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Effect of *Curcuma comosa* extracts on the functions of peptide transporter and P-glycoprotein in intestinal epithelial cells

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Curcuma comosa has been widely used as a herbal medicine in Thailand; however, it remains unclear whether *C. comosa* influences the absorption of drugs that are substrates for the transporters in the small intestine. In this study, we investigated the effect of *C. comosa* extracts on the functioning of peptide transporter 1 (PEPT1), an influx transporter, and P-glycoprotein (P-gp), an efflux transporter, in Caco-2 cells and rat intestine. In Caco-2 cells, the ethanolic extract of *C. comosa* (CCE) lowered the uptake of glycylsarcosine (Gly-Sar), a PEPT1 substrate, while it enhanced the uptake of rhodamine 123 (Rho123), a P-gp substrate, in a concentration-dependent manner. In addition, CCE inhibited apical-to-basal transport of Gly-Sar and basal-to-apical transport of Rho123. Furthermore, the absorption of cephalexin, another PEPT1 substrate, and the exsorption of Rho123 across the rat intestine were inhibited by CCE. Conversely, CCW, the hot water extract of *C. comosa*, suppresses the function of PEPT1 but not of P-gp in Caco-2 cells. These results suggest that *C. comosa* used as a herbal medicine in Thailand may affect the intestinal absorption of certain drugs.

1. Introduction

Curcuma comosa, commonly known in Thailand as *wan chak motluk*, is a perennial herb belonging to the Zingiberaceae family. The rhizome has been traditionally used for the treatment of post-partum uterine bleeding and inflammation in Thailand (Weerachayaphorn et al. 2010). In addition, *C. comosa* has pharmacological effects such as estrogenic, choleretic, and hypolipidemic activities (Winuthayanon et al. 2009; Piyachaturawat et al. 2000, 2002). Thus, the demand for herbal medicines has been increasing in recent years in Thailand. However, herbal medicines may affect the pharmacokinetics of co-administered conventional drugs (Shi and Klotz 2012). Therefore, information about herb-drug interactions is critical for obtaining the optimum therapeutic outcome with herbal medicines such as *C. comosa*.

There is some information about the pharmacokinetic properties of certain ingredients in *C. comosa*. Su et al. (2012) demonstrated that estrogenic diarylheptanoids in *C. comosa* were relatively well absorbed from rat gastrointestinal tract and distributed into various organs including brain, liver, and kidney. In addition, another compound, (3*R*)-1,7-diphenyl-(4*E*,6*E*)-4,6-heptadien-3-ol, in *C. comosa* increased the protein expression level of GLUT4, a facilitative glucose transporter, in skeletal muscle, resulting in enhancement of glucose uptake (Prasannarong et al. 2012). However, there has been little information concerning the interaction of *C. comosa* extract and/or its ingredients with drug transporters such as peptide transporter 1 (PEPT1) and P-glycoprotein (P-gp) in the intestine.

PEPT1 is localized on the brush-border membrane of the intestinal epithelial cells, and transports a variety of dipeptides, tripeptides, and peptide-like drugs in a proton-coupled manner (Takano et al. 2006). P-gp is also expressed on the brush-border membrane of the intestinal epithelial cells and it recognizes various structurally and pharmacologically unrelated neutral and positively charged hydrophobic compounds (Takano et al. 2006). Since oral drug administration is the most common route of administration, the interaction of herbal medicines with these transporters in the intestine may alter the absorption of co-administered drugs. Therefore, it is important to clarify the herb-drug interactions in order to avoid possible therapeutic failures and adverse effects (Awortwe et al. 2014).

Thai plant extracts have been studied in search for a new class of P-gp inhibitors to overcome the multi-drug resistance of tumor cells (Patanasethanont et al. 2007a, b). In addition, we established paclitaxel-resistant HepG2 (PR-HepG2) cells, which showed enhanced expression and function of P-gp (Takano et al. 2009). Using PR-HepG2 cells, we demonstrated that some extracts of Thai plants potentiated the cytotoxicity of anticancer drugs by inhibiting P-gp-mediated efflux (Kawami et al. 2010). Recently, we studied the P-gp modulating effect of the ethanolic extract of *C. comosa*, and reported that it showed P-gp inhibitory effect and potentiated the sensitivity of paclitaxel-resistant cancer cells to paclitaxel (Takano et al. 2014). However, it is not clear whether the extract affects the functions of PEPT1 and P-gp in the intestine. In the present study, we investigated the effect of 50% ethanolic extract of *C. comosa* (CCE) on the accumulation or transepithelial transport of PEPT1 and P-gp substrates in Caco-2 cells. In addition, the functions of PEPT1 and P-gp in the rat intestine were evaluated using an *in situ* closed loop study and an *in vivo* exsorption study, respectively. We also compared the effect of hot water extract of *C. comosa* (CCW) with that of CCE.

2. Investigations and results

2.1. Effect of CCE on the uptake and transepithelial transport of [^{3}H] glycylsarcosine (Gly-Sar) and Rho123 in Caco-2 cells

The effect of CCE on [^{3}H]Gly-Sar and Rho123 uptake in Caco-2 cells was examined at a concentration range of 100 to 1000 µg/mL. CCE reduces and enhances the uptake of [^{3}H]Gly-Sar and Rho123, respectively, in a concentration-dependent manner (Fig. 1A, B). In addition, we examined the effect of 100-1000 µg/mL CCE on the protein amount of Caco-2 cells. However, treatment of Caco-2 cells with the extract for one hour did not affect the protein amount

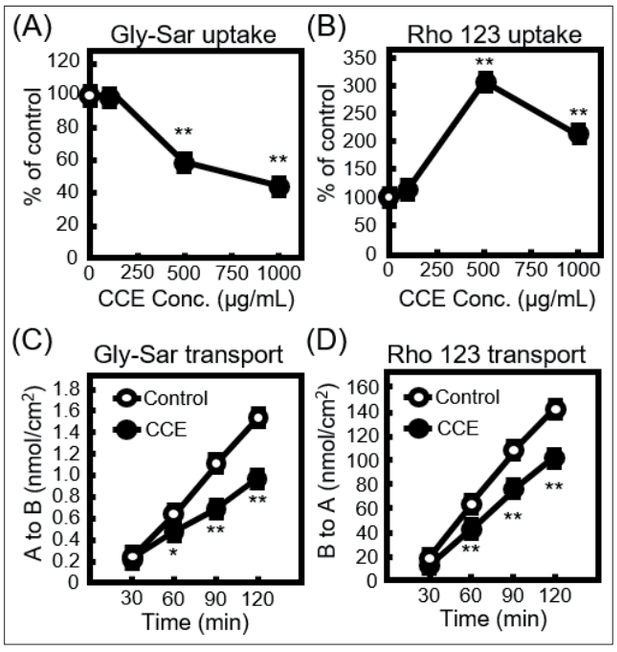

Fig. 1: Effect of CCE on the uptake and transepithelial transport of [^{3}H]Gly-Sar and Rho123 in Caco-2 cells. Caco-2 cells were incubated with [^{3}H]Gly-Sar (A) for 15 min and Rho123 (B) for 60 min in the absence (open circle; control) or presence (closed circles) of various concentrations of CCE. Transepithelial transport of [^{3}H]Gly-Sar (C) in the apical (A)-to-basolateral (B) direction and Rho123 (D) in B-to-A direction across Caco-2 monolayers cultured on Transwell® in the absence (open circles; controls) or presence (closed circles) of CCE (500 μg/mL) in apical side. Each value represents the mean ± S.E. (n=3). * $p < 0.05$, ** $p < 0.01$, significantly different from each control.

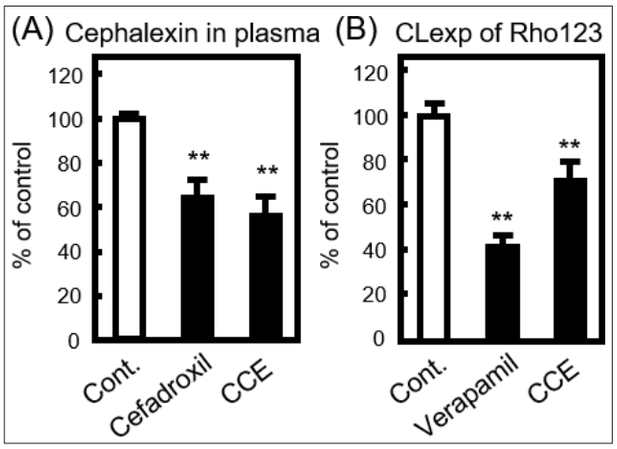

Fig. 2: Effect of CCE on the functions of PEPT1 and P-gp in rat intestine. (A) Cephalexin (3 mM) with (closed column) or without (open column; control) CCE (500 μg/mL) and cefadroxil (10 mM, a PEPT1 inhibitor) was administered into the closed loop in the upper intestine. Blood sample was collected at 20 min and the plasma concentration of cephalexin was measured. (B) Intestinal exsorption clearance of Rho123 under a steady state plasma concentration in the absence (open column; control) or presence (closed column) of CCE (300 μg/mL) and verapamil (100 μM, a P-gp inhibitor) in the intestinal perfusate. Each value represents the mean ± S.E. (n=3). ** $p < 0.01$, significantly different from each control.

of the cells (data not shown), indicating that the extract would not have apparent cell toxicity.

PEPT1 is expressed at the apical side of Caco-2 cell monolayers (Takano et al. 2006). In addition, transepithelial transport of Rho123 from the basolateral (B)-to-apical (A) direction across Caco-2 cell monolayers is much higher than that from A-to-B (Takano et al. 1998). Therefore, the effect of CCE on A-to-B transport of [^{3}H]Gly-Sar and B-to-A transport of Rho123 was examined in Caco-2 cells. The A-to-B transport of [^{3}H]Gly-Sar was inhibited by CCE (Fig. 1C). In addition, CCE administration to the apical side significantly inhibited the B-to-A transport of Rho123 (Fig. 1D). Such inhibition indicates that CCE suppresses the transepithelial transport of Gly-Sar and Rho123 by inhibiting the functions of PEPT1 and P-gp in Caco-2 cells, respectively.

2.2. Effect of CCE on the functions of PEPT1 and P-gp in rats

The effect of CCE on the intestinal absorption of cephalexin, a PEPT1 substrate, was examined by an *in situ* closed loop method in rats. The plasma concentration of cephalexin decreased following its coadministration with cefadroxil (10 mM) and CCE (500 μg/mL) (Fig. 2A).

The intestinal P-gp function in rats was determined by measuring *in vivo* exsorption clearance (CLexp) of Rho123 from blood to the intestinal lumen under steady state. The CLexp of Rho123 was significantly suppressed by CCE (300 μg/mL) as well as by verapamil (100 μM), a P-gp inhibitor (Fig. 2B).

2.3. Effects of Curcuma comosa active moieties on the uptake of [^{3}H]Gly-Sar and Rho123 in Caco-2 cells

Bisdemethoxycurcumin, 4-hydroxybenzaldehyde, rhododendrol, and vanillin are reportedly present in *C. comosa* extracts (Sodsai et al. 2007; Nakamura et al. 2008). Therefore, the effects of these molecules on the uptake of [^{3}H]Gly-Sar and Rho123 in Caco-2 cells were also examined. However, as shown in Fig. 3, all the molecules tested had no effect on the function of PEPT1 and P-gp at a concentration range from 0.1 to 10 μM.

2.4. Effect of CCW on the functions of PEPT1 and P-gp in Caco-2 cells and rats

It is important to determine the effect of CCW, as it is generally consumed orally after boiling *C. comosa* in water. Therefore, the effect of CCW on the functions of PEPT1 and P-gp in Caco-2 cells was determined. [^{3}H]Gly-Sar uptake in Caco-2 cells significantly decreased in the presence of CCW at a concentration of 1000 μg/mL, but not at 100 or 500 μg/mL (Fig. 4A). However, CCW had no effect on Rho123 uptake (Fig. 4B).

We further examined the effect of CCW on the intestinal absorption of cephalexin in rats by using an *in situ* closed loop method. CCW (500 μg/mL) suppressed the plasma concentration of cephalexin at 20 min post administration (Fig. 4C), indicating that CCW could inhibit the PEPT1-mediated absorption of cephalexin from the intestine under *in vivo* conditions.

3. Discussion

The rhizome of *C. comosa* has been widely used in Southeast Asia for the treatment of various diseases. In particular, the estrogenic effect of several diarylheptanoids purified from *C. comosa* was reported in cell culture studies (Suksamrarn et al. 2008). However, it is unclear whether *C. comosa* affects transporter function in the intestine. In this study, the effects of CCE and CCW on the transport of PEPT1 and P-gp substrates were examined using Caco-2 cells and rats.

In Caco-2 cells, we examined the effect of CCE on the uptake of [^{3}H]Gly-Sar, a PEPT1 substrate, and Rho123, a P-gp substrate. As shown in Fig. 1A and B, CCE reduced the uptake of [^{3}H]Gly-Sar and enhanced that of Rho123, indicating that CCE could have an inhibitory effect on the functions of PEPT1 and P-gp. Previously, we examined the effect of Z01 (ethanol extract from the same plant, *C. comosa*) on P-gp function in PR-HepG2 cells, and found that 100 μg/mL Z01 significantly increased the accumulation of Rho123 (Takano et al. 2014). However, 100 μg/mL CCE had no effect on Rho123 uptake in Caco-2 cells (Fig. 1B). The apparent discrepancy of inhibitory potency of CCE on P-gp between PR-HepG2 and Caco-2 cells may be attributable to the higher expression of P-gp in PR-HepG2 cells.

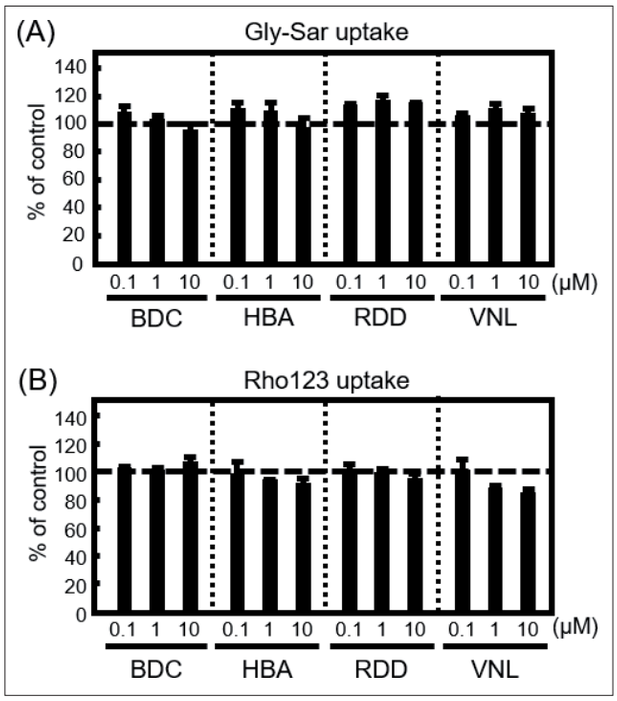

Fig. 3: Effects of compounds present in *Curcuma comosa* on the uptake of [^{3}H]Gly-Sar (A) and Rho123 (B) in Caco-2 cells. Caco-2 cells were incubated with [^{3}H]Gly-Sar (A) for 15 min and Rho123 (B) for 60 min in the absence or presence of various concentrations of bisdemethoxycurcumin (BDC), 4-hydroxybenzaldehyde (HBA), rhododendrol (RDD), and vanillin (VNL). The dashed line represents control vale. Each value represents the mean ± S.E. (n = 3).

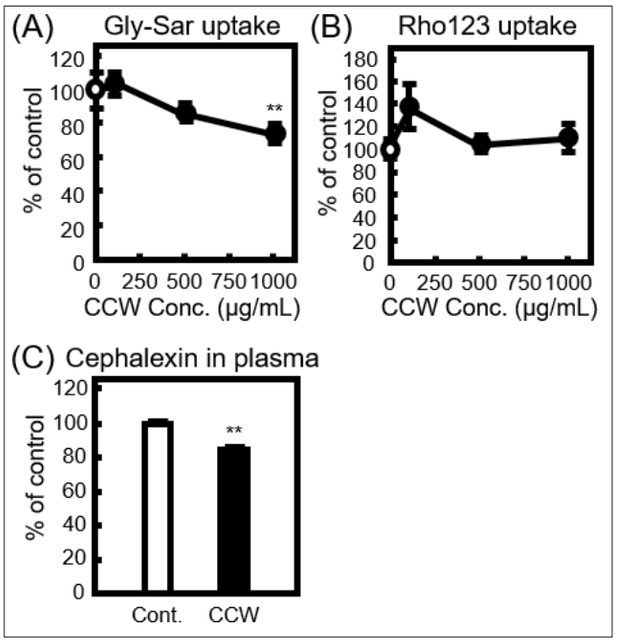

Fig. 4: Effect of CCW on the uptake of [^{3}H]Gly-Sar (A) and Rho123 (B) in Caco-2 cells and the absorption of cephalexin from rat intestine (C). Caco-2 cells were incubated with [^{3}H]Gly-Sar (A) for 15 min and Rho123 (B) for 60 min in the absence (closed column) or presence (open column; control) of various concentrations of CCW. (C) Cephalexin (3 mM) with or without CCW (500 μg/mL) was administered into the closed loop in the upper intestine. Blood sample was collected at 20 min and the plasma concentration of cephalexin was measured. Each value represents the mean ± S.E. (n=3). ** $p < 0.01$, significantly different from each control.

Thus far, Caco-2 cells have been used to predict the permeability and absorption of substrates for PEPT1 and P-gp (Hillgren et al. 1995). Liu et al. (2011) used Caco-2 cells to characterize the uptake and transepithelial transport of cyclo-trans-4-l-hydroxy-prolyl-L-serine (JBP485), which is a dipeptide with anti-hepatitis activity, and demonstrated that JBP485 is a substrate for PEPT1. Caco-2 cells were also employed as a model system for screening drug interaction with human P-gp at an early drug discovery stage (Balimane et al. 2004). The present results showed that the directional transport of [^{3}H]Gly-Sar and Rho123 across Caco-2 cell monolayers was prevented by CCE (Fig. 1C, D), indicating that CCE affects the absorption of PEPT1 and P-gp substrates in the human intestine.

On the other hand, there are some reports concerning the drawbacks of Caco-2 cells as a biorelevant model. Hayeshi et al. (2008) reported that there was large variability of Caco-2 cells in the expression and function of various transporters among different laboratories. In addition, there are some reports showing the difference in gene expression between Caco-2 cells and human small intestine (Ölander et al. 2016; Brück et al. 2016). Therefore, variability of Caco-2 cells and differences between human intestine and Caco-2 cells should be considered.

Based on *in vitro* studies using Caco-2 cells, we further examined the effect of CCE on the PEPT1 and P-gp functions in rat intestine (Fig. 2). So far, species differences in the expression level and amino acid sequence of some transporters have been discussed. Hilgendorf et al. (2007) compared species differences in mRNA expression of 20 orthologous drug transporters between human intestine and rat ileum. They showed that the mRNA expression level of PEPT1 in human intestine was comparable to that in rat ileum. Cao et al. (2006) also demonstrated that the expression level and regional (duodenum and colon) expression pattern of PEPT1 protein is similar between human and rat intestine. In addition, PEPT1 is highly homologous between human (Liang et al. 1995) and rat (Saito et al. 1995). Therefore, the effect of *C. comosa* extract on PEPT1 function in human intestine may be predictable from *in vivo* study in rat. On one hand, it has been demonstrated that human MDR1 sequence is 87 and 80% indentical to rat mdr1a and mdr1b isoforms, respectively (Mazur et al. 2012). However, the level of P-gp expression is 2.7-fold higher in duodenum than in the colon in human intestine, while P-gp expression was 7-fold higher in colon than in the duodenum in rat intestine (Cao et al. 2006). In addition, substrate specificity may not be the same between human MDR1 and rat mdr1b (Mazur et al. 2012). Although our studies concerning PEPT1 and P-gp functions in rat intestine corresponded to *in vitro* studies using Caco-2 cells (Fig. 2), above mentioned and other differences should be considered to further understand the effect of *C. comosa* extract on the transporter function in human intestine.

Generally, metabolizing enzymes in the liver can affect the blood concentration of many drugs. However, Kittichanun et al. (2010) reported that phase I drug metabolizing enzymes such as CYP1A1, 2E1, and 3A in rat liver were not affected by hexane and ethanolic extracts from *C. comosa*. In addition, Jiwapornkupt et al. (2010) examined the effect of an ethanolic extract from *C. comosa* on phase II drug metabolizing enzymes such as UDP-glucuronosyltransferase in rat liver, and found that the extract had no effect on these enzymes. These findings indicate that an ethanolic extract from *C. comosa* would not affect the activity of above mentioned metabolizing enzymes.

The ethanolic extract of *C. comosa* has many constituents that may interact with PEPT1 and P-gp. Bisdemethoxycurcumin, a natural dimethoxy derivative of curcumin, increased the sensitivity of KB-V1, which is a multidrug-resistant cervical carcinoma cell line, to vinblastine by modulating P-gp function (Mapoung et al. 2015). In addition, phenolic compounds, especially flavonoids, have been shown to affect P-gp activity and the uptake of anticancer drugs by binding to P-gp hydrophobic binding pocket surrounding the active site (Daddam et al. 2014). 4-Hydroxybenzaldehyde, rhododendrol, and vanillin are not flavonoids but phenolic compounds and may have an inhibitory effect on P-gp in Caco-2 cells. Therefore, the effects of these compounds on the function of PEPT1 and P-gp

in Caco-2 cells were determined and no effect was observed on PEPT1 and P-gp at the tested concentration range (Fig. 3). In addition, the synergic effect of these compounds on PEPT1 and P-gp was examined and combination treatment was also found to have no effect (data not shown), indicating that these compounds do not interact with PEPT1 and P-gp in Caco-2 cells.

Since *C. comosa* has various pharmacological effects, several extraction methods have been investigated. For example, Su et al. (2011) reported the estrogenic-like activity of *C. comosa* hexane extracts, which improved the spatial memory in ovariectomized rats. Moreover, the ethanol extracts of *C. comosa* significantly reduced cisplatin-induced nephrotoxicity attributable to its antioxidant capacity and anti-inflammatory activity (Jariyawat et al. 2009). However, in Thailand, *C. comosa* rhizome is commonly used after boiling it in water. Therefore, we examined the effect of a hot water extract of *C. comosa* on PEPT1 and P-gp functions in Caco-2 cells. Although CCW had no effects on Rho123 uptake in Caco-2 cells, [^{3}H]Gly-Sar uptake was slightly but significantly suppressed by CCW (Fig. 4A, B). Furthermore, the plasma concentration of cephalexin was examined using an *in situ* closed loop method and found to be significantly reduced by CCW (Fig. 4C), indicating that the oral administration of the hot water extract of *C. comosa* affects the intestinal absorption of PEPT1 substrate drugs.

In conclusion, *in vitro* and *in vivo* studies showed that the ethanolic extract of *C. comosa* alters the functioning of PEPT1 and P-gp. The inhibitory effects of the ethanolic extract of *C. comosa* on PEPT1 and P-gp were more potent than those of the hot water extract. Although the water extract had no effect on P-gp function, it inhibits Gly-Sar uptake in Caco-2 cells and absorption of cephalexin in the rat intestine, indicating that the clinical use of *C. comosa* would affect PEPT1 function in the intestine. These findings may provide useful information for the herb-drug interaction in the intestine.

4. Experimental

4.1. Chemicals and reagents

[^{3}H] Gly-Sar was obtained from Moravek Biochemicals (Brea, CA, USA). Unlabeled Gly-Sar, cephalexin, cefadroxil, Rho123, and verapamil hydrochloride were purchased from Tokyo Chemical Industry (Tokyo, Japan), Wako Pure Chemical (Osaka, Japan), Sigma-Aldrich (St. Louis, MO, USA), Kanto Chemical (Tokyo, Japan), and Nacalai Tesque (Kyoto, Japan), respectively. All other chemicals were of the highest grade commercially available.

4.2. Cell culture

Caco-2 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 1% non-essential amino acids, 2 mM l-glutamine, 100 units/mL penicillin G, and 100 µg/mL streptomycin at 37 °C in humidified environment with 5% CO_2-95% air, as described previously (Takano et al. 1998).

4.3. Curcuma comosa extracts

For CCE preparation, 25 g of dried rhizome was macerated in 250 mL of 50% ethanol for 7 days at room temperature. The evaporated fraction was then dissolved in DMSO at a concentration of 100 mg/mL. For CCW preparation, 25 g of dried rhizome was boiled in 250 mL of water for 30 min. Then, the fraction was freeze-dried and further dissolved in dH_2O at a concentration of 100 mg//mL. CCE and CCW were stored at -20 °C.

4.4. Uptake of [^{3}H]Gly-Sar and Rho 123 in Caco-2 cells

After removal of the culture media, cells were washed with phosphate buffered saline (PBS) (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.1 mM $CaCl_2$, and 0.5 mM $MgCl_2$, pH 7.4) supplemented with 5 mM d-glucose (PBS-G) and preincubated at 37 °C for 15 min. The cells were incubated with [^{3}H]Gly-Sar or Rho123 in the presence or absence of CCE and CCW at 37 °C for 15 min and 60 min, respectively, and were rapidly rinsed three times with stop solution UF (5 M urea and 15% formaldehyde in PBS), and ice-cold PBS in case of [^{3}H]Gly-Sar and Rho123 uptake study, respectively. The uptake amounts of [^{3}H]Gly-Sar and Rho123 were estimated using a procedure reported previously (Takano et al. 2015; Yumoto et al. 2003).

4.5. In vitro transepithelial transport across Caco-2 cell monolayers

Caco-2 cells were grown for 21 days on the polycarbonate filter (1.6 x 10^5 cells/cm^2) in a Transwell chamber (Costar, Cambridge, MA, USA). Monolayers with transepithelial electrical resistance (TEER) values higher than 200 Ω•cm^2 were used. The transported amounts of [^{3}H]Gly-Sar and Rho123 were measured as reported previously (Takano et al. 2015; Yumoto et al. 2003).

4.6. In vivo intestinal exsorption of Rho123 under steady-state plasma concentration

Experiments with Sprague-Dawley rats were performed in accordance with the Guide for Animal Experimentation from Hiroshima University and the guidelines of the Committee of Research Facilities for Laboratory Animal Sciences, Hiroshima University. *In vivo* intestinal exsorption clearance of Rho 123 at steady state in presence of CCE (300 µg/mL) and verapamil (100 µM, a P-gp inhibitor) were estimated as reported previously (Yumoto et al. 2003).

4.7. In situ closed loop study

After flushing the small intestine with saline, the upper segment (a 10-cm-long jejunal segment; 5-15 cm below the bile duct opening) of the small intestine was ligated to form a closed loop. Cephalexin (3 mM) with or without CCE, CCW (500 µg/mL), and cefadroxil (10 mM, a PEPT1 inhibitor) were administered into the loop as 0.5 mL of isotonic PBS (pH 6.5). Blood was collected after 20 min *via* a cannula placed at femoral artery and the plasma concentration of cephalexin was determined using HPLC (Takano et al. 2015).

4.8. Statistical analysis

Data were expressed as mean±S.E. All statistical analyses were performed by Student's *t*-test or by Tukey's test for multiple comparisons. The level of significance was set at $*p < 0.05$ or $**p < 0.01$.

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Conflicts of interest: None declared.

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