

Utility of P-Glycoprotein and Organic Cation Transporter 1 Double-Transfected LLC-PK1 Cells for Studying the Interaction of YM155 Monobromide, Novel Small-Molecule Survivin Suppressant, with P-Glycoprotein^[S]

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ABSTRACT:

1-(2-Methoxyethyl)-2-methyl-4,9-dioxo-3-(pyrazin-2-ylmethyl)-4,9-dihydro-1*H*-naphtho[2,3-*d*]imidazolium bromide (YM155 monobromide), a novel small molecule that downregulates survivin and exhibits potent antitumor activity, is hydrophilic and cationic. Although previous studies have shown that influx transporters play important roles in the uptake of YM155 into hepatocytes and possibly into cancer cells, efflux transporters have yet to be investigated. In this study, we assessed the interaction of YM155 with P-glycoprotein [multidrug resistance 1 (MDR1)/ATP-binding cassette B1] using two kinds of transcellular transport systems: Caco-2 and MDR1-expressing LLC-PK1 cells (LLC-MDR1). We also used a newly established LLC-OCT1/MDR1 cell line, which expresses basal YM155 uptake transporter organic cation transporter1 (OCT1) and apical MDR1. Direct interaction between YM155 and MDR1 and other efflux transporters was evaluated using transporter-expressing membrane vesicles. A bidirectional

transporter assay using Caco-2 and LLC-MDR1 cells showed low permeability and no vectorial transport of YM155, suggesting that YM155 is not a substrate of MDR1. However, vectorial transport across LLC-OCT1/MDR1 cells was identified, which was inhibited by the MDR1 inhibitor cyclosporine A, clearly indicating that YM155 is in fact a substrate of MDR1. Insufficient expression of basal uptake transporter of YM155 in Caco-2 and LLC-MDR1 might have confounded conclusions regarding YM155 and MDR1. Using the transporter-expressing vesicles, MDR1-mediated transport was most significantly involved in YM155 transport among the efflux transporters examined. In conclusion, these findings suggest that YM155 is a substrate of MDR1, and that MDR1 may play an important role in the pharmacokinetics of YM155. Transcellular assays lacking basal uptake transporters may be inaccurate in the assessment of hydrophilic compounds that have poor membrane permeability by passive diffusion.

Introduction

Survivin, a member of the inhibitor of apoptosis protein family, is a key regulator of mitosis and apoptotic cell death and is regarded as a novel and attractive target for cancer chemotherapy (Zaffaroni and Daidone, 2002; Mita et al., 2008). The novel small-molecule survivin suppressant 1-(2-methoxyethyl)-2-methyl-4,9-dioxo-3-(pyrazin-2-ylmethyl)-4,9-dihydro-1*H*-naphtho[2,3-*d*]imidazolium bromide (YM155

monobromide) (Minematsu et al., 2009), a hydrophilic organic cation, is now being tested as an anticancer drug in phase II clinical trials in combination with other agents. YM155 given as a single agent has shown modest antitumor activity against non-Hodgkin's lymphoma (Tolcher et al., 2008) and advanced nonsmall lung cancer (Giaccone et al., 2009). In addition, it has also exhibited antitumor activity against a broad spectrum of human cancer cell lines and in various human-derived tumor xenograft mouse models (Nakahara et al., 2007, 2011).

In clinical and nonclinical studies, YM155 is delivered as a continuous intravenous administration. The compound is excreted as unchanged drug both in the urine via tubular active secretion (Minematsu et al., 2010) and feces (Sohda et al., 2007; Tolcher et al., 2008; Satoh et al., 2009). Transporters are considered to play an important role in membrane permeation of the hydrophilic YM155 during its distribution and excretion.

We previously demonstrated the involvement of several uptake transporters in the pharmacokinetics of YM155. In the liver, organic

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ABBREVIATIONS: YM155 monobromide, 1-(2-methoxyethyl)-2-methyl-4,9-dioxo-3-(pyrazin-2-ylmethyl)-4,9-dihydro-1*H*-naphtho[2,3-*d*]imidazolium bromide; OCT, organic cation transporter; ABC, ATP-binding cassette; MDCKII, Madin-Darby canine kidney II; MPP, 1-methyl-4-phenylpyridinium; MRP, multidrug resistance-associated protein; P-gp/MDR1, P-glycoprotein/multidrug resistance 1; BCRP, breast cancer resistance protein; CysA, cyclosporine A.

cation transporter (OCT) 1-mediated uptake accounts for the in vitro hepatic uptake and in vivo nonrenal clearance of YM155 (Iwai et al., 2009). OCT2, which is mainly expressed in the renal proximal tubules, transports YM155 and may mediate the first step in urinary excretion (Minematsu et al., 2010). Various human cancer cell lines show cationic transporter-mediated uptake of YM155 (Minematsu et al., 2009), although the transporter responsible has yet to be identified. In contrast to uptake transporters, no study has investigated efflux transporters of YM155.

Among ATP binding cassette (ABC) transporters, P-glycoprotein [P-gp; multidrug resistance 1 (MDR1)] is one of the most clinically important transporters in humans (Zhou, 2008). MDR1 is located on the apical membrane of intestinal epithelial cells, bile canaliculi, renal tubular cells, and placenta as well as on the luminal surface of capillary endothelial cells in the brain and testes. It protects the human body from xenobiotics by suppressing intestinal absorption and excretion into the urine and bile and limits central nervous system entry (Thiebaut et al., 1987). MDR1 shows a wide spectrum of substrate specificities, and many substrates are hydrophobic organic cations, including anticancer drugs such as vinca alkaloids and taxanes (Shirakawa et al., 1999; Borst and Elferink, 2002). For this reason, overexpression of MDR1 in cancer cells is the primary cause of multidrug resistance to its substrate drugs (Gottesman and Pastan, 1993; Ambudkar et al., 1999).

The interaction of a drug candidate with MDR1 is routinely examined in drug development programs (Balimane et al., 2006). The bidirectional transcellular transport system is regarded as a definitive method of identifying MDR1 substrates or inhibitors in the U.S. Food and Drug Administration Drug Interaction Guidance released in September 2006 (see <http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm072101.pdf>) (Huang et al., 2007; Zhang et al., 2008). Recommended cells for this purpose include human colon cancer-derived Caco-2 cells, which endogenously express MDR1 along with other efflux transporters [breast cancer resistance protein (BCRP) and multidrug resistance-associated proteins (MRPs)] (Hilgendorf et al., 2007; Hayeshi et al., 2008; Ahlin et al., 2009) and MDR1-expressing polarized cell lines such as Madin-Darby canine kidney II (MDCKII)-MDR1 cells and LLC-MDR1 cells. Therefore, we initially decided to use Caco-2 and LLC-MDR1 cells for our studies. Double-transfected polarized cell lines that simultaneously express both basal uptake and apical efflux transporters have been reported to be useful in evaluating the interaction between hydrophilic anions and uptake and efflux transporters (Sasaki et al., 2002; Mita et al., 2006; Nies et al., 2008). Using OCT1/MDR1 double-transfected MDCKII cells, Nies and colleagues (2008) identified a new MDR1 substrate, the cationic plant alkaloid berberine. However, the application of OCT1/MDR1 double-transfected cells in interaction studies of drug candidates with MDR1 has not been reported.

Here, we tested for bidirectional transcellular transport by constructing LLC-OCT1/MDR1 cells, in addition to the use of Caco-2 and LLC-MDR1 cells. We also confirmed the interaction of YM155 with MDR1 using inside-out membrane vesicles prepared from MDR1-expressing cell lines, in which YM155 has direct access to MDR1. Finally, we evaluated the interaction of YM155 with other efflux transporters via vesicular transport.

Materials and Methods

Materials. [14 C]YM155 monobromide (specific activity 3.05 and 3.27 MBq/mg; radiochemical purity $\geq 98.2\%$) was synthesized by Sekisui Medical Co. Ltd. (Ibaraki, Japan). Unlabeled YM155 monobromide (assay value

100.1%) was synthesized by Astellas Pharma Inc. (Tokyo, Japan). [3 H]Digoxin, [3 H]-1-methyl-4-phenylpyridinium (MPP), and [3 H]mannitol were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). [3 H]Vinblastine was obtained from GE Healthcare Bio-Sciences (Little Chalfont, Buckinghamshire, UK). *N*-Methylquinidine, MDR1-expressing membrane vesicles, and control vesicles for MDR1 were purchased from GenoMembrane Inc. (Kanagawa, Japan). MRP1, MRP2, MRP3, BCRP, and control vesicles for these transporters were purchased from Solvo Biotechnology (Budapest, Hungary). All other chemicals and reagents used were commercially available and their purity was guaranteed.

Construction of Cells Expressing OCT1 and MDR1. The construction of stable transfectants expressing human OCT1 and MDR1 was performed as follows. Human OCT1 cDNA (GenBank accession number X98332) was subcloned into expression vector pcDNA3.1/Zeo(+) (Invitrogen, Carlsbad, CA) to produce OCT1-pcDNA3.1/Zeo(+). MDR1 cDNA clone (American Type Culture Collection, Manassas, VA) was confirmed to have the same sequence as that represented by the GenBank accession number M14758 and subcloned into pcDNA3.1(+) (Invitrogen) to produce MDR1-pcDNA3.1(+). We selected LLC-PK1 cells as the host cells because endogenous porcine Mdr1 expression is low and not altered by the expression of human MDR1 in LLC-MDR1 cells; this is in contrast with the high endogenous Mdr1 expression in parental MDCKII cells, the expression of which is considerably reduced in MDCKII-MDR1 cells (Kuteykin-Teplyakov et al., 2010). LLC-PK1 cells were transfected using Lipofectamine 2000 (Invitrogen) with OCT1-pcDNA3.1/Zeo(+), MDR1-pcDNA3.1(+), or empty vectors (pcDNA3.1/Zeo[+] and pcDNA3.1[+]) according to the manufacturer's instructions. Stable clones were selected in media containing antibiotics (zeocin for pcDNA3.1/Zeo[+] and G418 for pcDNA3.1[+]). After antibiotic selection, MDR1-expressing cells were rescreened in media containing colchicine. The following four transfectants were constructed: empty vectors-transfected LLC-mock (empty pcDNA3.1/Zeo[+] and empty pcDNA3.1[+]), LLC-OCT1 (OCT1-pcDNA3.1/Zeo[+] and empty pcDNA3.1[+]), LLC-MDR1 (empty pcDNA3.1/Zeo[+] and MDR1-pcDNA3.1[+]), and LLC-OCT1/MDR1 (OCT1-pcDNA3.1/Zeo[+] and MDR1-pcDNA3.1[+]). The expression of OCT1 and MDR1 was confirmed by reverse transcriptase polymerase chain reaction (Supplemental Fig. 1). The cellular localization of OCT1 and MDR1 on the basolateral and apical membrane, respectively, was confirmed by immunocytochemistry using specific antibodies.

Cell Culture. Caco-2 cells were obtained from American Type Culture Collection and grown in Dulbecco's modified Eagle's medium with L-glutamate and supplemented with heat-inactivated fetal bovine serum (10% v/v), nonessential amino acids (1% v/v), penicillin (100 U/ml), and streptomycin (100 μ g/ml).

Transfected LLC-PK1 cells were cultured in medium 199 with 100 μ g/ml zeocin, 800 μ g/ml G418, and fetal bovine serum (10% v/v). All cells were cultured at 37°C in humidified air with 5% CO₂.

Transcellular Transport Experiments. Caco-2 cells were seeded onto 12-well Transwell membrane inserts (0.4- μ m pore size; Corning Life Sciences, Lowell, MA) at a density of approximately 7×10^4 cells/well. The cells were cultured for 21 days to allow for enterocyte-like differentiation. To initiate the transcellular transport experiment, cell culture medium was replaced by Hanks' balanced salt solution with 25 mM HEPES (pH 7.4) solution in either the apical or basal compartment, and Hanks' balanced salt solution with 25 mM HEPES containing a radiolabeled ligand was added to the other side.

One week before the transcellular transport experiments, LLC-mock, LLC-OCT1, LLC-MDR1, and LLC-OCT1/MDR1 cells were seeded onto 24-well Transwell membrane inserts (0.4- μ m pore size; Corning Life Sciences) at a density of approximately 1.4×10^5 cells/well. One day before the experiments, medium in each well was replaced by Medium 199 with 10% fetal bovine serum and sodium butyrate (5 mM). Cell culture medium was replaced by HEPES Krebs-Henseleit buffer (11.1 mM D-glucose, 1.17 mM MgSO₄, 1.18 mM KH₂PO₄, 4.69 mM KCl, 143 mM NaCl, 2.54 mM CaCl₂, and 10 mM HEPES, pH 7.4), and cells were preincubated for at least 15 min at 37°C before initiating transport experiments. Transcellular transport was initiated by adding HEPES Krebs-Henseleit buffer containing a radiolabeled ligand to the apical or basal compartment and HEPES Krebs-Henseleit buffer to the other side.

At designated times after the initiation of the experiments at 37°C, 100- or 200- μ l samples in the receiver compartment were aliquoted to a scintillation

vial for measurement of radioactivity. Except for the last time points, an equal volume of buffer was added to the sampling compartment immediately after sampling. Radioactivity was measured with a scintillation counter using the external standard method.

To confirm monolayer integrity, transcellular transport of [^3H]mannitol was also performed. In the presence of 1 μM YM155 (same concentration as in the transcellular transport study), the basal-to-apical flux in LLC-OCT1 cells increased after more than 60 min of incubation, indicating disruption of the tight junction of cells during the incubation. The basal-to-apical flux in other cell lines and the apical-to-basal flux in all cell lines were comparable to those under control conditions. In the transcellular transport study, the steady-state velocity was achieved after the first sampling time point (30 min) in all cell lines, which indicated sufficient monolayer integrity and therefore had no effects on the results.

Vesicular Transport Experiments. Vesicular transport was measured using a rapid filtration technique (Ito et al., 1998). Frozen vesicles were quickly thawed at 37°C and diluted by assay buffer containing 50 mM MOPS-Tris (pH 7.0), 70 mM KCl, and 7.5 mM MgCl_2 . When MRP-expressing vesicles were used, 2 mM GSH was also added. After a 3-min preincubation at 37°C, vesicular transport was initiated by mixing membrane vesicle solutions and probe substrate/inhibitor solutions with or without ATP, and the mixture was then incubated at 37°C for the designated periods. The final concentrations of the membrane vesicles and AMP/ATP were 50 $\mu\text{g}/\text{sample}$ and 4 mM, respectively. The transport reactions were stopped by adding ice-cold wash buffer containing 40 mM MOPS-Tris (pH 7.0) and 70 mM KCl. To reduce the nonspecific binding of [^{14}C]YM155, a wash buffer containing YM155 (1 mM) was used for the evaluation of its transport. To evaluate MDR1-mediated transport, the stopped reaction mixture was rapidly filtered through a GF/B glass fiber filter on 96-well plates (Unifilter; Whatman, Clifton, NJ). Filters were washed with 200 μl of ice-cold wash buffer 4 times, and the test compounds on the filters were eluted twice by centrifugation after the addition of 50 μl of 10% SDS solution. To measure *N*-methylquinidine concentration, 100 μl of 0.05 μM sulfuric acid was added to the eluate and the intensity was measured using a microplate reader (excitation 360 nm, emission 465 nm, Genious; Tecan Japan Co. Ltd., Kanagawa, Japan). To measure radioactivity, the eluate was diluted with 100 μl of MilliQ water and transferred into scintillation vials with 2 ml of the scintillation cocktail. Radioactivity was measured with a scintillation counter using the external standard method. To evaluate MRP- and BCRP-mediated transport, a GF/F glass fiber filter (Whatman) was also used as a filter. Wash buffer volume was adjusted accordingly, and elution of radioactivity was conducted by placing the filter directly into a scintillation vial containing the scintillation cocktail.

Data Analysis and Estimation of Kinetic Parameters. Permeability (P) of the substrate was calculated from the cumulative amount of radioactivity transported across the cell monolayers from the basal to apical side (P_{ba}) and from the apical to basal side (P_{ab}) during the transport assay using the following equation:

$$P = (dQ/dt)/(A \times C_0)$$

where dQ/dt is the permeability rate, A is the surface area of growth on the insert, and C_0 is the initial concentration of the substrate. The flux ratio was calculated as the ratio of P_{ba} divided by P_{ab} .

The Michaelis-Menten constant (K_m) and V_{max} values were calculated using WinNonlin version 4.1 (Pharsight, Mountain View, CA) by simultaneously fitting the following equations to the YM155 concentration ($[S]$; μM) versus uptake velocity (V ; $\text{pmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$) data in the presence of ATP and AMP:

$$\text{ATP: } V = V_{\text{max}} \times [S]/(K_m + [S]) + P_{\text{dif}} \times [S]$$

$$\text{AMP: } V = P_{\text{dif}} \times [S]$$

where P_{dif} ($\mu\text{l} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$) is the uptake clearance by passive diffusion and adsorption to the membrane.

Results

Transcellular Transport across Caco-2 Cells. Transcellular transport of [^{14}C]YM155 (2.9 μM) across Caco-2 cells was examined.

YM155 showed low permeability across monolayers, and the basal-to-apical flux was similar to the apical-to-basal flux, with a flux ratio of 1.20 ± 0.42 . In addition, the presence of 100 μM (\pm)-verapamil, a MDR1 inhibitor, in the transport medium did not affect the transport of YM155 (data not shown). MDR1 activity was confirmed by the transcellular transport of [^3H]vinblastine (1 μM) with a flux ratio of 27.1 ± 9.1 .

Transcellular Transport across LLC-Mock, LLC-OCT1, LLC-MDR1, and LLC-OCT1/MDR1 Monolayers. The transport activity of OCT1 was confirmed by the uptake of [^3H]MPP, a typical substrate of OCTs, whereas the transport activity of MDR1 was confirmed using [^3H]digoxin, a typical substrate for MDR1. Transport activities of these typical substrates were higher in transporter transfected cells than in mock cells and were similar between single (LLC-OCT1 or LLC-MDR1 cells) and double (LLC-OCT1/MDR1 cells) transfected cells. The transport of [^3H]MPP is 18.2 ± 0.5 and $12.2 \pm 3.3 \mu\text{l} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ in LLC-OCT1 and LLC-OCT1/MDR1 and 2.7 ± 0.3 and $4 \pm 0.4 \mu\text{l} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ in LLC-mock and LLC-MDR1, respectively. The transcellular transport of [^{14}C]YM155 across LLC-mock, LLC-OCT1, LLC-MDR1, and LLC-OCT1/MDR1 monolayers is shown in Fig. 1. The apical-to-basal flux of YM155 was higher than the basal-to-apical flux in LLC-mock cells, and the flux ratio was 0.2 after an incubation of 180 min. The flux ratio in LLC-OCT1 and LLC-MDR1 cells approached 1. In contrast, the basal-to-apical flux in LLC-OCT1/MDR1 cells was much higher than that in the opposite direction, and the flux ratio was 16.6 after incubation for 180 min.

Cyclosporine A (CysA) and MPP are known to inhibit MDR1 and OCT1, respectively, and not to cross-inhibit the other transporter. The inhibitory effects of these compounds on YM155 transport are shown in Fig. 2. The flux ratio across LLC-OCT1/MDR1 cells decreased from 13.2 to 0.3 and 1.4 in the presence of CysA and MPP, respectively. With regard to the apical-to-basal flux, these values markedly increased in the presence of CysA compared with control conditions in LLC-MDR1 and LLC-OCT1/MDR1 cells, suggesting that MDR1 was sufficiently inhibited by CysA. These results supported the notion that YM155 is a substrate for OCT1 and MDR1. The inhibitory effects of YM155 and CysA on [^3H]digoxin transport are shown in Fig. 3. In the absence of any inhibitor, flux ratios in LLC-MDR1 and LLC-OCT1/MDR1 cells were 5.7 and 5.6, respectively. The basal-to-apical flux of [^3H]digoxin was inhibited in the presence of CysA in LLC-MDR1 and LLC-OCT1/MDR1 cells, and the flux ratio approached approximately 1. In contrast, the basal-to-apical flux and flux ratio were not affected by 100 μM YM155, indicating that YM155 does not inhibit MDR1-mediated transport of digoxin at the concentrations tested.

Vesicular Transport of YM155 into MDR1-Expressing Membrane Vesicles. Figure 4, A and B, shows the time profile of ATP-dependent transport of [^{14}C]YM155 at 10 μM into control and MDR1-expressing membrane vesicles. The uptake volume of [^{14}C]YM155 in the presence of ATP into MDR1-expressing membrane vesicles was higher than that in the absence of ATP, and ATP-dependent transport was higher than that observed in control membrane vesicles. The ATP-dependent and MDR1-mediated transport increased in a time-proportional manner, at least up to 0.5 min. Eadie-Hofstee plots for concentration-dependence of the 0.5-min transport of YM155 into MDR1-expressing membrane vesicles are shown in Fig. 4C. The K_m , V_{max} , and P_{dif} values were $64.4 \pm 20.9 \mu\text{M}$, $692.5 \pm 189.8 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$, and $1.90 \pm 0.39 \mu\text{l} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$, respectively. The inhibitory effect of CysA on YM155 transport was also evaluated. The MDR1-mediated transport of YM155 was inhibited by 20 μM CysA; when expressed as a percentage relative to the control experiment without CysA, it was 38.4%.

Figure 4D shows the effect of YM155 on the MDR1-mediated transport of *N*-methylquinidine. ATP-dependent transport was inhibited in the presence of

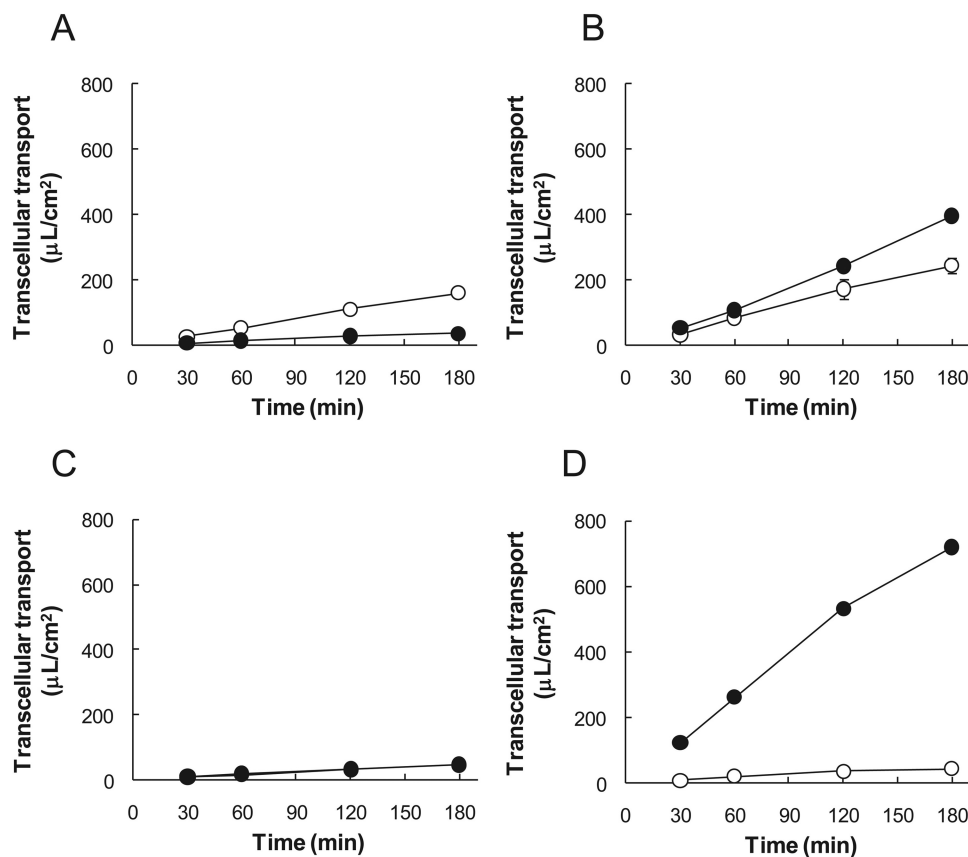


FIG. 1. Time profiles for the transcellular transport of [^{14}C]YM155 (1 μM) across mock- (A), OCT1- (B), MDR1- (C), and OCT1/MDR1-expressing LLC-PK1 (D) cells. ○, apical-to-basal flux; ●, basal-to-apical flux. Data are expressed as the mean \pm S.D. of triplicate experiments.

20 μM CysA, a positive control inhibitor, and the percentage of remaining activity relative to the control experiment was 9.08%. However, it was only slightly inhibited in the presence of YM155 up to 300 μM , and the percentage of remaining transport activity in the presence of 300 μM relative to the control experiments was approximately 50%.

Vesicular Transport of YM155 into MRP1-, MRP2-, MRP3-, and BCRP-expressing Membrane Vesicles. Vesicular transport of YM155 into MRP1-, MRP2-, MRP3-, and BCRP-expressing membrane vesicles is shown in Fig. 5. Transport activities of vesicles were confirmed using their typical substrates and inhibitors. No or only slight differences were noted in the ATP-dependent transport of their substrates in the presence or absence of 1

mM of YM155, suggesting that YM155 has no or very low affinity for these transporters (Supplemental Fig. 2). Thus, vesicular transport of [^{14}C]YM155 into these vesicles was conducted at 100 μM YM155. Compared with control vesicles, no ATP-dependent transport was observed in MRP3- or BCRP-expressing membrane vesicles. YM155 transport increased slightly in the presence of ATP in MRP1- and MRP2-expressing membrane vesicles.

Discussion

We investigated the interaction of YM155, a hydrophilic organic cation and novel small-molecule survivin suppressant, with MDR1 and

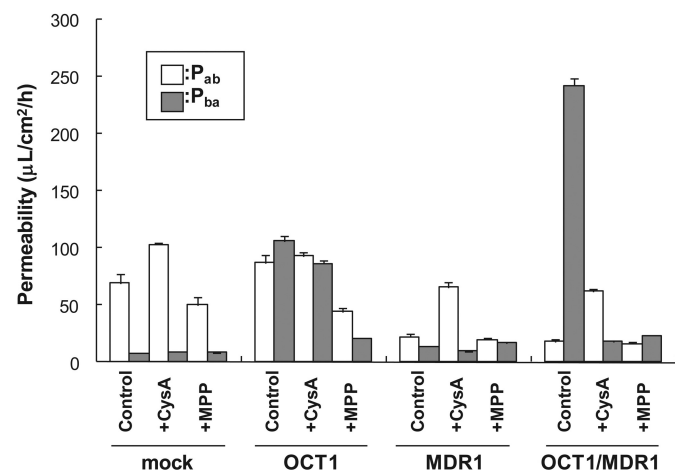


FIG. 2. Inhibitory effects of CysA (10 μM) and MPP (1 mM) on the transcellular transport of [^{14}C]YM155 (1 μM) across mock-, OCT1-, MDR1-, and OCT1/MDR1-expressing LLC-PK1 cell monolayers at 120 min. □, apical-to-basal flux; ■, basal-to-apical flux. Data are expressed as the mean \pm S.D. of triplicate experiments. Control experiments were performed in the absence of any inhibitors.

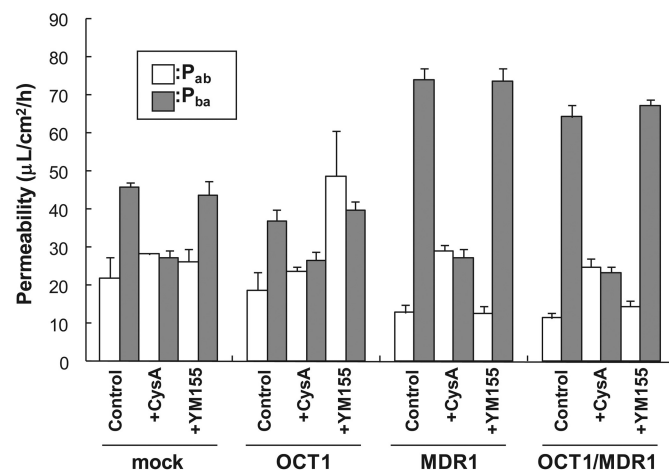


FIG. 3. Inhibitory effects of CysA (10 μM) and YM155 (100 μM) on the transcellular transport of [^3H]digoxin (1 μM) across mock-, OCT1-, MDR1-, and OCT1/MDR1-expressing LLC-PK1 cell monolayers at 120 min. □, apical-to-basal flux; ■, basal-to-apical flux. Data are expressed as the mean \pm S.D. of triplicate experiments. Control experiments were performed in the absence of any inhibitors.

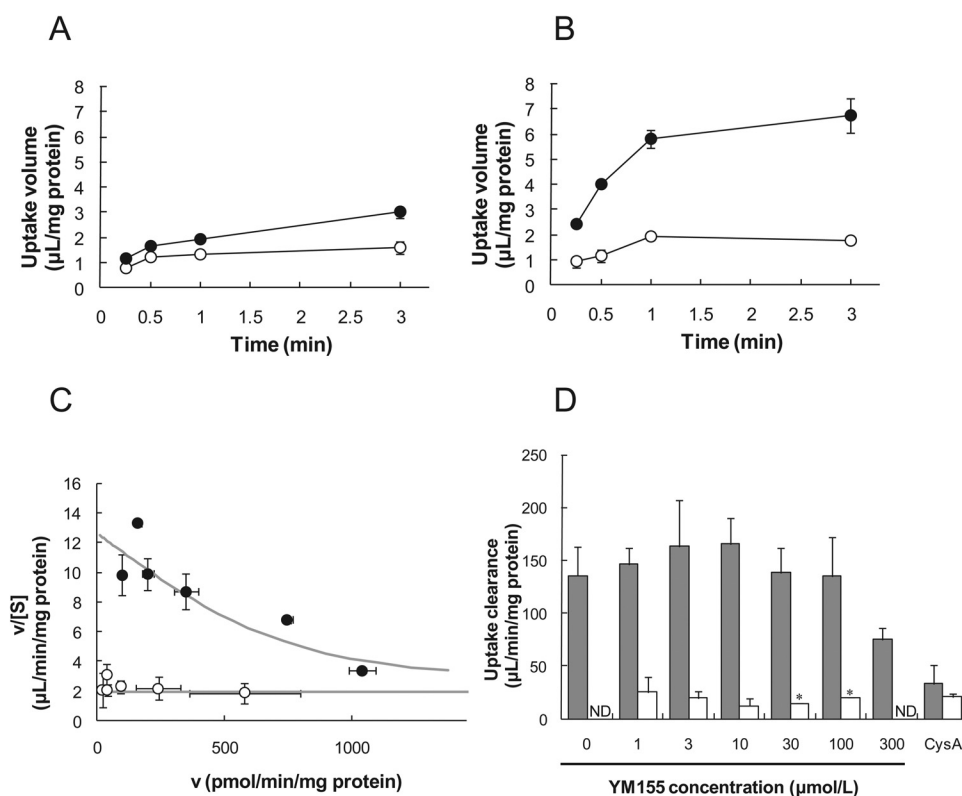


Fig. 4. Time profiles for the vesicular uptake of [14 C]YM155 (10 μ M) into control vesicles (A) and MDR1-expressing vesicles (B). Eadie-Hofstee plot of MDR1-mediated [14 C]YM155 uptake into membrane vesicles (0.5 min) (C) and inhibitory effect of YM155 and CysA (20 μ M) on MDR1-mediated transport of *N*-methylquinidine (5 μ M) (D). Data represent the uptake in the absence (○) or in the presence (●) of ATP. Data are expressed as the mean \pm S.D. of triplicate experiments. Gray lines were drawn using the estimated parameters (C). The uptake clearance was calculated in the presence (■) or absence (□) of ATP. Data are expressed as the mean \pm S.D. of triplicate experiments (D). ND, not detected (two or more samples were below detection limit). *, one sample was below detection limit, thus only mean values were calculated.

several other ABC transporters. First, Caco-2 cells and LLC-MDR1 cells were used, which are the transcellular transport system recommended by the U.S. Food and Drug Administration for assessing the interaction of a drug candidate with MDR1. In addition, we generated OCT1/MDR1 double-transfected LLC-PK1 cells, which express the basal YM155 uptake transporter OCT1 and apical MDR1. The interaction of YM155 with MDR1 and other efflux transporters was also evaluated using transporter-expressing membrane vesicles. Using the double-transfected cells and vesicular transport assay, we found that YM155 is a substrate of MDR1.

Nakahara et al. (2011) reported that MCF-7/MDR1 and MCF-7/mdr-1 cells, which express MDR phenotypes, were less sensitive to YM155-dependent cell growth inhibition than wild type MCF-7 cells, suggesting that YM155 is excreted by MDR1 in MCF-7/MDR1 and MCF-7/mdr-1 cells. We did not anticipate the results from Caco-2 and LLC-MDR1 cells, which suggest that YM155 is not transported by MDR1. In particular, the permeability of YM155 from the basal to apical side was similar to that in the opposite direction in both Caco-2 and LLC-MDR1 cells (Fig. 1C). In addition, MDCKII-MDR1 cells, which are another MDR1-expressing polarized cell line, showed a similar YM155 transport profile (data not shown).

In contrast, further studies using OCT1/MDR1 double-transfected LLC-PK1 cells allowed us to observe the transport of YM155 by MDR1. Uptake into cells is the first and rate-limiting step of transcellular transport of hydrophilic compounds with low membrane permeability, and uptake transporters are known to be important for transcellular transport of these compounds. Because OCT1 is involved in the hepatic uptake of YM155 (Iwai et al., 2009), we constructed OCT1/MDR1 double-transfected LLC-PK1 cells, which express OCT1 basolaterally and MDR1 apically, thus mimicking the system of biliary excretion of cations. In the transcellular transport study, the newly established LLC-OCT1/MDR1 cells clearly showed vectorial transport of YM155 (Fig. 1D), in contrast with LLC-MDR1 and Caco-2 cells. This vectorial transport was not observed in LLC-mock or LLC-OCT1 cells. The transport of YM155 in LLC-OCT1/MDR1

cells was inhibited by an MDR1 inhibitor (CysA) and an OCT1 inhibitor (MPP), indicating that YM155 is a dual substrate of both transporters and that expression of both transporters is indispensable for the vectorial transport of YM155 (Fig. 2). Limited expression of basal uptake transporters in Caco-2 cells and LLC-MDR1 cells may lead to insufficient interaction between YM155 and apical-expressed MDR1. Because most reported MDR1 substrates are hydrophobic and, therefore, can access the apical transporter by passive diffusion from the basal side, Caco-2 and MDR1 single-transfected cells can be used for their assessment (Gottesman and Pastan, 1993), in contrast to YM155. The inhibitory effect of YM155 on the MDR1-mediated transport of [3 H]digoxin was also evaluated; results showed no effect on transport even in the presence of 100 μ M of YM155, suggesting a low inhibitory effect of YM155 on MDR1-mediated transport (Fig. 3).

The vesicular transport experimental system was applied to examine efflux transporter-mediated transport of YM155. In a recently published review from the International Transporter Consortium, inside-out membrane vesicles prepared from transporter-expressing cell lines were regarded as another useful tool in evaluating the interaction between drug candidates and MDR1 and BCRP (Giacomini et al., 2010). However, a few reports have clearly shown the involvement of MDR1-mediated transport in the system because of the influence of high lipophilicity on high background values (Hooiveld et al., 2002). We therefore used the vesicular transport assay to evaluate the direct interaction between YM155 and MDR1. YM155 was taken up into MDR1 vesicles in an ATP-dependent manner, and the uptake volume was much higher than that in control vesicles (Fig. 4). The K_m value was estimated to be 64 μ M, which was much higher than the steady-state plasma concentration (21 nM) at the maximal tolerated dose (4.8 mg/m 2 per day) in a clinical phase I study (Tolcher et al., 2008). Using YM155 as an inhibitor, an inhibitory study on the MDR1 vesicular transport of *N*-methylquinidine also suggested low affinity of YM155 with a IC_{50} value exceeding 300 μ M, a finding consistent with that of the LLC-OCT1/MDR1 study using [3 H]digoxin as a substrate.

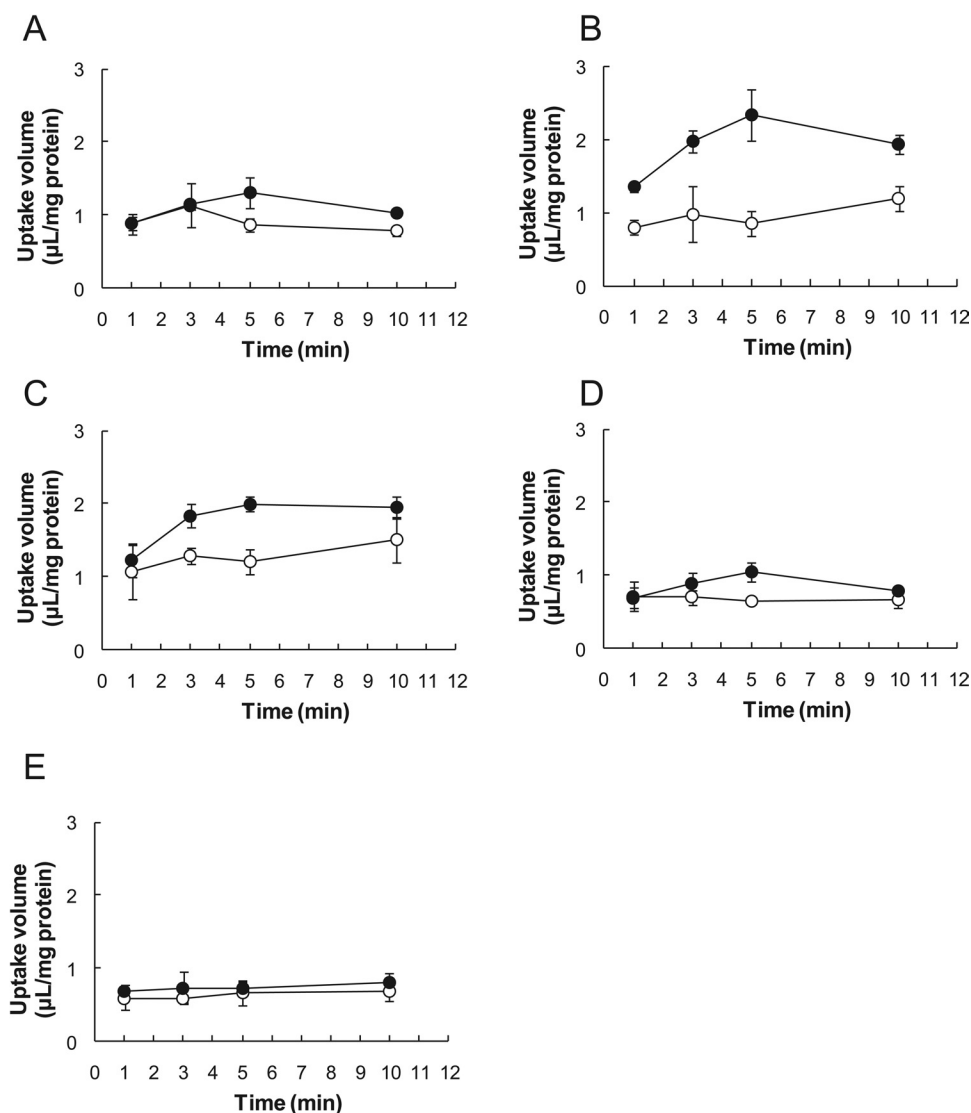


FIG. 5. Time profiles for the vesicular uptake of [14 C]YM155 (100 μ M) into control vesicles (A), MRP1- (B), MRP2- (C), MRP3- (D), and BCRP- (E) expressing vesicles. Data represent the uptake in the absence (○) or presence (●) of ATP. Data are expressed as the mean \pm S.D. of triplicate experiments.

These data suggested that there is an interaction between YM155 and MDR1. Because of the low affinity of YM155 to MDR1, we expect MDR1-mediated transport to be linear under clinical conditions, and that MDR1-mediated drug-drug interaction by YM155 as a MDR1 inhibitor cannot occur. OCT1 and MDR1 have been suggested to have sequential roles in hepatic uptake and biliary excretion of YM155 in the same way as LLC-OCT1/MDR1 cells under clinical conditions. Apart from biliary excretion, it is possible that MDR1 works coordinately with renally expressed OCT2 and unidentified cancer cation transporters in urinary excretion and tumor distribution, respectively. Furthermore, the limited distribution of YM155 into the brain in animal studies (T. Sonoda, unpublished observations) may partly be due to MDR1. In cancer cell lines, the overexpression of MDR1 may be a factor in the drug resistance of YM155 in MCF-7/MDR1 and MCF-7/mdr-1 (Nakahara et al., 2011). Presently, no data on drug resistance under clinical conditions are available.

The interaction of YM155 with other efflux transporters was also evaluated using the vesicular transport assays (Fig. 5). These transporters are known to play a role in the multidrug resistance of their substrate drugs. Furthermore, MRP2 and BCRP are expressed at the apical membrane of hepatocytes and excrete their substrates into bile. Whereas MRP3 and BCRP showed no ATP-dependent transport of YM155, slightly enhanced transport of YM155 in MRP1 and MRP2

vesicles was observed in the presence of ATP, although MDR1-mediated transport was the most significant. These findings suggest that YM155 may interact with MRP1 and MRP2 in addition to MDR1, although the interaction is weaker than that of MDR1. These transporters are also known to be expressed in excretory organs such as the liver and kidneys and in pharmacological target tissues of YM155, namely tumors. Further studies are necessary to elucidate the impact of these transporters on pharmacokinetics and the pharmacological effect of YM155.

In conclusion, we found that YM155 is transported by MDR1 and that MDR1 may play an important role in the pharmacokinetics of YM155. As in the case of Caco-2 cells and LLC-MDR1 cells, trans-cellular assays that lack a basal uptake transporter may lead to erroneous conclusions in the assessment of hydrophilic compounds such as YM155. To our knowledge, ours is the first study to use OCT1/MDR1 double-transfected cells and a vesicular transport system to assess the interaction of a drug candidate with MDR1. These assay methods are also applicable to drug metabolites, which are generally more hydrophilic than the parent drug.

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Authorship Contributions

Participated in research design: Iwai, Minematsu, Li, and Usui.

Conducted experiments: Iwai, Minematsu, and Li.

Performed data analysis: Iwai and Li.

Wrote or contributed to the writing of the manuscript: Iwai, Minematsu, Li, Iwatsubo, and Usui.

References

- Ahlin G, Hilgendorf C, Karlsson J, Szegedy CA, Uhlén M, and Artursson P (2009) Endogenous gene and protein expression of drug-transporting proteins in cell lines routinely used in drug discovery programs. *Drug Metab Dispos* **37**:2275–2283.
- Ambudkar SV, Dey S, Hrycyna CA, Ramachandra M, Pastan I, and Gottesman MM (1999) Biochemical, cellular, and pharmacological aspects of the multidrug transporter. *Annu Rev Pharmacol Toxicol* **39**:361–398.
- Balimane PV, Han YH, and Chong S (2006) Current industrial practices of assessing permeability and P-glycoprotein interaction. *AAPS J* **8**:E1–E13.
- Borst P and Elferink RO (2002) Mammalian ABC transporters in health and disease. *Annu Rev Biochem* **71**:537–592.
- Giaccone G, Zatloukal P, Roubec J, Floor K, Musil J, Kuta M, van Klaveren RJ, Chaudhary S, Gunther A, and Shamsili S (2009) Multicenter phase II trial of YM155, a small-molecule suppressor of survivin, in patients with advanced, refractory, non-small-cell lung cancer. *J Clin Oncol* **27**:4481–4486.
- Giacomini KM, Huang SM, Tweedie DJ, Benet LZ, Brouwer KL, Chu X, Dahlin A, Evers R, Fischer V, Hillgren KM, et al. (2010) Membrane transporters in drug development. *Nat Rev Drug Discov* **9**:215–236.
- Gottesman MM and Pastan I (1993) Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu Rev Biochem* **62**:385–427.
- Hayeshi R, Hilgendorf C, Artursson P, Augustijns P, Brodin B, Dehertogh P, Fisher K, Fossati L, Hovenkamp E, Korjamo T, et al. (2008) Comparison of drug transporter gene expression and functionality in Caco-2 cells from 10 different laboratories. *Eur J Pharm Sci* **35**:383–396.
- Hilgendorf C, Ahlin G, Seithel A, Artursson P, Ungell AL, and Karlsson J (2007) Expression of thirty-six drug transporter genes in human intestine, liver, kidney, and organotypic cell lines. *Drug Metab Dispos* **35**:1333–1340.
- Hooiveld GJ, Heegsma J, van Montfort JE, Jansen PL, Meijer DK, and Müller M (2002) Stereoselective transport of hydrophilic quaternary drugs by human MDR1 and rat Mdr1b P-glycoproteins. *Br J Pharmacol* **135**:1685–1694.
- Huang SM, Temple R, Throckmorton DC, and Lesko LJ (2007) Drug interaction studies: study design, data analysis, and implications for dosing and labeling. *Clin Pharmacol Ther* **81**:298–304.
- Ito K, Suzuki H, Hirohashi T, Kume K, Shimizu T, and Sugiyama Y (1998) Functional analysis of a canalicular multispecific organic anion transporter cloned from rat liver. *J Biol Chem* **273**:1684–1688.
- Iwai M, Minematsu T, Narikawa S, Usui T, and Kamimura H (2009) Involvement of human organic cation transporter 1 in the hepatic uptake of 1-(2-methoxyethyl)-2-methyl-4,9-dioxo-3-(pyrazin-2-ylmethyl)-4,9-dihydro-1H-naphtho[2,3-d]imidazolium bromide (YM155 monobromide), a novel, small molecule survivin suppressant. *Drug Metab Dispos* **37**:1856–1863.
- Kuteykin-Teplyakov K, Luna-Tortós C, Ambroziak K, and Löscher W (2010) Differences in the expression of endogenous efflux transporters in MDR1-transfected versus wildtype cell lines affect P-glycoprotein mediated drug transport. *Br J Pharmacol* **160**:1453–1463.
- Minematsu T, Iwai M, Sugimoto K, Shirai N, Nakahara T, Usui T, and Kamimura H (2009) Carrier-mediated uptake of 1-(2-methoxyethyl)-2-methyl-4,9-dioxo-3-(pyrazin-2-ylmethyl)-4,9-dihydro-1H-naphtho[2,3-d]imidazolium bromide (YM155 monobromide), a novel small-molecule survivin suppressant, into human solid tumor and lymphoma cells. *Drug Metab Dispos* **37**:619–628.
- Minematsu T, Iwai M, Umehara K, Usui T, and Kamimura H (2010) Characterization of human organic cation transporter 1 (OCT1/SLC22A1)- and OCT2 (SLC22A2)-mediated transport of 1-(2-methoxyethyl)-2-methyl-4,9-dioxo-3-(pyrazin-2-ylmethyl)-4,9-dihydro-1H-naphtho[2,3-d]imidazolium bromide (YM155 monobromide), a novel small molecule survivin suppressant. *Drug Metab Dispos* **38**:1–4.
- Mita AC, Mita MM, Nawrocki ST, and Giles FJ (2008) Survivin: key regulator of mitosis and apoptosis and novel target for cancer therapeutics. *Clin Cancer Res* **14**:5000–5005.
- Mita S, Suzuki H, Akita H, Hayashi H, Onuki R, Hofmann AF, and Sugiyama Y (2006) Vectorial transport of unconjugated and conjugated bile salts by monolayers of LLC-PK1 cells doubly transfected with human NTCP and BSEP or with rat Ntcp and Bsep. *Am J Physiol Gastrointest Liver Physiol* **290**:G550–G556.
- Nakahara T, Kita A, Yamanaka K, Mori M, Amino N, Takeuchi M, Tominaga F, Kinoyama I, Matsuhisa A, Kudou M, et al. (2011) Broad spectrum and potent antitumor activities of YM155, a novel small-molecule survivin suppressant, in a wide variety of human cancer cell lines and xenograft models. *Cancer Sci* **102**:614–621.
- Nakahara T, Takeuchi M, Kinoyama I, Minematsu T, Shirasuna K, Matsuhisa A, Kita A, Tominaga F, Yamanaka K, Kudou M, et al. (2007) YM155, a novel small-molecule survivin suppressant, induces regression of established human hormone-refractory prostate tumor xenografts. *Cancer Res* **67**:8014–8021.
- Nies AT, Herrmann E, Brom M, and Keppler D (2008) Vectorial transport of the plant alkaloid berberine by double-transfected cells expressing the human organic cation transporter 1 (OCT1, SLC22A1) and the efflux pump MDR1 P-glycoprotein (ABCB1). *Naunyn-Schmiedeberg Arch Pharmacol* **376**:449–461.
- Sasaki M, Suzuki H, Ito K, Abe T, and Sugiyama Y (2002) Transcellular transport of organic anions across a double-transfected Madin-Darby canine kidney II cell monolayer expressing both human organic anion-transporting polypeptide (OATP2/SLC21A6) and Multidrug resistance-associated protein 2 (MRP2/ABCC2). *J Biol Chem* **277**:6497–6503.
- Satoh T, Okamoto I, Miyazaki M, Morinaga R, Tsuya A, Hasegawa Y, Terashima M, Ueda S, Fukuoka M, Ariyoshi Y, et al. (2009) Phase I study of YM155, a novel survivin suppressant, in patients with advanced solid tumors. *Clin Cancer Res* **15**:3872–3880.
- Shirakawa K, Takara K, Tanigawara Y, Aoyama N, Kasuga M, Komada F, Sakaeda T, and Okumura K (1999) Interaction of docetaxel (“Taxotere”) with human P-glycoprotein. *Jpn J Cancer Res* **90**:1380–1386.
- Sohda K, Suzuki K, Nakamura E, Minematsu T, Hashimoto T, Imai H, Usui T, and Kamimura H (2007) Metabolism of YM155 monobromide, a novel survivin suppressant: in vitro species comparison and identification of metabolites in rats. *Drug Metab Rev* **39** (Suppl 1):235.
- Thiebaut F, Tsuruo T, Hamada H, Gottesman MM, Pastan I, and Willingham MC (1987) Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues. *Proc Natl Acad Sci USA* **84**:7735–7738.
- Tolcher AW, Mita A, Lewis LD, Garrett CR, Till E, Daud AI, Patnaik A, Papadopoulos K, Takimoto C, Bartels P, et al. (2008) Phase I and pharmacokinetic study of YM155, a small-molecule inhibitor of survivin. *J Clin Oncol* **26**:5198–5203.
- Zaffaroni N and Daidone MG (2002) Survivin expression and resistance to anticancer treatments: perspectives for new therapeutic interventions. *Drug Resist Updat* **5**:65–72.
- Zhang L, Zhang YD, Strong JM, Reynolds KS, and Huang SM (2008) A regulatory viewpoint on transporter-based drug interactions. *Xenobiotica* **38**:709–724.
- Zhou SF (2008) Structure, function and regulation of P-glycoprotein and its clinical relevance in drug disposition. *Xenobiotica* **38**:802–832.

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