Pharmacokinetic Characterization of Transcellular Transport and Drug Interaction of Digoxin in Caco-2 Cell Monolayers

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To characterize the intestinal absorption of digoxin, its transcellular transport and drug interaction activity was investigated using Caco-2 cell monolayers. We examined digoxin transport in the presence and absence of ouabain to determine whether digoxin binding to Na^+,K^+-ATPase affects its transcellular digoxin transport, and evaluated its influx and efflux clearance by model-dependent pharmacokinetic analysis. Transcellular transport in the basal-to-apical direction was greater than that in the opposite direction. In addition, ouabain decreased the cellular accumulation of digoxin, but it did not alter its transcellular transport profile. The observations for transcellular transport and cellular accumulation in the presence of ouabain were used for the pharmacokinetic analysis, which showed that the efflux clearance of digoxin on the apical side of the monolayer was 15 times greater than that on the basal side. Apical-to-basal transport was increased by carvedilol and pimobendan, and these compounds suppressed the efflux clearance on the apical side and the influx clearance on the basal side. These findings indicate that the intestinal absorption of digoxin is primarily dominated by the efflux process on the luminal side of the intestine, and that carvedilol and pimobendan may vary the rate of intestinal digoxin absorption mainly by inhibiting its exsorptive transport.

Key words digoxin; intestinal absorption; carvedilol; pimobendan; Caco-2 cell monolayer

The cardiac glycoside digoxin is one of the most commonly used compounds for treating congestive heart failure, and its treatment efficacy is maximal at a serum concentration of 0.8—2.0 ng/ml.[1,2] However, the margin between its effective and toxic doses is narrower and less well defined than those of other therapeutic compounds.[1—3] In some cases, digoxin toxicity is readily perceived even at a concentration of less than 2 ng/ml. It is therefore recommended that the digoxin dosage should be carefully determined according to the patient's clinical conditions and other medications simultaneously being administered with the digoxin treatment.

The serum digoxin concentration is increased by various therapeutic compounds. Some such as carvedilol interact with digoxin,[4] whereby the plasma digoxin concentration is increased when carvedilol and digoxin are orally administered, while carvedilol does not affect the digoxin concentration when digoxin is intravenously administered.[4] Erythromycin,[5] clarithromycin[5—7] and talinolol[8] also interact in a similar way with digoxin. Together, these observations suggest that the increased digoxin concentration is not due to a decrease in its renal excretion, but rather an increase in bioavailability, and that the increased bioavailability is probably a consequence of these compounds enhancing the intestinal digoxin absorption.[9,10] Therefore, in the present study we characterized the intestinal absorption of digoxin to elucidate the factor which is primarily responsible for digoxin bioavailability. We also examined whether cardiovascular compounds such as the β-blocking agent carvedilol and the inotropic agent pimobendan affect its intestinal absorption.

During intestinal drug absorption, therapeutic compounds first enter intestinal epithelial cells from their apical side, then pass through the epithelia to the basal side, and finally appear in the blood stream. Therefore, to investigate intestinal drug absorption, it is important to separately assess these sequential processes. One useful approach to studying intestinal drug absorption is to use Caco-2 cells monolayers, which can be prepared on a porous filter as a polarized monolayer.[11—15] The drug concentrations on the apical and basal sides of the monolayer can be readily determined along with the intracellular drug accumulation. The directional specificity of transcellular digoxin transport in Caco-2 cells has been mentioned in various reports.[16,17] However, in many cases, digoxin transport on the apical and basal sides of the monolayer were not separately examined, and the influx and efflux clearance rates of digoxin were not evaluated.[16,17]

For the characterization of transcellular drug transport, a pharmacokinetic approach is useful.[18—20] Transcellular drug transport in LLC-PK_1 and OK cell monolayers can be analyzed in detail in a model-dependent manner,[18,19] where their drug concentration–time profiles on both sides of the monolayer can be assessed by curve fitting calculations and the influx and efflux clearance of the monolayer separately evaluated. Thus, when transcellular drug transport is examined under the condition where the unlabeled drug concentration in the monolayer is equilibrated with that of the incubation medium in the apical and basal chambers, the transport data for a small amount of radio-labeled drug can be analyzed using a linear pharmacokinetic model.[15,18]

MATERIALS AND METHODS

Materials Radio-labeled digoxin ([^{3}H]-digoxin, 1.37 TBq/mmol) and mannitol ([1-^{14}C]-D-mannitol, 1.96 GBq/mmol) were purchased from PerkinElmer (Boston, MA, U.S.A.) and Moravek Biochemicals (Brea, CA, U.S.A.), respectively, and unlabeled digoxin and ouabain were obtained from Nacalai Tesque (Kyoto, Japan). Quinidine was obtained from Sigma (St. Louis, MO, U.S.A.), and carvedilol was supplied by Daiichi Pharmaceutical (Tokyo, Japan), and pimobendan (Acardi Capsule® 1.25) was purchased from Nip-

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pon Boehringer Ingelheim (Kawanishi, Japan) and further purified by ethanol extraction. All other chemicals were of the finest grade available.

Cell Culture and Preparation of Monolayers Caco-2 cells at passage number 40 were obtained from the Riken Bioresource Center (Tsukuba, Japan), and were maintained in plastic dishes with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Valley Biochemical Inc., Winchester, VA, U.S.A.) in an atmosphere of 5% CO_2–95% air at 37 °C. All experiments were carried out with cells between passages 48—73. The medium was changed every second or third day, and when the cells reached 80—90% confluence they were removed using a 0.05% trypsin/0.02% EDTA solution. They were then washed with phosphate buffered saline and seeded at 5×10^5 cells/cm^2 on a 0.9-cm^2 porous membrane in a Falcon^{TM} cell culture insert (BD Biosciences, Bedford, MA, U.S.A.). The pore size of the membrane was 0.4 μm in diameter and the pore density was 1.6×10^6/cm^2. The seeded cells were maintained for 3 weeks to prepare the cell monolayers. One milliliter of the culture media was supplied to the chamber on the apical side of the monolayer (the apical chamber), and 2-ml was supplied to the chamber on the other side, the basal chamber. The culture medium was changed every other day, and maturity of the monolayer was judged by transepithelial electrical resistance (TEER). TEER was measured using a Millicell-ERS resistance system (Millipore, Bedford, MA, U.S.A.). Caco-2 cell monolayers whose TEER was above 900 $\Omega\cdot$cm^2 were used for experiments.

Transport of Digoxin and Mannitol in Caco-2 Cell Monolayers The transcellular transport of digoxin in Caco-2 cell monolayers was examined according to a previously reported method.[18,19] All transport experiments were conducted in an atmosphere of 5% CO_2–95% air at 37 °C. In brief, the monolayer was first pre-incubated with the experiment medium in the apical and basal chambers to equilibrate the digoxin concentration. The experiment medium was composed of cell culture medium containing unlabeled digoxin at 10 nM. After a 60 min equilibration period, ^{3}H-digoxin was applied to the apical chamber to examine the apical-to-basal transcellular transport of digoxin. The medium in the basal chamber was then collected after 60, 120 and 180 min. Following the last medium collection, the porous membrane on which the Caco-2 cell monolayer was prepared was immediately washed three times with ice-cold phosphate buffer, and then the cells were removed and collected. The Caco-2 cells were used to evaluate digoxin accumulation, where the amounts of ^{3}H-digoxin were determined using a liquid scintillation counter and normalized against the initially applied doses. The profile for digoxin transport in the opposite direction (basal-to-apical) was also examined in the same manner. To evaluate the paracellular transport of digoxin, mannitol transport was examined using ^{14}C-mannitol.

To determine whether digoxin binding to Na^+,K^+-ATPase affects the transcellular transport of digoxin, another series of experiments as described above was carried out in the presence of ouabain, which was dissolved in the experiment medium in the apical and basal chambers at a final concentration of 100 μM 60 min before the addition of ^{3}H-digoxin.[21]

The inhibitory effects of carvedilol and pimobendan on transcellular digoxin transport were examined in the same manner as described above, for which carvedilol was dissolved in the experiment medium in the apical and basal chambers at a final concentration of 1 or 5 μM 60 min before the addition of ^{3}H-digoxin. Pimobendan was used at a final concentration of 5 or 50 μM. For the inhibition study, we used quinidine as a positive inhibition control, since it potently inhibits P-glycoprotein.[22] Quinidine was dissolved in the experiment medium at a concentration of 5 μM.

Estimation of Cell Volume of the Caco-2 Cell Monolayer The volume of the monolayer was calculated from the amount of sulfanilamide that accumulated in the monolayer by simple diffusion under equilibrium conditions.[23] Briefly, the Caco-2 cell monolayer was incubated for 60 min in 2 mg/ml sulfanilamide solution, and washed three times with ice-cold phosphate buffer, and the monolayer was collected to be homogenized. The sulfanilamide concentration in the homogenized sample was spectrophotometerically determined at 550 nm after diazotization.

Pharmacokinetic Analysis The transcellular transport of digoxin was analyzed in a model-dependent manner using the method of non-linear least squares.[18—20] Assuming that digoxin was transported as shown in Fig. 1, the following mass balance equations were prepared for the pharmacokinetic analysis:

$$\frac{dX_A}{dt} = -\frac{CL_{AC}}{V_A}\cdot X_A + \frac{CL_{CA}}{V_C}\cdot X_C - \frac{CL_{PARA}}{V_A}\cdot X_A + \frac{CL_{PARA}}{V_B}\cdot X_B \quad (1)$$

$$\frac{dX_B}{dt} = -\frac{CL_{BC}}{V_B}\cdot X_B + \frac{CL_{CB}}{V_C}\cdot X_C + \frac{CL_{PARA}}{V_A}\cdot X_A - \frac{CL_{PARA}}{V_B}\cdot X_B \quad (2)$$

$$\frac{dX_C}{dt} = \frac{CL_{AC}}{V_A}\cdot X_A + \frac{CL_{BC}}{V_B}\cdot X_B - \frac{(CL_{CB}+CL_{CA})}{V_C}\cdot X_C \quad (3)$$

where X_A, X_B and X_C are the amount of digoxin in the apical chamber, the basal chamber and the monolayer determined at time t, respectively. V_A and V_B indicate the volume of the api-

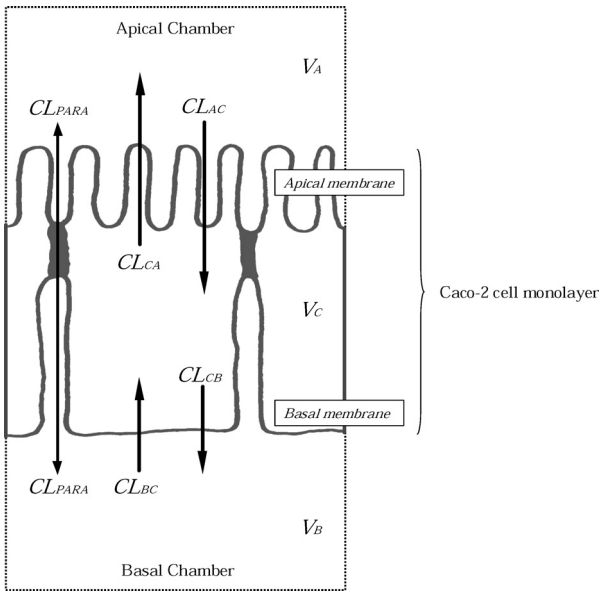

Fig. 1. Schematic of the Three-Compartment Model Used to Analyze Transcellular Digoxin Transport in Caco-2 Cell Monolayers

Digoxin influx and efflux clearance on the apical side of the monolayer are designated CL_{AC} and CL_{CA}, respectively, and influx and efflux clearance on the basal side are designated CL_{BC} and CL_{CB}, respectively. CL_{PARA} stands for the paracellular clearance of digoxin. The volumes of the apical and the basal chambers and the intracellular volume of the monolayer are indicated by V_A, V_B and V_C, respectively.

cal and basal chambers, respectively, and the distribution volume of digoxin in the monolayer is designated V_C. The influx and efflux clearance of digoxin on the apical side of the monolayer are designated CL_{AC} and CL_{CA}, respectively, and these clearance parameters for the basal side of the monolayer are designated CL_{BC} and CL_{CB}, respectively. Paracellular transport clearance is denoted as CL_{PARA}.

Paracellular clearance of digoxin (CL_{PARA}) was estimated by analyzing the mannitol transport profile using the mass balance equations described below:

$$\frac{dX_{MA}}{dt} = -\frac{CL_{PARA}}{V_A} \cdot X_{MA} + \frac{CL_{PARA}}{V_B} \cdot X_{MB} \quad (4)$$

$$\frac{dX_{MB}}{dt} = \frac{CL_{PARA}}{V_A} \cdot X_{MA} - \frac{CL_{PARA}}{V_B} \cdot X_{MB} \quad (5)$$

where X_{MA} and X_{MB} are the amount of mannitol in the apical and basal chambers determined at time t, respectively.

The calculations were conducted using the UCSF NONMEM program,[24] and with the POSTHOC directive option the clearance parameters for each experiment were obtained by Bayesian estimation.

Data Analysis Data were expressed as means±S.E. derived from 6 experiments. For multiple comparisons against a single control group, Bartlett's test for homogeneity of the variances was first performed. Then, significant differences were evaluated by analysis of the variance followed by Dunnett's test. If homogeneity of the variance could not be assumed, significance was evaluated by Kruskal–Wallis nonparametric analysis of the variance followed by Dunnett-type test. For the comparison of two means, Student's t-test was used to judge the significance, where $p<0.05$ was considered to be statistically significant.

RESULTS

Transcellular Transport and Cellular Accumulation of Digoxin in Caco-2 Cell Monolayers in the Presence and Absence of Ouabain We first examined the transcellular transport of digoxin in Caco-2 cell monolayers at a digoxin concentration of 10 nM in the presence and absence of 100 μM ouabain, and determined whether digoxin binding to Na^+,K^+-ATPase affected the transcellular digoxin transport. As shown in Fig. 2A, digoxin was transported in a direction-specific manner, where the amount of digoxin transported in the basal-to-apical direction within 180 min was 6—7 times greater than that transported in the opposite direction (Fig. 2A). In addition, the digoxin transport profiles for 100 nM were almost identical to those examined for 10 nM, indicating that digoxin transport was not concentration-dependent over this concentration range (data not shown). Ouabain did not affect the amount of digoxin transported in either direction (Fig. 2A). However, cellular digoxin binding was displaced in the presence of excess ouabain, and ouabain significantly decreased ($p<0.05$) the amount of digoxin remaining in the monolayer (0.145±0.019% *vs.* 0.091±0.010% for A-to-B,

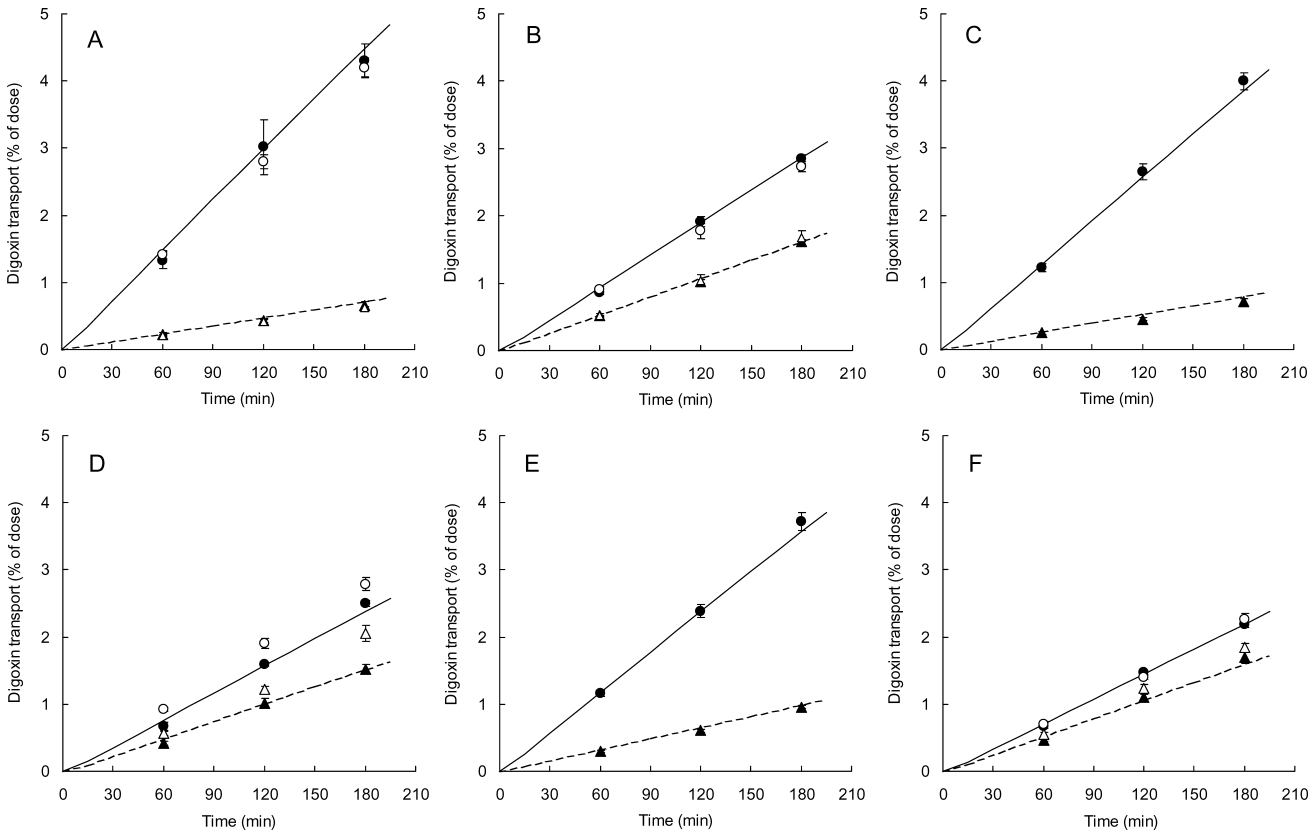

Fig. 2. Transcellular Transport Profiles of Digoxin in Caco-2 Cell Monolayers with or without Various Compounds

Transcellular digoxin transport was examined at a concentration of 10 nM (panel A). The effects of various compounds on digoxin transport were studied using 5 μM quinidine (panel B), 1 μM and 5 μM carvedilol (panels C and D), and 5 μM and 50 μM pimobendan (panels E and F). Digoxin transport in the basal-to-apical and apical-to-basal directions is indicated with closed circles and triangles, respectively. The digoxin profiles determined in the absence of ouabain are shown with open symbols. The solid and dotted lines indicate the simulation curves obtained from the pharmacokinetic analysis for basal-to-apical and apical-to-basal digoxin transport, respectively.

Table 1. Digoxin Accumulation after 180 min in Caco-2 Cell Monolayers in the Presence and Absence of Ouabain

	Without ouabain (%)		With ouabain (%)	
	A-to-B	B-to-A	A-to-B	B-to-A
Digoxin 10 nM	0.145±0.019	0.162±0.017	0.091±0.010	0.058±0.006
+5 μM quinidine	0.423±0.044**	0.434±0.035**	0.057±0.005	0.099±0.006**
+1 μM carvedilol	N.D.	N.D.	0.087±0.012	0.061±0.003
+5 μM carvedilol	0.466±0.048**	0.508±0.035**	0.173±0.008**	0.091±0.004**
+5 μM pimobendan	N.D.	N.D.	0.074±0.012	0.063±0.003
+50 μM pimobendan	0.490±0.041**	0.399±0.017**	0.111±0.006	0.096±0.005**

The values are expressed as means±S.E. for 6 experiments. $**p<0.01$; significantly different from 10 nM digoxin.

Table 2. Influx and Efflux Clearance of Digoxin for Transcellular Digoxin Transport in Caco-2 Cell Monolayers in the Presence of Ouabain

	Clearance (μl/min/cm^2)			
	CL_{CA}	CL_{CB}	CL_{AC}	CL_{BC}
Digoxin 10 nM	1.023±0.045	0.065±0.001	0.437±0.013	0.279±0.011
+5 μM quinidine	0.369±0.011*	0.327±0.012**	0.181±0.007**	0.309±0.007
+1 μM carvedilol	0.885±0.023	0.076±0.001	0.380±0.013	0.250±0.004
+5 μM carvedilol	0.339±0.006**	0.102±0.003	0.340±0.006	0.176±0.003**
+5 μM pimobendan	0.759±0.009	0.120±0.003*	0.310±0.012	0.246±0.005
+50 μM pimobendan	0.282±0.005**	0.178±0.002**	0.223±0.003**	0.195±0.005*

The values are expressed as means±S.E. for 6 experiments. $*p<0.05$, $**p<0.01$; significantly different from 10 nM digoxin.

and 0.162±0.017% *vs*. 0.058±0.006% for B-to-A) (Table 1). These results indicate that the transcellular transport and cellular accumulation data for the presence of ouabain could be used for pharmacokinetic analysis.

Pharmacokinetic Analysis of the Transcellular Transport and Cellular Accumulation of Digoxin We first determined the mean distribution volume in the monolayer to be 2.50 μl/cm^2 to represent the clearance parameters in units of μl/min/cm^2. Then, to characterize transcellular digoxin transport, we analyzed the digoxin profiles obtained in the presence of ouabain. The influx and efflux digoxin clearance parameters determined from the analysis are shown in Table 2. For the control (10 nM digoxin) study, the efflux clearance on the apical side of the monolayer (CL_{CA}) was 15.7 times greater than that on the basal side (CL_{CB}); 1.023±0.045 *vs*. 0.065±0.001 μl/min/cm^2 (Table 2), indicating that digoxin in the monolayer was mainly excreted through the apical membrane. The paracellular digoxin (mannitol) clearance (CL_{PARA}) was 0.016±0.002 μl/min/cm^2, which was smaller than any other clearance parameter for digoxin transport (Table 2), indicating that the paracellular transport of digoxin is insignificant compared with its transcellular transport.

Effects of Quinidine, Carvedilol and Pimobendan on the Transcellular Transport and Cellular Accumulation of Digoxin To evaluate whether the cardiovascular compounds carvedilol and pimobendan affect intestinal digoxin absorption, their inhibition effects on transcellular digoxin transport were examined and compared to those of the positive inhibition control compound quinidine. Digoxin transport was affected by 5 μM quinidine (Fig. 2B). In contrast, carvedilol did not affect digoxin transport at a concentration of 1 μM, although it altered the digoxin transport profiles when applied at 5 μM (Figs. 2C, D). Pimobendan at 5 μM did not affect digoxin transport (Fig. 2E), but changed the transport profiles considerably when its concentration was increased to 50 μM (Fig. 2F). These compounds decreased the basal-to-apical transport of digoxin and increased its apical-to-basal transport, indicating that the direction specificity of digoxin transport has been reduced. Carvedilol and pimobendan also increased the intracellular accumulation of digoxin (Table 1).

Quinidine changed the clearance parameters considerably (Table 2). CL_{CA} and CL_{AC}, which were large for the control study were decreased, while the parameter with a small value (CL_{CB}) was increased (Table 2). Carvedilol and pimobendan did not significantly affect the clearance parameters at low concentrations, as opposed to high concentrations (Table 2). These cardiovascular compounds decreased the digoxin efflux clearance on the apical side of the monolayer (CL_{CA}) to one-third that of the control value (Table 2). Carvedilol and pimobendan also decreased the digoxin influx clearance on the basal side of the monolayer (CL_{BC}), while quinidine did not affect it (Table 2).

DISCUSSION

To analyze the characteristics of transcellular digoxin transport in detail, we followed a pharmacokinetic approach in which a three-compartment model was utilized to assess the influx and efflux of digoxin on both sides of the monolayer (Fig. 1).[18—20] We also examined ^{3}H-digoxin transport at the constant concentration of 10 nM unlabeled digoxin so that the influx and efflux parameters of ^{3}H-digoxin would not change in a time- and concentration-dependent manner during the experiments.[18,19] Na^+,K^+-ATPase is expressed on the basal side of the Caco-2 cell monolayer,[25,26] and digoxin potently binds to Na^+,K^+-ATPase. Therefore, we also used experiment conditions under which digoxin binding to Na^+,K^+-ATPase was minimized. As a result, 100 μM ouabain significantly decreased cellular digoxin accumulation, although it had little effect on the digoxin transport profile (Fig. 2A, Table 1). We then analyzed the data for transcellular digoxin

transport in the presence of ouabain,[21] and quantitatively evaluated digoxin transport based on the influx and efflux clearances.

By characterizing the transcellular digoxin transport and also calculating the rate of digoxin clearance, we found that digoxin, which enters the monolayer from the apical side, is excreted in large part back into the apical chamber (Fig. 1, Table 2). In contrast, digoxin which enters the monolayer from the basal side passed through the monolayer to the apical side (Fig. 1, Table 2). The direction specificity of transcellular digoxin transport can be explained by the observation that digoxin efflux clearance on the apical side of the monolayer (CL_{CA}) was larger than that on the basal side (CL_{CB}). ATP-binding cassette (ABC) transporters such as MDR1/P-glycoprotein and MRP2 are involved in digoxin exsorption,[16,17,22,27] and these transporters are expressed on the apical membrane of Caco-2 cell monolayers.[28,29] Thus, they are probably responsible for digoxin efflux on the apical side of the monolayer.

Five micromolar carvedilol and 50 μM pimobendan decreased transcellular digoxin transport in the basal-to-apical direction (Figs. 2D, F). These compounds decreased the digoxin efflux clearance on the apical side of the monolayer (CL_{CA}) to the greatest degree among all of the clearance parameters (Table 2). Therefore, it is likely that carvedilol and pimobendan inhibit the ABC transporters, and suppress digoxin efflux on the apical membrane.[30] They also increased the amount of digoxin transported in the apical-to-basal direction. Furthermore, carvedilol inhibited digoxin transport more potently than pimobendan, and its effect was comparable to that of quinidine with respect to the clearance parameters at 5 μM (Table 2). It is unlikely that the blood concentration of these cardiovascular compounds can rise to such high levels in clinical situations,[31,32] and therefore the renal excretion of digoxin may not be affected by these compounds. However, their concentrations might reach such high levels in the gastrointestinal tract when they are orally administered.

As listed in Table 2, quinidine, carvedilol and pimobendan increased efflux clearance on the basal side of the monolayer (CL_{CB}) at the concentration for which they affected transcellular digoxin transport, but the reason for this is unclear. These compounds may increase the unbound digoxin fraction in the Caco-2 cell monolayer by displacing digoxin bound to cellular components and/or transporters. The increased unbound fraction may then partly contribute to increased efflux clearance of digoxin (CL_{CB}). On the other hand, digoxin influx clearance on the apical side (CL_{AC}) was found to be larger than that on the basal side (CL_{BC}): 0.437 ± 0.013 *vs.* 0.279 ± 0.011 μl/min/cm^2 (Table 2). In addition to this, pimobendan and quinidine decreased CL_{AC} more significantly compared with CL_{BC}, while carvedilol significantly decreased CL_{BC} (Table 2). These results suggest that the mechanisms responsible for the digoxin influx on the apical side of the monolayer are probably different from those for the basal side. Further investigation is required to clarify the molecular aspects of digoxin influx in Caco-2 cell monolayers and/or intestinal tissues.

In summary, we studied the transcellular transport and drug interaction of digoxin in Caco-2 cell monolayers to characterize intestinal digoxin absorption. We first examined digoxin transport in the presence and absence of ouabain, and found that the observations for transcellular digoxin transport in Caco-2 cell monolayers in the presence of ouabain is appropriate for pharmacokinetic analysis. Pharmacokinetic analysis showed that the efflux of digoxin on the apical side of the monolayer is greater than that on the basal side. Carvedilol and pimobendan increased the apical-to-basal transport of digoxin, and these compounds suppressed digoxin efflux on the apical side and digoxin influx on the basal side of the monolayer. These findings indicate that efflux on the apical side of the monolayer is the process primarily responsible for transcellular digoxin transport, and that carvedilol and pimobendan may affect the intestinal absorption of digoxin.

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