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Effect of Probenecid on the Biliary Excretion of Belotecan

Eun-Mi Namkoong[1,2], In-Wha Kim[1,2], Dae-Duk Kim[1], Suk-Jae Chung[1], and Chang-Koo Shim[1,2]

[1]Research Institute of Pharmaceutical Sciences & Department of Pharmaceutics, College of Pharmacy, Seoul National University, Seoul 151-742, Korea and [2]National Research Laboratory for Transporters Targeted Drug Design, College of Pharmacy, Seoul National University, Seoul 151-742, Korea

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The purpose of this study was to investigate the effect of probenecid, an inhibitor of the MRP2/ABCC transporter, on the pharmacokinetics and transport of belotecan (7-[2-(*N*-isopropylamino)ethyl]-(20S)-camptothecin). The effect of probenecid on the pharmacokinetics of belotecan was studied in rats. When belotecan was injected as a bolus dose of 5 mg/kg after probenecid was infused at a rate of 42.8 mg/2 mL/h/kg, the cumulative biliary excretion amounts and biliary clearance (CL_b) of belotecan decreased (28.29 ± 2.83 versus 19.96 ± 1.45% of dose and 161.01 ± 26.95 versus 92.66 ± 1.45 mL/min/kg), whereas the systemic pharmacokinetics did not change. This indicates that the MRP2 transporter is involved in the biliary excretion of belotecan. The involvement of MRP2 in the secretory transport was further characterized using Caco-2 cell monolayers expressing MRP2. The apparent permeability across Caco-2 cell monolayers from basolateral to apical was 2.3 times greater than that from the apical to the basolateral side at the 50 μM belotecan. In addition, probenecid significantly decreased the basolateral-to-apical transport of belotecan (52.9%). These results indicate that MRP2 is involved in the secretory transport of belotecan in biliary excretion.

Key words: Belotecan, Biliary Excretion, Probenecid, Caco-2, MRP2

INTRODUCTION

Belotecan (7-[2-(*N*-isopropylamino)ethyl]-(20S)-camptothecin, Fig. 1), a semi-synthetic derivative of camptothecin, has recently developed by Chong Keun Dang Pharmaceutical Co. (Seoul, Korea) and is marketed in Korea as Camtobell®. Belotecan is an anti-cancer drug for the treatment of ovarian and small-cell lung cancers (Jew *et al.*, 1998, Lee *et al.*, 1998) based on a series of successful clinical studies (Lee *et al.*, 2000). 20-(s)-camptothecin (CPT), a plant alkaloid isolated from a tree found in China (*Camptotheca accuminata*), is a novel anti-tumor agent that exerts its activity exclusively by inhibiting topoisomerase I (Kim *et al.*, 1992, Slichenmyer *et al.*, 1993, Tanizawa *et al.*, 1994). The major toxicity of irinotecan, a derivative of camptothecin, is myelosuppression and diarrhea (Ohno *et al.*, 1994). Several reports showed that this diarrhea correlates with the biliary excretion of irinotecan and/or its metabolites (Gupta *et al.*, 1994, Takasuna *et al.*, 1998, 1995, 1996). It has been reported that the multi-drug resistance-associated protein 2 (MRP2) was responsible for the biliary excretion of the irinotecan and its metabolites (Chu *et al.*, 1997a, 1997b, 1998). In addition, the co-administration of probenecid, an MRP2 inhibitor, reduced irinotecan-induced late-onset toxicity in the gastrointestinal tissues due to inhibiting the biliary excretion of irinotecan and SN-38 (Horikawa *et al.*, 2002). It also has been reported that diarrhea is a side effect of belotecan (Lee *et al.*, 2000).

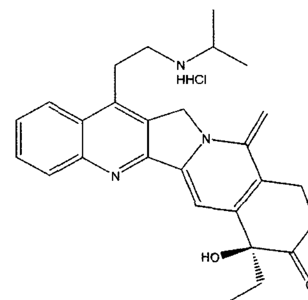

Fig. 1. Chemical structure of belotecan

Correspondence to: Chang-Koo Shim, Research Institute of Pharmaceutical Science and Department of Pharmaceutics, and National Research Laboratory for Transporters Targeted Drug Design, College of Pharmacy, Seoul National University, Seoul 151-742, Korea
Tel: 82-2-880-7873, Fax: 82-2-888-5969
E-mail: shimck@snu.ac.kr

Therefore, in this study our group investigated whether probenecid could also change the biliary excretion of belotecan and examined its mechanism using the human intestinal cell line Caco-2.

MATERIALS AND METHODS

Materials

Belotecan was kindly provided by Chong Kun Dang Pharm. (Seoul, Korea). Fetal bovine serum was purchased from Hyclone Laboratories (Logan, Utah, U.S.A.). Trypsin-EDTA was purchased from Gibco Laboratories (Gaithersburg, Md, U.S.A.). Dulbecco's Modified Eagle's medium, non-essential amino acid solution, L-glutamine, penicillin-streptomycin, Hank's balanced salt solution (HBSS), N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), probenecid, hexane-1-sulfonic acid, phosphoric acid, and N,N,N',N'-tetramethyl-ethylenediamine were purchased from Sigma Chemical Co. (St. Louis, Mo, U.S.A.). All other reagents were analytical grade.

Animals

Male Sprague-Dawley (SD) rats (Dae-Han Biolink, Eumsung, Korea), weighing 260-290 g, were used for the *in vivo* pharmacokinetic studies. All animal experiments were performed according to the guidelines in Animal Care and Use, Seoul National University.

Pharmacokinetics study

The SD rats were fasted overnight prior to the experiments; although they did have free access to water. The rats were anesthetized with ketamine (Yuhan Co., Seoul, Korea). The femoral vein and artery were cannulated with PE-50 polyethylene tubing (Clay Adams, Parsippany, N.J., U.S.A.), and the bile duct was cannulated with PE-10 polyethylene tubing. After the rats recovered from the anesthesia, probenecid dissolved in 100 mM phophosphate buffered saline (PBS) was infused into a femoral vein through a catheter at a rate of 42.8 mg/2 mL/h/kg. As a control experiment, PBS was administered in the same manner. One hour after starting the probenecid infusion, belotecan was injected as a bolus dose of 5 mg/kg. Blood samples (150 μL) were taken at 0.0833, 0.5, 1, 2, 3, 5, 8, and 12 h. The 3% mannitol as an intravascular expander was infused at a rate of 3 mL/min until the end of the experiments. Bile samples were collected at 1 h intervals. The concentration of belotecan in each sample was determined by HPLC.

Cell cultures

The human colonic epithelial cell line (Caco-2 cells) was obtained from American Type Culture Collection (Rockville, Md, U.S.A.). Cells were routinely grown in Dulbecco's Modified Eagle's medium containing 10% fetal bovine serum, a 1% non-essential amino acid solution, 100 units/mL penicillin and 0.1 mg/mL streptomycin at 37°C in an atmosphere of 5% CO_2 and 90% relative humidity. The Caco-2 cells from passage numbers 45 to 55 were seeded on the permeable polycarbonate filter insert (1 cm^2, 0.4 mm pore size: Corning Costar Corp., Cambridge, Mass, U.S.A.) in 12-Transwell plates at a density of $1.3\text{-}1.5\times10^5$ cells/insert and were grown for 18-25 days (Li *et al.*, 2001). The integrity of the cell monolayers was evaluated prior to the transport experiments by measuring transepithelial electrical resistance (TEER) and/or [^{14}C]mannitol permeability across the monolayers. TEER values in the range of 300-500 $\Omega\cdot cm^2$ cell monolayers or the transport permeability values of [^{14}C]mannitol $< 1\times10^{-6}$ cm/sec were used in the transport experiment.

Transport study

Before the transport experiment, the cell monolayers were washed three times with an incubation medium (pH 7.4, HBSS containing 25 mM HEPES and 25 mM glucose). After each wash, the plates were incubated in the incubation medium for 30 min at 37°C, and the TEER value was then measured (Li *et al.*, 2001). For the measurement of the apical to the basolateral (AP-BL, absorptive) transport, 0.5 mL of the incubation medium containing 50 μM and 100 μM belotecan was added on the apical side of the cell monolayers in the presence or absence of 1 mM and 5 mM probenecid, and 1.5 mL of the incubation medium without the drug was added on the basolateral side. The inserts were moved to wells containing fresh incubation medium (1.5 mL) at appropriate intervals over a 2 h period. At each time point (i.e., 30, 60, 90, and 120 min), a 1 mL aliquot of the incubation medium was taken from the basolateral side, and the concentration of the drug in each sample was determined by HPLC. For the measurement of the basolateral to the apical transport (BL-AP, secretory), 1.5 mL of the incubation medium containing 50 and 100 μM of belotecan was added on the basolateral side in the presence or absence of 1 mM and 5 mM probenecid, and 0.5 mL of the incubation medium without the drug was added on the apical side. A 0.3 mL aliquot of the incubation medium in the apical side was taken at appropriate intervals over a 2 h period, and was replaced with 0.3 mL of fresh incubation medium. The concentration of the drug in each sample was determined by HPLC.

HPLC analysis

The concentration of belotecan in the sample was determined by a modified reversed phase HPLC method (Cho *et al.*, 2003; Vali *et al.*, 2005). For the analysis of plasma samples, an aliquot of 50 μL of the sample was spiked with 50 μL of 0.25% (w/v) tetracycline (in acetonitrile), 50

μL acetonitrile and 50 μL of 7% (w/v) perchloric acid and mixed. The mixture was then centrifuged, and 100 μL of the supernatant was injected for HPLC analysis. To analyze the samples from the transport studies, 100 μL of the samples were acidified with 200 μL of phosphoric acid (30%, w/v). Acid (perchloric acid for plasma samples and phosphoric acid for transport samples) was added to ensure the conversion of each drug to its respective lactone form. HPLC was performed using a C_{18} column (XTerra™, 250 mm × 4.6 mm, 5 μm, Waters, Milford, Mass, U.S.A.). The mobile phase was a mixture of methanol, 0.1M hexane-1-sulfonic acid in methanol, and 0.01M N,N,N',N'-tetramethyl-ethylenediamine in purified water at pH 6.0 adjusted with phosphoric acid (40:1:59, v/v/v). The flow rate was maintained at 1 mL/min. A Shimazu RF-535 fluorescence detector was operated at an excitation wavelength of 370 nm and an emission wavelength of 430 nm.

Data analysis

The area under the plasma concentration time curve from zero to infinity ($AUC_{0\text{-}\infty}$) was calculated by the trapezoidal rule, followed by an extrapolation method using Winnonlin 3.1 (Pharsight, Cary, N.C., U.S.A.). To determine the elimination clearance (CL) and apparent volume of distribution at a steady state (V_{ss}) for belotecan, Eqs. (1) and (2) were then used.

$$CL = Dose/AUC \quad (1)$$

$$V_{ss} = MRT \cdot CL \quad (2)$$

$$MRT = AUMC/AUC \quad (3)$$

To determine the biliary clearance (CL_b), the cumulated amount in bile (X_b) was calculated and used to calculate the biliary clearance (CL_b) by Eq. (4).

$$CL_b = X_b/ AUC \quad (4)$$

For each transport assay, the mean transport rate was calculated from the linear portion of a plot of the total amount of drug transported versus time. The apparent permeability values, P_{app}, of the drug across the Caco-2 cell monolayers, expressed as cm/s, were calculated as $\Delta Q/\Delta t \times 1/60 \times 1/A \times 1/C_0$, where $\Delta Q/\Delta t$ is the permeability rate (mmole/min); A is the surface area of the membrane (cm^2, 1 in the present study); and C_0 is the initial concentration of the drug in the donor chamber (mmole/mL) (Artursson *et al.*, 1991). An efflux ratio was used to represent the extent of efflux and was calculated according to the following equation: efflux ratio = $P_{app(basolateral\ to\ apical)}/P_{app(apical\ to\ basolateral)}$. All data are expressed as the mean ± S.D. of the three experiments. The statistical significance of differences between treatments was evaluated using unpaired Student's *t* tests, with a value of $p<0.05$ considered statistically significant.

RESULTS AND DISCUSSION

Pharmacokinetics of belotecan

Temporal profiles for the plasma concentration of belotecan after it was administered intravenously to rats at a dose of 5 mg/kg are shown in Fig. 2. The profile of the drugs followed a multi-exponential decline after the *IV* administration. The pharmacokinetic parameters are summarized in Table I. Probenecid did not affect the systemic pharmacokinetic profile of belotecan. However, the mean accumulation amounts of belotecan in bile and biliary clearance (CL_b) decreased in rats treated with probenecid compared to control rats (28.29 ± 2.83 vs 19.96 ± 1.45% of dose and 161.01 ± 26.95 vs 92.66 ± 3.97 mL/min/kg, Fig. 3. and Table I). This suggests that the excretion of this compound across the bile canalicular membrane is inhibited by probenecid and the primary active transport of this compound, which is mediated by MRP2. This result is consistent with probenecid reducing the biliary excretion of irinotecan

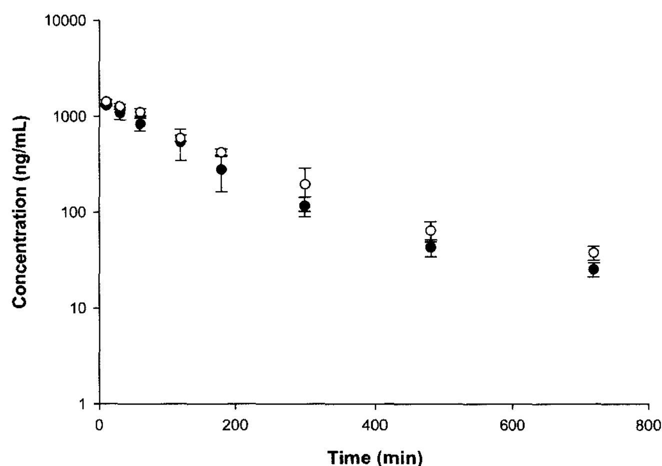

Fig. 2. Effect of probenecid on the plasma concentration of belotecan. Probenecid was administered intravenously at a rate of 0 (closed symbol) or 42.8 mg/h/kg (open symbol), and belotecan was administered intravenously as a dose of 5 mg/kg bolus. Each data point represents the mean ± S.D. of three different rats.

Table I. The effect of probenecid on the pharmacokinetics of belotecan after IV administration (5 mg/kg)

Pharmacokinetic parameters	Control	Treatment
$AUC_{0\text{-}\infty}$ (ng·min/mL)	183300 ± 29360	238600 ± 25180
CL_t (mL/min/kg)	27.78 ± 4.70	21.11 ± 2.10
V_{ss} (mL/kg)	4214 ± 675	3509 ± 57
MRT (min)	152.05 ± 9.17	167.3 ± 16.32
Cumulative biliary excretion (% of dose)	28.29 ± 2.83	19.96 ± 1.45*
CL_b (mL/min/kg)	161.01 ± 26.95	92.66 ± 3.97*

Data represents the mean ± S.D. of three different rats.
*$p<$ 0.05, significantly different from the control.

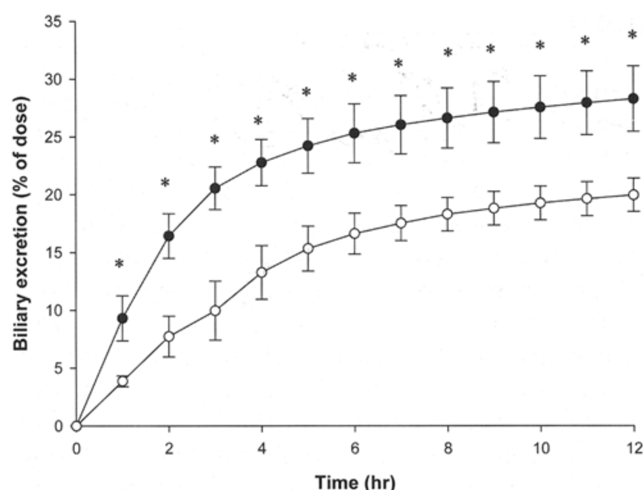

Fig. 3. Effect of probenecid on the biliary excretion of belotecan. Probenecid was administered intravenously at a rate of 0 (closed symbol) or 42.8 mg/h/kg (open symbol) and belotecan was administered intravenously as a dose of 5 mg/kg bolus. Each data point represents the mean ± S.D. of three different rats *$p<0.05$, significantly different from the control.

(Horikawa *et al.*, 2002). The reason for no change in the AUC seems to be the inhibition of the metabolic pathway or decrease in urinary excretion of belotecan by probenecid like irinotecan and SN-38 (Horikawa *et al.*, 2002).

Transport of belotecan in Caco-2 cell monolayers

To understand the involvement of the transporter in the efflux of belotecan, an *in vitro* cell monolayer system, Caco-2 cell monolyers, was used (Sha *et al.*, 2004). The transport of belotecan, both in the apical to the basolateral (AP-BL, absorptive) and in the basolateral to the apical (BL-AP, secretory) directions, was determined as a function of time. The transport of belotecan was observed to be much faster in the BL-AP direction compared to the AP-BL direction (Fig. 4). The apparent permeability coefficient of belotecan in the BL-AP direction ($13.1 \pm 0.574 \times 10^{-6}$ cm/s) was significantly greater than that in the AP-BL direction ($5.62 \pm 0.26 \times 10^{-6}$ cm/s) at a concentration of 50 μM. The ratio of $P_{appBL\text{-}AP}/P_{appAP\text{-}BL}$ of belotecan was 2.3 at a concentration of 50 μM, and decreased to 1.5 at a concentration of 100 μM (Table II). This suggests that the carrier-mediated system is involved in the transport of belotecan in Caco-2 cells.

Effect of the inhibitors on transport of belotecan

It is well known that MRP2 is expressed in the apical membrane of Caco-2 cells and plays a role in secreting a variety of xenobiotics into the lumen (Prime-Chapman *et al.*, 2004, Taipalensuu *et al.*, 2001). The relationship between the active transport of belotecan and the MRP2 transporter was examined using Caco-2 cell monolayers.

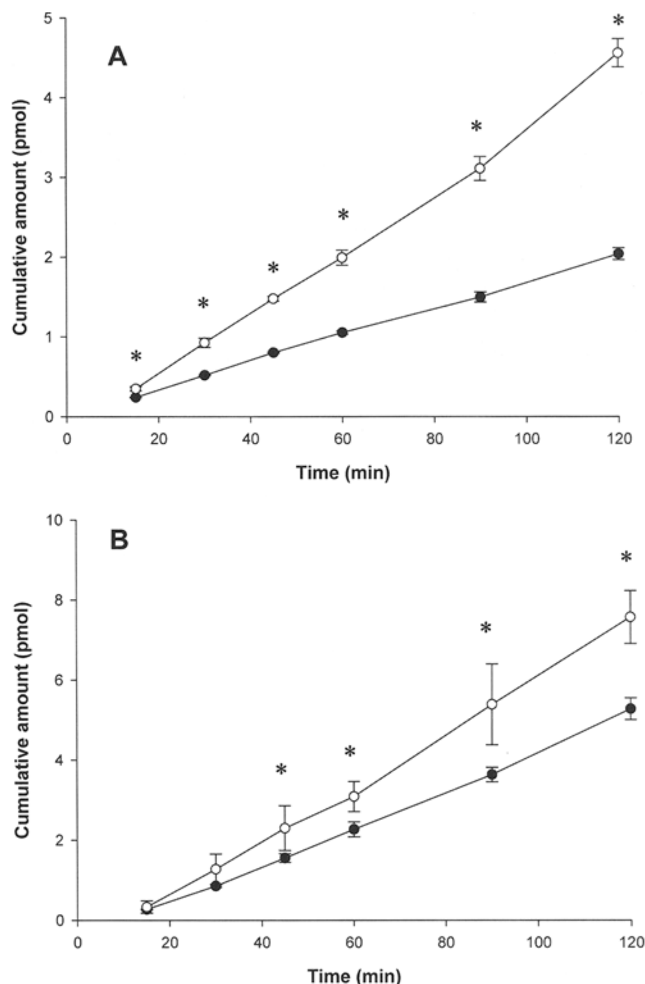

Fig. 4. Cumulative amount of belotecan found at the receiver side as a function of time in the apical to basolateral (AP to BL, ●) or basolateral to apical (BL to AP, ○) transport of belotecan. The concentration of belotecan in the transport studies was 50 μM (A) and 100 μM (B). Each point represents the mean ± S.D. of three monolayers.

For this purpose, the bidirectional transport of belotecan (50 and 100 μM) was examined in the absence and presence of an MRP2 inhibitor-probenecid (1 and 5 mM) on both the apical and basal sides. The presence of the above MRP2 inhibitor had no significant effect on the TEER values for the Caco-2 cell monolayers. The probenecid (5 and 1 mM) reduced the BL-AP flux of belotecan to 47.1% and 64.4% of control at the concentration of 50 and 100 μM, respectively (Table II). The ratio of $P_{appBL\text{-}AP}/P_{appAP\text{-}BL}$ of belotecan at a concentration of 50 μM significantly decreased in the presence of 5 mM probenecid (2.3 to 1.7). This suggests that belotecan transports across Caco-2 cell monolayers via MRP2 and that an active carrier-mediated active mechanism is operative. On the other hand, the AP-BL fluxes of belotecan at concentrations of 50 and 100 μM also decreased when probenecid was added (5 and 1 mM) by 64.6% and 83.2% of control,

Table II. The effect of probenecid on the transport of belotecan across Caco-2 cell monolayers

	$P_{app} \times 10^6$ (cm/s)		Ratio BL-AP/ AP-BL	AP-BL permeation (% of control)	BL-AP permeation (% of control)
	AP-BL	BL-AP			
Belotecan (50 μM)	5.62 ± 0.259	13.1 ± 0.796*	2.33	-	-
Belotecan (100 μM)	7.94 ± 0.299	11.5 ± 1.05*	1.45	-	-
Belotecan (50 μM) + Probenecid (5 mM)	3.57 ± 0.530	6.17 ± 0.574*	1.73	64.6 ± 7.48	47.1 ± 6.44*
Belotecan (100 μM) + Probenecid (1 mM)	6.61 ± 0.584	7.26 ± 1.85	1.10	83.2 ± 7.25	64.4 ± 4.76*

Data represents the mean ± S.D. of three monolayers.
*$p < 0.05$, significantly different from apical to basolateral permeation.

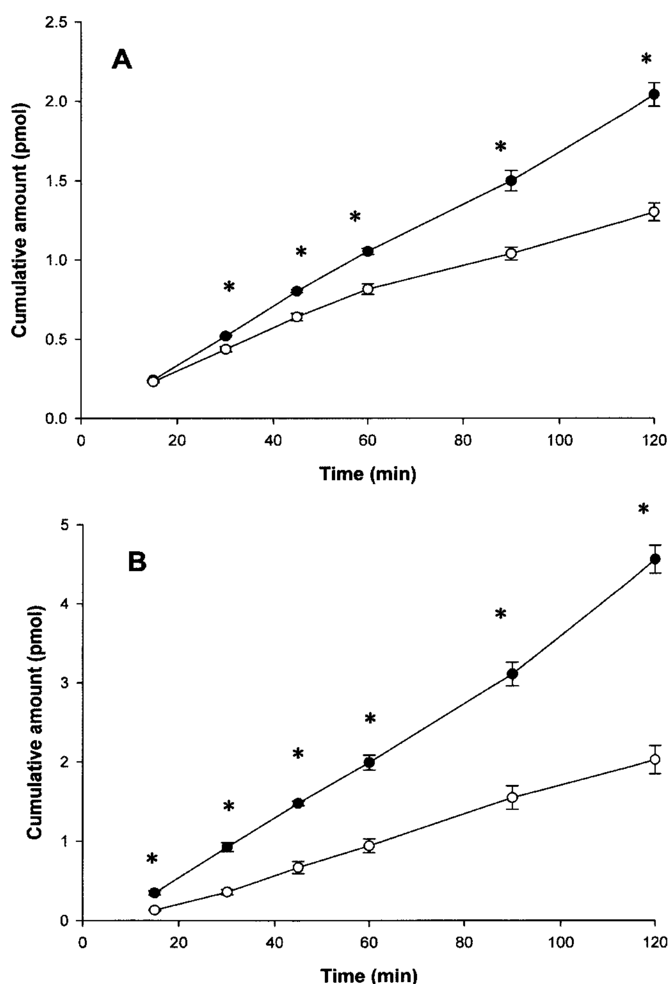

Fig. 5. Effect of probenecid on belotecan permeation across Caco-2 cell monolayers. Apical to basolateral (A) and basolateral to apical (B) permeation of 50 μM belotecan was investigated in the presence (○) and absence (●) of 5 mM probenecid. Each point represents the mean ± S.D. of three monolayers. *p<0.05, transport flux (with inhibitor) is significantly different from the control (without inhibitor).

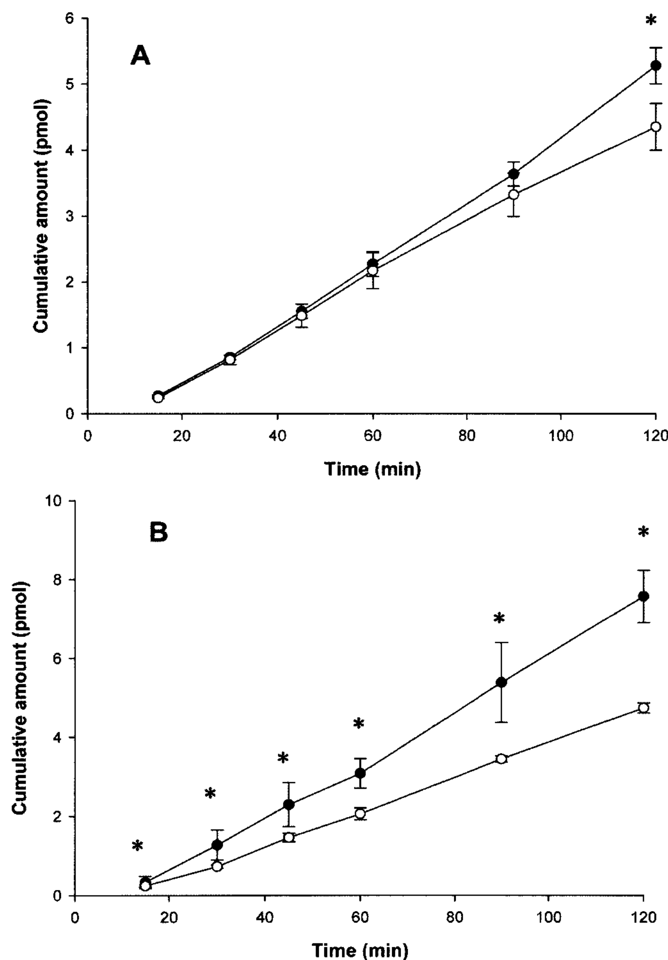

Fig. 6. Effect of probenecid on belotecan permeation across Caco-2 cell monolayers. Apical to basolateral (A) and basolateral to apical (B) permeation of 100 μM belotecan was investigated in the presence (○) and absence (●) of 1 mM probenecid. Each point represents the mean ± S.D. of three monolayers. *p<0.05, transport flux (with inhibitor) is significantly different from the control (without inhibitor).

respectively. This result was unexpected. It suggests that belotecan and probenecid share the absorptive transporter expressed in Caco-2 cell monolayers.

Information about the relationship between transporters and new drug candidates is important for improvements in drug delivery and drug design. It is thought that the regulation of transporter functions will enable the development of highly efficient drugs with ideal pharmacokinetic profiles. Our group examined the effect of probenecid on

the hepatobiliary transport of belotecan in rats and Caco-2 cell monolayers. Probenecid inhibited biliary excretion of belotecan via an MRP2 transporter, suggests that it could be used to reduce side effects, such as diarrhea. In addition, probenecid inhibited both the secretive and absorptive transport of belotecan in Caco-2 cell monolayers. Further studies are needed to elucidate the transport mechanism's contribution to the absorption of belotecan.

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