μl/designated time/mg; Cfilter is the radioactivity of the membrane filter in the presence of membrane vesicles (dpm/designated time); Bfilter is the radioactivity of the membrane filter in the absence of membrane vesicles (dpm/designated time); Cmedium is the radioactivity concentration in the medium (dpm/μl); and Protein is the protein concentration (mg/μl).

**Net Permeability Coefficient.** The net permeability coefficient (Papp) was calculated from the initial radiolabeled ligand concentration in the donor compartment and the amount of transport using the equation given below:

\[
P_{\text{app}} = \frac{1}{A \times C_D} \times \frac{V_R \times \Delta C_R}{\Delta t}
\]

where Papp is the permeability coefficient (cm/s); C₀ is the radioactivity in the donor compartment at time 0 (dpm/ml); A is the area of the filter (cm²); Vᵣ is the volume in the receiver compartment (ml); and ∆Cᵣ/Δt is the change in substance concentration over time in the receiver compartment (dpm/ml-s). The transport rate (Vᵣ × ∆Cᵣ/Δt) 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:

\[
\text{Efflux ratio} = \frac{P_{\text{app,BtoA,transf}}/P_{\text{app,AtoB,transf}}}{P_{\text{app,BtoA,parent}}/P_{\text{app,AtoB,parent}}}
\]

where Papp,BtoA,transf is the BtoA transport in transfected cells; Papp,AtoB,transf is the AtoB transport in transfected cells; Papp,BtoA,parent is the BtoA transport in parental cells; and Papp,AtoB,parent is the AtoB transport in parental cells.

**Kinetic Parameters.** The kinetic parameters, Kₘ value, were calculated using the computer program WinNonlin (version 4.1; Pharsight, Sunnyvale, CA), assuming Michaelis-Menten kinetics for the active transport. The permeability coefficient for passive transport is independent of drug concentration.

\[
P_{\text{app,AtoB}} = P_0 + \frac{(P_{\text{max}} - P_0) \times C_b}{C_b + K_m}
\]

**TABLE 2**

Inhibition of substrate uptake for each solute carrier transporter by 100 μM linagliptin

<table>
<thead>
<tr>
<th>Transporter</th>
<th>Substrate</th>
<th>Linagliptin</th>
<th>Prototypical Inhibitor</th>
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<tbody>
<tr>
<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</td>
<td>8.33</td>
</tr>
<tr>
<td>OAT3</td>
<td>[3H]E-sul (0.1 μM)</td>
<td>0–(5.70)</td>
<td>8.71</td>
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<tr>
<td>OAT4</td>
<td>[3H]E-sul (0.1 μM)</td>
<td>0–(26)</td>
<td>7.98</td>
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<tr>
<td>OAPT1B1</td>
<td>[3H]E217βG (0.1 μM)</td>
<td>35.5</td>
<td>3.93</td>
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<tr>
<td>OAPT1B3</td>
<td>[3H]CCK8 (0.1 μM)</td>
<td>3.39</td>
<td>5.78</td>
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<tr>
<td>OCTN1</td>
<td>[3H]Ergothioneine (1 μM)</td>
<td>19.4</td>
<td>3.60</td>
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<td>OCTN2</td>
<td>[3H]Carnitine (0.1 μM)</td>
<td>15.5</td>
<td>1.25</td>
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</tbody>
</table>

**Notes:** CCK8, cholecystokinin-8; E217βG, estradiol 17β-α-glucuronide; E-sul, estrone 3-sulfate; OAT, organic anion transporter; OAPT, organic anion-transporting polypeptide; OCTN, organic cation/carnitine transporter; PAH, aminopyrine acid; SD, standard deviation.
\[ P_{\text{app}} \text{BtoA} : P = P_0 - \frac{(P_{\text{max}} - P_0) \times C^h}{C^h + K_m^h} \]

where \( P \) is the observed \( P_{\text{app}} \) of substrate (cm/s); \( P_{\text{max}} \) is the \( P_{\text{app}} \) of substrate at \( C = 0 \) (cm/s); \( P_0 \) is the \( P_{\text{app}} \) of substrate at \( C = 0 \) (cm/s); \( C \) is the concentration of substrate in donor compartment (µM); \( K_m \) is the Michaelis-Menten constant (µM); and \( h \) is the slope factor.

**50% Inhibition of Drug Transport (Apparent IC50).** The apparent IC50 was calculated as given in the equation below using a nonlinear least-squares regression analysis assuming Michaelis-Menten kinetics for active transport and a nonsaturable process independent of drug concentration for passive transport.

\[ E = E_{\text{max}} - \frac{(E_{\text{max}} - E_{\text{min}}) \times 1^h}{IC_{50}^h + 1^h} \]

where \( E \) is the observed transporter-mediated uptake of substrate (µl/designated time/mg) in the presence of an inhibitor; \( E_{\text{max}} \) is the maximum transporter-mediated uptake of substrate in the absence of an inhibitor (µl/designated time/mg); \( E_{\text{min}} \) is the nonsaturable uptake of substrate in the presence of an inhibitor (µl/designated time/mg); \( I \) is the inhibitor concentration (µM); \( IC_{50} \) is the inhibitor concentration for 50% inhibition of transporter-mediated uptake (µM); and \( h \) is the slope factor.

**Statistical Analysis**

Data are presented as mean ± 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 [14C]linagliptin uptake between HEK293 cells transfected with OAT1, OAT3, OAT4, OATP1B1, OATP1B3, OCT1, OCTN1, OCTN2, or the vector alone (Fig. 1). A statistically significant difference (\( P < 0.05 \)) in [14C]linagliptin uptake was observed only in HEK293 cells transfected with OCT2 plasmid compared with cells transfected with vector plasmid (Fig. 1, E 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 [14C]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. 2, A and B), [14C]metformin (Fig. 2, C and D), and [ethyl 1-14C]TEA (Fig. 2, E 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 IC50 values of 41 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) and [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 of 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 to 500 µM in the donor compartment. There was a concentration-dependent effect on the \( P_{\text{app}} \) values in both directions in P-gp-expressing LLC-PK1 cells (Fig. 3A). The mean AtoB \( P_{\text{app}} \) increased with linagliptin concentration from 4.21 × 10⁻⁶ cm/s at 2 µM to 26.7 × 10⁻⁶ cm/s at 500 µM, whereas
the mean BtoA $P_{\text{app}}$ decreased from $39.4 \times 10^{-6}$ cm/s at 2 μM to $15.7 \times 10^{-6}$ cm/s at 500 μM (Fig. 3B). These data indicate that, although the mean $P_{\text{app}}$ 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. On the basis of these data, the calculated mean $K_m$ of P-gp-associated transport was 187 μM (Table 3). There was no significant change in $P_{\text{app}}$ 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 $P_{\text{app}}$ 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 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 $P_{\text{app}} = 11.9 \times 10^{-6}$ cm/s, AtoB $P_{\text{app}} = 1.06 \times 10^{-6}$ cm/s, 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$_{217}$βG 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 μM MK-571 and 50 μM prazosin, respectively.

![Fig. 4.](image-url) Dose-dependent effect of linagliptin on the $P_{\text{app}}$ values (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.](image-url) 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 3

<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/s)</td>
<td>$27.4 \times 10^{-6}$</td>
<td>$25.1 \times 10^{-6}$</td>
<td>-</td>
</tr>
<tr>
<td>$P_m$ (cm/s)</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>

AtoB, apical-to-basal; AtoA, basal-to-apical; h, slope factor; $P_c$, transporter-mediated active membrane permeability; $P_m$, nontransporter-related passive membrane permeability.
Potential Drug-Drug Interactions of Linagliptin. The calculated apparent IC50 values for linagliptin inhibition of specific substrate transport by P-gp, OCT1, and OCT2 were 66, 41, and 80 μM, respectively (Table 4). Linagliptin did not appear to inhibit substrate transport by OAT3 or OATP1B1 since the calculated apparent IC50 for both transports was >100 μM (Table 4). The unbound Cmax calculated based on the total steady-state Cmax 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 attributed 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 Cmax (2.2 nM) to the apparent IC50 of linagliptin for P-gp-mediated substrate transport (66 μM) was 0.000033, well below the limit of 0.1 set by the Consortium. On the basis of 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 μM, and its ratio to the apparent IC50 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 (Aytton 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; 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 assessed 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 is 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 is 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 on 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 area under the curve after a 5-mg single dose was 2-fold higher than that without coadministration with 200-mg ritonavir twice daily, although no dose adjustment is recommended (Boehringer Ingelheim Pharmaceuticals, 2011). Furthermore, the P-gp inducer rifampin decreased linagliptin area under the curve to 60% after repeated once-daily dosing of 600-mg rifampin until 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 IC50 values generally are 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 μM, assuming that the compound is completely dissolved in a volume of 250 ml of intestinal fluid. We therefore used 1 μM digoxin as the probe substrate concentration for determining the apparent IC50 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 Cmax of linagliptin to its apparent IC50 for P-gp-mediated transport and the ratio of the theoretical maximal intestinal concentration of linagliptin to its apparent IC50 for P-gp-mediated transport. The values for both of these ratios were well below the threshold set by the ITC, indicating that although 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 a substrate of P-gp and linagliptin as a putative P-gp inhibitor at an 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 physiologic response to high doses of linagliptin, such as used in a clinical phase I study (Hütter 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; b; Retlitch et al., 2009), or humans administered a single oral dose of linagliptin of up to 600 mg (Hütter et al., 2008) or an intravenous dose of 10 mg (Retlitch 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. On the basis of the criteria set by the ITC for transporter substrates, our data seem to indicate that linagliptin also does not exhibit a risk for DDIs due to its being a substrate for OCT2 because of the very minor contribution of the renal excretion process to overall disposal of linagliptin from the body at therapeutic doses. In contrast to linagliptin, active renal clearance accounts for approximately 70% of MPP+ disposal in healthy human subjects (Bergman et al., 2007). Therefore, although it has not yet been tested in a clinical situation, linagliptin exposure may be increased by interactions with other drugs such as ibuprofen, fenofibrate, 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 with any compound being evaluated. The DDI assessment using the decision tree established by the ITC and our apparent IC50 values of linagliptin using metformin as a 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 for Drug Evaluation and Research, 2003; Boehringer Ingelheim Pharmaceuticals, 2011), suggesting no clinical DDI between these two drugs.

Overall, our results are consistent with the clinical findings for linagliptin pharmacokinetics and excretion. On the basis of these data, there seems to be a low risk for linagliptin to demonstrate significant DDIs in clinical use.

Acknowledgments

The excellent technical assistance of Yugo Miki and Masahito Takatani in conducting the in vitro experiments is gratefully acknowledged. [14C]Linagliptin was kindly provided by Ralf Kiesling, head of the isotope chemistry laboratory, Boehringer Ingelheim, Germany. The authors were fully responsible for all content and editorial decisions. They were involved at all stages of manuscript development and have approved the final version. Medical writing assistance, supported financially by Boehringer Ingelheim, was provided by Mukund Nori, CMMP, of Envision Scientific Solutions during the preparation of the manuscript.

Authorship Contributions

Participated in research design: Ishiguro, Kishimoto, Ebner. Conducted experiments: Shimizu, Kishimoto. Contributed new reagents or analytic tools: Shimizu. Performed data analysis: Shimizu, Kishimoto. Wrote or contributed to the writing of the manuscript: Ishiguro, Kishimoto, Ebner, Schafer.

References

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**Address correspondence to:** Naoki Ishiguro, Nippon Boehringer Ingelheim Co., Ltd., 6–7–5 Minatojima-Minamimachi, Chuo-ku, Kobe, Hyogo, Japan 650-0047.

E-mail: naoki.ishiguro@boehringer-ingelheim.com