Effects of pharmaceutical excipients on the intestinal transport and absorption of breast cancer resistance protein substrates

Ph. D. Dissertation

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Abbreviations

5-ASA	5-Aminosalicylic acid
ABC	ATP binding cassette
ATP	Adenosine triphosphate
AUC	Area under the concentration versus time curve
BCRP	Breast cancer resistance protein transporter
BSA	Bovine serum albumin
Caco-2	Colon adenocarcinoma-2
CBB	Coomassie brilliant blue G-250
cDNA	Complementary deoxyribonucleic acid
CF	5(6)-Carboxyfluorescein
C_{max}	Maximum concentration
CMC	Critical micelle concentration
CYP3A4	Cytochrome P subtype 3A4
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DPH	1,6-Diphenyl-1,3,5-hexatriene
DNA	Deoxyribonucleic acid
E2	Ethinyl estradiol
EC_{50}	Effective concentration of inhibitor that reduce drug resistance by 50%
EC_{90}	Effective concentration of inhibitor that reduce drug resistance by 90%
EMA	European Medicines Agency
ER	Efflux ratio
FTC	Fumitremorgin C
HBSS	Hank's balance salt solution
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
hGAPDH	Human glyceraldehyde-3-phosphate dehydrogenase
HLB	Hydrophilic lipophilic balance
HPLC	High performance liquid chromatography
IC_{50}	Half maximal inhibitory concentration
LDH	Lactate dehydrogenase
MDCK-II	Madin-Darby canine kidney cell-2
MDR	Multidrug resistance
mRNA	Messenger ribonucleic acid
MRP	Multidrug resistance protein
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenpyl-tetrazolium bromide

MW	Molecular weight
MWCO	Molecular weight cut off
N.A.	Not applicable
N.S.	Not statistically different
NADH	reduced pyridine nucleotide cofactor
NBD	Nucleotide binding domain
OD	Optical density
P-gp	P-glycoprotein
P_{app}	Apparent permeability coefficient
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PBS	Phosphate buffered saline
PEO	Polyethylene oxide
PL	Polarization
PPO	Polypropylene oxide
RNA	Ribonucleic acid
RT-PCR	Reverse-transcription polymerase chain reaction
S.E.	Standard error
SNP	Single nucleotide polymorphism
TEER	Transepithelial electrical resistance
TKI	Tyrosine kinase inhibitor
tma-DPH	N,N,N-Trimethyl-4-(6-phenyl-1,3,5-hexatrien-1-yl)phenylammonium p-toluene sulfonate
T_{max}	Time to maximum plasma concentration
TMD	Transmembrane domain
USFDA	United States Food and Drug Administration
W/V	Weight by volume

Abstract

The breast cancer resistance protein (BCRP) transporter is a second member of an ATP binding cassette (ABC) transporter subfamily G. It is an ATP driven efflux transporter that pushes its substrates to the secretory direction regardless of the concentration gradient. BCRP is highly expressed in the apical membrane of intestinal epithelial cells and was reported to be a significant factor that can reduce the intestinal absorption of its substrates. Presently, hundreds of drugs were identified as BCRP substrates and the number is still increasing. This low intestinal absorption of BCRP substrates should be overcome for the drug development in the pharmaceutical industries. For instance, US Food and Drug Administration (FDA) is currently recommending that all new drugs in the development pipelines should be evaluated by *in vitro* transport studies if their drug candidate is a potential substrate of BCRP. Meanwhile, several inhibitors have been developed to reduce the function of BCRP. Among these inhibitors, pharmaceutical excipients are considered to be promising candidates to inhibit the function of BCRP. In this study, sulfasalazine and topotecan were used as model substrates of BCRP and the effects of pharmaceutical excipients on the transport and absorption of these substrates were evaluated by *in vitro* and *in situ* experiments. Moreover, possible mechanisms of BCRP inhibition by these pharmaceutical excipients were elucidated by evaluating their interactions to the biomembranes. Lastly, the toxicity of these pharmaceutical excipients was studied.

Chapter I: Effects of various pharmaceutical excipients on the intestinal transport and absorption of sulfasalazine, a model substrate of BCRP transporter

Sulfasalazine, which is actively effluxed by BCRP and has less than 15% oral bioavailability, was used as a typical model for BCRP transporter studies. Our experiment showed that sulfasalazine was preferentially transported into a secretory direction (serosal to mucosal side of a membrane) by an *in vitro* diffusion chamber method using the isolated rat small intestinal membranes. Later, Ko143, a selective BCRP inhibitor, was used as a positive control. Results showed that the directional transport of sulfasalazine was completely neutralized in the presence of Ko143 (Efflux ratio (ER)=1.17), suggesting that sulfasalazine is a substrate of Bcrp1 and Ko143 can be used as a typical inhibitor of rodent Bcrp1. Next, the effects of several pharmaceutical excipients on the intestinal absorption of sulfasalazine were examined. These pharmaceutical excipients including BL-9EX, Brij97, Labrasol, and Tween 20 significantly decreased the secretory transport of sulfasalazine, suggesting that Bcrp1 might be inhibited by these excipients. Because these pharmaceutical excipients were reported to open the tight junction and increase the transport of drugs via a paracellular route, the transport of 5(6)-carboxyfluorescein (CF), a non-BCRP substrate paracellular transport marker, was measured. CF was equally transported in both direction (ER=1.14). Ko143 as well as other pharmaceutical excipients did not affect the transport parameters of CF, suggesting that the decrease in sulfasalazine secretory transport might be due to the inhibition of Bcrp1-mediated efflux transport by these pharmaceutical

excipients, while the paracellular permeation was not altered. Next, the qualified pharmaceutical excipients were further examined using an *in situ* closed loop intestinal absorption method. BL-9EX at 0.1% increased $AUC_{(0-4h)}$ value of sulfasalazine by over 2-fold without any significant alteration of CF absorption. Brij97 at the same concentration also increased $AUC_{(0-4h)}$ value of sulfasalazine by 1.79-fold, but the author observed that Brij97 also increased CF absorption, suggesting that Brij97 might increase the transport of sulfasalazine via a paracellular pathway. These results suggested that BL-9EX could be used as a BCRP inhibitor in an oral drug formulation.

Chapter II: Effects of various pharmaceutical excipients on the intestinal transport and absorption of topotecan, an orally active BCRP substrate

In this chapter, the author examined the effects of pharmaceutical excipients on the intestinal transport and absorption of topotecan, an orally active anticancer drug with low bioavailability (30%-40%). It was also reported to be a substrate of BCRP. The author first evaluated the transport of topotecan across Caco-2 cell monolayers. Topotecan was preferentially transported into a secretory direction (ER=16). This directional transport of topotecan was neutralized in the presence of 1 μM Ko143 but not with 50 μM verapamil, a P-gp inhibitor. These findings suggested that topotecan was mainly transported by BCRP. Results also showed that most pharmaceutical excipients significantly decreased the efflux transport of topotecan but had no effect on the transport of CF, suggesting that the decrease in the transport of topotecan might be caused by the inhibition of BCRP by the pharmaceutical excipients. Next, the intestinal absorption of topotecan was examined by the *in situ* closed loop method. Tween 20 and Cremophor EL at 0.05% significantly increased the $AUC_{(0-4h)}$ values of topotecan by over 2-fold. Labrasol and Pluronic F68 also increased the absorption of topotecan, although the increased absorption was not statistically significant. Therefore, their inhibitory effect against rodent Bcrp1 might be weaker than with human BCRP. Lastly, none of our tested excipient significantly increased the CF absorption across rat small intestine, suggesting that the pharmaceutical excipients might inhibit the function of Bcrp1 and increase the permeation of topotecan via a transcellular pathway.

Chapter III: Possible inhibitory mechanisms of BCRP transporters by the pharmaceutical excipients and their safety

All tested pharmaceutical excipients in this study have amphipathic characteristics. They tend to form a micelle at the concentration above their critical micellar concentration (CMC). The author found that at ≤0.1% concentration, interactions between sulfasalazine and pharmaceutical excipients were minimal (micellar ratio <10%). Therefore, this finding indicates that the highest applicable concentration is up to 0.1% for all tested pharmaceutical excipients. Additionally, since each drug was entrapped into the micelle in a different degree, the author suggest that it should be evaluated for the micellar interaction to avoid the excessive micellar entrapment which could interfere with the absorption of the drug.

In general, changes in membrane fluidity are one of the most important mechanisms to inhibit the function of ABC transporters by pharmaceutical excipients. Therefore, the effects of pharmaceutical excipients on the membrane fluidity of Caco-2 cells were evaluated. In this study, tma-DPH and DPH were used as fluorescence probes to measure the changes in membrane fluidity. Interestingly, most pharmaceutical excipients increased the membrane fluidity in the inner lipid bilayers, while only Pluronic F68 decreased the membrane fluidity in the outer lipid bilayers. These changes are in an accordance with the changes in topotecan permeation across Caco-2 cell monolayers, suggesting that the key mechanism of these pharmaceutical excipients was to modify the membrane microenvironment which could subsequently disrupt the efflux ability of BCRP transporters.

Lastly, the membrane toxicity of these pharmaceutical excipients after their administration to the intestine was examined. The author did not observe any elevation in LDH activities or protein amount after the exposure to the pharmaceutical excipients in our *in situ* closed loop experiments, suggesting that these pharmaceutical excipients are safe and did not cause any significant damage to the small intestinal membrane.

Summary

In conclusion, the author demonstrated that several pharmaceutical excipients could inhibit the BCRP transporter and improve the intestinal absorption of its substrates. Especially, BL-9EX, Tween 20, and Cremophor EL, increased the intestinal absorption of BCRP substrates and did not cause any significant damage to the small intestine. These findings suggested that these pharmaceutical excipients could be used to improve the intestinal absorption of BCRP substrates.

Introduction

In 1998, Doyle *et al.* published their discovery of a new multidrug resistance (MDR) transporter. This new transporter was found to be highly expressed in a specially resistance strain of breast cancer cell.[1] According to its origin, they called it a breast cancer resistance protein (BCRP) transporter. This transporter was registered as a second member of an ATP Binding Cassette (ABC) subfamily G and was encoded by the ABCG2 gene.[1] BCRP is an efflux pump which uses the energy from ATP hydrolysis to push its substrates into a secretory direction regardless of the concentration gradients.[2] This multidrug transporter is capable of transporting the virtually structurally-unrelated compounds[1,3,4]

Several models of BCRP were proposed in the past decade. At the time of writing, a newest ribbon model of ABCG2 is shown in Fig.1 (a). It is generally understood that unlike ABCB1/P-gp transporter, BCRP is a half-transporter which is comprised of 655 amino acids.[4,5] As shown in Fig.1 (b), BCRP has single nucleotide binding domain (NBD) and single transmembrane domain (TMD) with 6 α-helices.[6,7] It also carries a distinctive domain organization where NBD precedes TMD.[7] The functional form of BCRP is a homodimer with a total molecular weight of approx. 144 kDa.[4,6,8] BCRP resides on a biomembranes in an "inward-facing" configuration with opening cavity facing-in. This cavity represents a substrate binding site and open to the cytoplasm and to the inner leaflet of the lipid bilayer.[6] It was believed that the substrate binds to the BCRP at its substrate binding site from within the lipid bilayer.[9] After that, ATP binding to NBD triggers a closure of NBD and a subsequent conversion of substrate-bound inward-facing conformation to an outward-facing state which the substrate could be released to the outside by a means of hydrophobic mismatch. This release mechanism is similar to the substrate release mechanism of P-gp and MRP1.[10,11] The transporter then returns to its inward-facing conformation after the hydrolysis of ATPs.[6,11] It was also believed that BCRP has multiple substrate binding sites which explained its broad substrate selectivity and suggested that the use of the specific BCRP inhibitors could be in substrate dependent manner.[12]

The BCRP was also reported to express on various physiological barriers across the body including the blood brain barrier, mammary glands, placenta, kidney, testis and intestinal mucosa.[5,13–17] It is responsible for the regulation of endogenic substances as well as exogenic compounds. BCRP is regarded as a self-defense mechanism for the hematopoietic stem cells.[18,19] Most importantly, BCRP is highly expressed on the intestinal epithelial membrane and is a major obstacle for the absorption of its substrates.[13,20–22] Up to date, hundreds of virtually structure-unrelated substrates such atorvastatin, rosuvastatin, lamivudine, zidovudine, norfloxacin, ciprofloxacin, SN-38, mitoxantrone, topotecan, doxorubicin, and several flavonoids were reported to be a substrate of BCRP.[19,23–26] BCRP, P-gp and MRP2 are all expressed on the apical side of the small intestinal epithelial cells. Their presence is a serious concern for the pharmaceutical industries as well as other regulation agencies. For example, the U.S. Food and Drug Administration (USFDA) and European Medicines Agency (EMA) are currently

recommending that all new drugs in the development pipelines should be evaluate *in vitro* transport studies if their drug candidate is a potential substrate of these transporters which could heavily affect its bioavailability.[26]

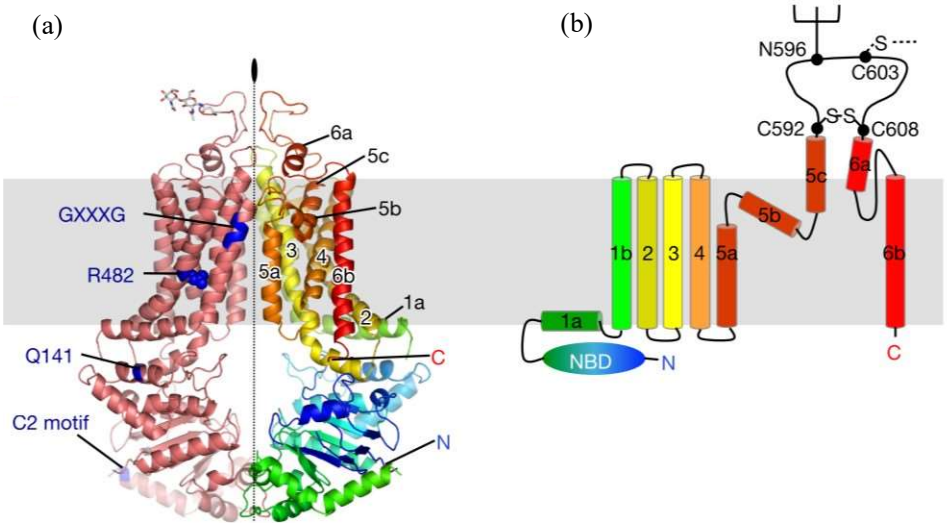


Figure 1. Ribbon structure and membrane topology of ABCG2. (a) Ribbon structure of ABCG2 with one monomer colored in pink. Motifs and residues associated with single nucleotide polymorphisms are colored blue and labelled. The other ABCG2 monomer is colored as a rainbow spectrum ranging from blue to red. (b) Topology of the ABCG2 TMD colored as in (a) . The image was derived from Taylor *et. al.,* Nature, 2017, 546.[6]

Since the discovery of ABC transporters, multiple strategies have been developed to diminish their effects. Especially, in the MDR cancers, ABCB1 or P-gp transporter gained massive attention after its discovery because P-gp is an important factor in MDR cancers due to its ability to recognize and transport various types of drugs that are differ in structure and pharmacological action.[27,28] The first-generation P-gp modulators are generally the pharmacologically active drugs. For example, calcium channel blocker verapamil was first reported to restore the sensitivity of MDR cancer to vincristine.[29] Immunosuppressant cyclosporin A was frequently used as a model P-gp inhibitor in the laboratories. It was also reported to undergo several phase I/II clinical studies as a modulator in MDR cancers.[30,31] However, these first-generation P-gp modulators were not suitable to be used as clinical P-gp inhibitors because of their pharmacological effects. The second-generation modulators such as dexverapamil and PSC-833 were reported to have higher P-gp specificity, lower pharmacological activity, but their interactions with cytochrome P450 enzymes and other transporters still persisted. The third-generation modulators were developed using structure-activity relationships to target against specific MDR mechanisms. These modulators are the high-potency, P-gp selective inhibitors including elacridar (GF120918), tariquidar, and laniquidar.[32,33] These third-generation inhibitors did not have several limitations like the first or second generation. Unfortunately, none of them was ever approved to be used as P-gp inhibitors in humans.

BCRP modulators were less studies than P-gp modulators in comparison. The first BCRP inhibitor, fumitremorgin C (FTC), was isolated from *Aspergillus fumigatus* and was reported to reverse drug resistance in human carcinoma cell line that do not express P-gp or MRP.[34] FTC is a highly BCRP-specific inhibitor and the half maximum inhibitory concentration (IC_{50}) of FTC against BCRP was reported to be approximately 1 μM.[35] Unfortunately, FTC is not suitable for *in vivo* applications because it induces tremors or convulsions in mice and other animals through toxicity to the central nervous system, similar to many other members of the fumitremorgin/verruculogen/tryprostatin class of alkaloids.[36,37] Later, non-neurotoxic FTC derivatives Ko132, Ko134, and Ko143 were developed. In Bcrp1-mediated topotecan resistant MEF3.8/Bcrp1 cells (IC_{50}=1,750 ± 50 nM), Ko143 showed highest potency with effective concentration of inhibitor that reduces topotecan resistance by 90% (EC_{90}) at 26±5 nM.[38] Therefore, Ko143 is intensively used as a positive BCRP inhibitor. However, like FTC, Ko143 is also unsuitable for *in vivo* applications because it is unstable in plasma and is rapidly metabolized by liver enzymes.[39] The dose required to achieve *in vivo* inhibition is too high and in that circumstance, Ko143 is likely to lose its BCRP selectivity.[40] Additional classes of BCRP inhibitors such as tyrosine kinase inhibitors (TKIs) imatinib, erlotinib, nilotinib as well as pyridines, and dihydropyridines such as nicardipine were also reported to effectively inhibit BCRP *in vitro* and *in vivo*.[41] However, none of them was approved to be used as a BCRP inhibitor in humans.

The pharmaceutical excipient is substance which is incorporated into a pharmaceutical product to improve the performance of a drug (e.g., physicochemical stability, dissolution rate, bioavailability) to make the manufacturing process more efficient and to prolong the product shelf life.[3] These pharmaceutical excipients were initially categorized as "Generally Recognized as Safe" by USFDA because they were regarded as inert substances and did not have any pharmacological activity.[3] In 1972, Tween 80 was the first pharmaceutical excipient which was reported to resensitize the resistant in Chinese Hamster cells to actinomycin D and daunomycin.[42] After the discovery of P-gp in 1976, the mechanism behind this phenomena was later identified as P-gp inhibition caused by Tween 80.[27,43,44] Since then, several pharmaceutical excipients were reported to modulate P-gp/BCRP transporters and reverse P-gp/BCRP-mediated MDR cancers, suggesting that they could be used as potential MDR modulators.[45–52] However, the effects of these pharmaceutical excipients on BCRP transporter, especially the intestinal BCRP transporter was far less documented.

In this study, the author examined several pharmaceutical excipients for their ability to modulate intestinal BCRP transporter in order to improve the intestinal absorption of its substrates. Several pharmaceutical excipients which were reported to modulate P-gp, BCRP or MRP2 transporters were chosen for the study. The physicochemical properties and their interactions with transporters are summarized in Table 1.

Table 1. Physicochemical properties of various pharmaceutical excipients and their interaction with transporters

Excipients	Generic or chemical name	MW (average)	HLB	CMC (%w/v)	Transporters inhibition			References
					P-gp	BCRP	MRP2	
Brij92	Polyoxyethylene 2 oleyl ether	312.53	4.9	0.0071	●			53
Brij97	Polyoxyethylene 10 oleyl ether	700	12.4	0.029	●			53,54
BL-9EX	Polyoxyethylene 9 lauryl ether	1,200	14.5	0.08	●	●		53,54
Cremophor EL	Polyoxyethylene 35 castor oil	2,500	12-14	0.02	●	●	●	49,55,56
Labrasol	Caprylocaproyl macrogol-8 glycerides	425-1,160	12	0.01	●			51,57
Tween 20	Polyoxyethylene sorbitan monolaurate	1,200	16.7	0.03	●	●		49,58
Pluronic F68	PEO_{80}-PPO_{27}-PEO_{80}	7,680-9,510	>24	0.77-0.95	●	●	●	50,56,57
Pluronic F127	PEO_{101}-PPO_{56}-PEO_{101}	12,600	18-23	0.004	●		●	56,59
Vitamin E acetate	Tocopheryl acetate	472.74	N.A.	0.02				

CMC, critical micelle concentration; HLB, hydrophilic lipophilic balance; MW, molecular weight; N.A., not applicable; PEO, polyethylene oxide; PPO, polypropylene oxide

Data are summarized from *J. Pharm. Sci.,* **107,** 2946-2956 (2018); Table 1 and *J. Pharm. Sci*., **108,** 1315-1325 (2019); Table 1.

Two model BCRP substrates (sulfasalazine and topotecan) were selected to this study. The pharmaceutical excipients were initially screened for their *in vitro* BCRP inhibitory activity using a diffusion chambers with rat intestinal membrane or a Caco-2 permeation method. Any excipient which showed its ability to modulate BCRP transporter was subjected for an *in situ* closed loop intestinal absorption experiment in rats. Because the pharmaceutical excipients were known to open the intercellular tight junctions, they might increase the paracellular permeation of the drugs.[60–62] Therefore, all transport parameters of model compounds were compared with the transport parameters of 5(6)-carboxyfluorescein (CF), a non-BCRP substrate and a paracellular transport marker using the identical experiment configurations. The inhibitory mechanisms of these pharmaceutical excipients were studied by measuring the membrane fluidity and the BCRP mRNA expression. In addition, the micellar interactions between pharmaceutical excipients and the model drugs were also evaluated. Lastly, the toxicity of these pharmaceutical excipients on the intestinal epithelial cells was also evaluated after the *in situ* closed loop experiments.

Chapter I: Effects of various pharmaceutical excipients on the intestinal transport and absorption of sulfasalazine, a model substrate of BCRP transporter

Sulfasalazine, a drug used for the treatment of inflammatory bowel diseases such as ulcerative colitis and Crohn's disease, was chosen as a model substrate of BCRP transporter in this chapter. The chemical structures of sulfasalazine and its metabolites are shown in Fig. 2. Despite of its calculated log P value of 3.88, which indicates a high permeability compound, sulfasalazine has less than 15% oral bioavailability as it remains unabsorbed until reaching a large intestine where it is broken down by an intestinal flora into its active metabolite 5-aminosalicylic acid (5-ASA or mesalazine) and a byproduct sulfapyridine.[21,63,64] The pathway of sulfasalazine after oral administration is summarized in Fig. 3. Sulfasalazine is reported to be a high-affinity substrate of BCRP transporter but not metabolized by CYP3A4.[64] These properties make sulfasalazine a perfect model for BCRP transporter studies.

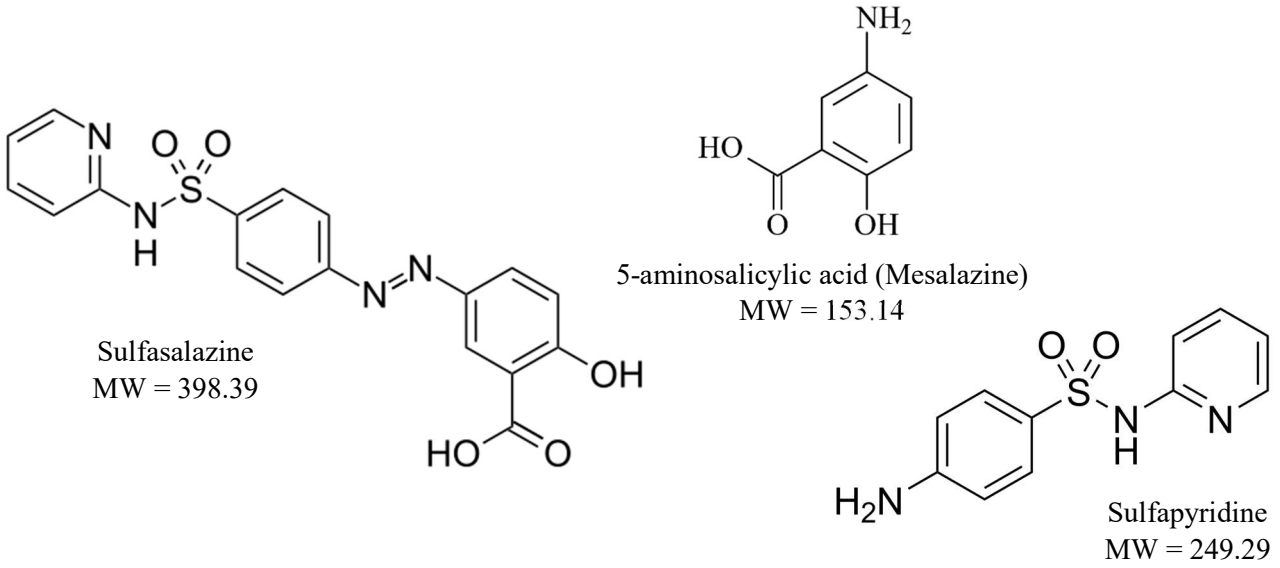


Figure 2. Chemical structures of sulfasalazine and its metabolites

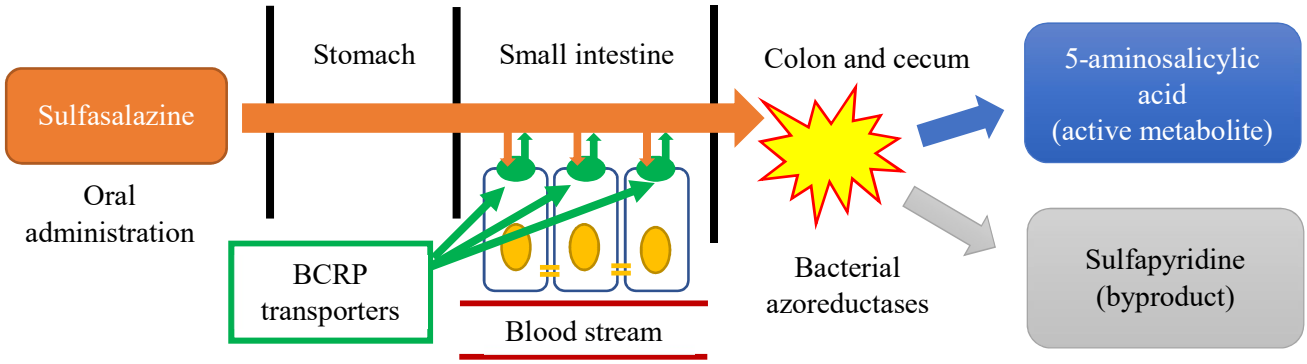


Figure 3. Pathway of sulfasalazine after its oral administration in humans

Researchers had examined and discussed about the effects of pharmaceutical excipients on the BCRP transporter. For example, Yamagata *et al.* reported that Cremophor EL and Tween 20 significantly increased the uptake of [^{3}H]mitoxantrone in BCRP expressing MDCK-II cells.[49] Therefore, both pharmaceutical excipients were included in this study. However, the information on how other pharmaceutical excipients modulate BCRP transporter was limited. In contrast, several pharmaceutical excipients were reported to inhibit P-gp transporters. For example, several polyoxyethylene alkyl ether derivatives (Brij92, Brij97, and BL-9EX) were reported to effectively inhibit P-gp transporter in rats.[53,54] Their main mechanisms involved the increase of membrane fluidity as well as the inhibition of P-gp ATPase enzymes.[54] These findings suggested that they might be also effective against BCRP transporters. Other pharmaceutical excipients including Labrasol and Pluronic F127 were included because they were reported to inhibit P-gp transporters.[51,59] Vitamin E (tocopheryl acetate) was included, because its derivative (D-α-tocopheryl polyethylene glycol 1000 succinate, vitamin E TPGS) was reported to inhibit P-gp via the similar mechanism as the polyoxyethylene alkyl ether derivatives.[47,48] Its result might be useful to identify crucial part of this excipient for its inhibitory activity. Furthermore, Ko143 and pantoprazole, BCRP inhibitors, were used as positive inhibitors of BCRP transporters. Additionally, the effect of these pharmaceutical excipients on the metabolism of sulfasalazine in the small intestine was also evaluated by measuring the plasma level of 5-ASA, an active metabolite of sulfasalazine.

The goal of this chapter is to identify the BCRP modulating potential from 8 different types of pharmaceutical excipients. The pharmaceutical excipients were initially screened using the diffusion chamber method. Small intestinal membranes were isolated from rats and were used as a permeation barrier. Further examinations were conducted using an *in situ* closed loop method.

1.1 Materials and methods

1.1.1 Materials

Brij92, Brij97, BL-9EX, and vitamin E acetate were obtained from Nikko Chemical Co., Ltd. (Tokyo, Japan). Tween 20, piroxicam, and sulfapyridine were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). CF, sulfasalazine, Cremophor EL, and Pluronic F127 were purchased from Sigma Aldrich Chemical Co., Ltd. (St. Louis, MO). Labrasol was kindly gifted from Gattefossé (Cedex, France). Pantoprazole sodium was purchased from LKT Laboratories, Inc. (St. Paul, MN., USA) Ko143 was purchased from Abcam Plc. (Cambridge, UK). LDH from chicken heart was purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). HPLC-grade acetonitrile and methanol were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). All other reagents were of laboratory grade.

1.1.2 Animals

Male Wistar rats weighing 240-260 g were purchased from Japan SLC Inc. (Shizuoka, Japan) and were maintained in a climate-controlled breeding facility equipped with 12-h automatic ambient light cycle control. Rats were fed with regular lab chow and given free access to tap water. The handling of animals and all experiments involving them were carried out in accordance with the "Regulations on Performing Animal Experiments 2nd Edition" and approved by the Animal Care and Use Committee at Kyoto Pharmaceutical University.

1.1.3 *In vitro* bi-directional transport assay of sulfasalazine and CF across rat intestinal membrane by a diffusion chamber method

Rats were fasted at least 16 h prior to the experiment. Tap water was given *ad libitum* during this fasting period. The author measured the bi-directional transport of model compounds by the method as described previously,[53,65] with minor modifications. The detailed procedure is described as follows. After rat was sedated with intraperitoneal injection of sodium pentobarbital (40 mg/kg), the whole abdomen section was exposed through the midline incision. The small intestinal section was exposed at approximately 1.5 cm below the pyloric sphincter and the 2.5 cm polyethylene tube (inner diameter 3 mm) was inserted and locked in place by using surgical suture. The other end of the small intestine was exposed at approximately 1.5 cm above the iliocecal junction. Pre-warmed pH 7.4 phosphate buffer saline (PBS) 37°C was used to flush this section to remove any remaining residue in the small intestine. After that, the rat was sacrificed, the small intestinal section was quickly removed and submerged in the PBS which was constantly supplied with carbogen (95% O_2 and 5% CO_2). The small intestinal section was cut into 2-cm pieces with the Peyer's patches intentionally excluded. Then, these 2-cm pieces of the small intestine were cut-open through the mesenteric line and the muscle layer was gently removed from the serosal side by using sharp-edge forceps. The intestinal membrane was immersed in a carbogen-saturated, ice-cold blank transport medium (Hank's balanced salt solution (HBSS) pH 7.4) before mounting on the diffusion chamber.

The transport of sulfasalazine or CF was initiated by filling the donor side of the chamber with 7 ml pre-warmed drug solution (100 μM or 10 μM for sulfasalazine or CF, respectively) and the same volume of pre-warmed blank transport medium to the receiver chamber. The chambers were maintained at 37°C and constantly supplied with carbogen. The samples were taken from the receiver side at a predetermined interval and the equal volume of blank transport medium was immediately replenished. The samples were kept at -20°C until analysis. The schematic of the experiment is illustrated in Fig. 4.

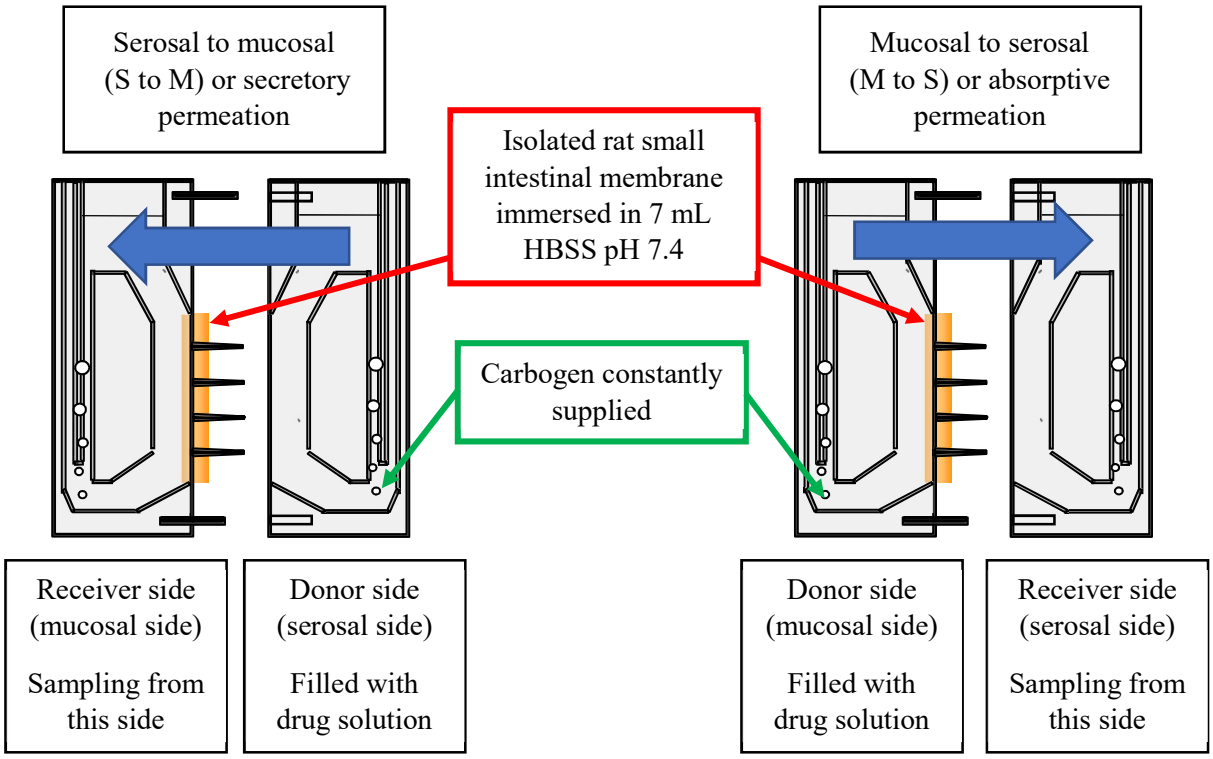


Figure 4. Schematic illustration of a diffusion chamber experiment. Blue arrows indicating the directions of the transport.

1.1.4 Intestinal absorption of sulfasalazine and CF by an *in situ* closed loop method in rats

Male Wistar rats weighing 240-260 g. were treated and anesthetized using the same procedure as described in the previous section. The small intestinal section was exposed at the same locations and was rinsed with pre-warmed PBS. Instead of only one polyethylene tube at below the pyloric sphincter, another tube was inserted at the other end of the small intestine above the iliocecal junction. Both tubes were tied with surgical suture to form a closed loop. Sulfasalazine solution (0.1 mg/mL in PBS) was administered to this small intestinal loop at 1 mg/kg. The blood samples were taken from the jugular vein at the predetermined-time intervals and were centrifuged at 13,000 rpm for 5 min. to separate a plasma. The plasma samples were kept frozen at -20°C until analysis. After 4 h absorption period, the small intestinal loop was flushed with ice-cold PBS and the rats were sacrificed. The intestinal content obtained during this last period was collected for further toxicity analysis.

For CF absorption experiment, CF solution (0.05 mg/mL in PBS) was administered to the small intestinal loop at 0.5 mg/kg. Blood samples were collected, centrifuged to separate the plasma and were kept in the light-protected ice-box. The plasma samples of CF were analyzed within 1 h. The schematic of this *in situ* closed loop experiment is illustrated in Fig. 5.

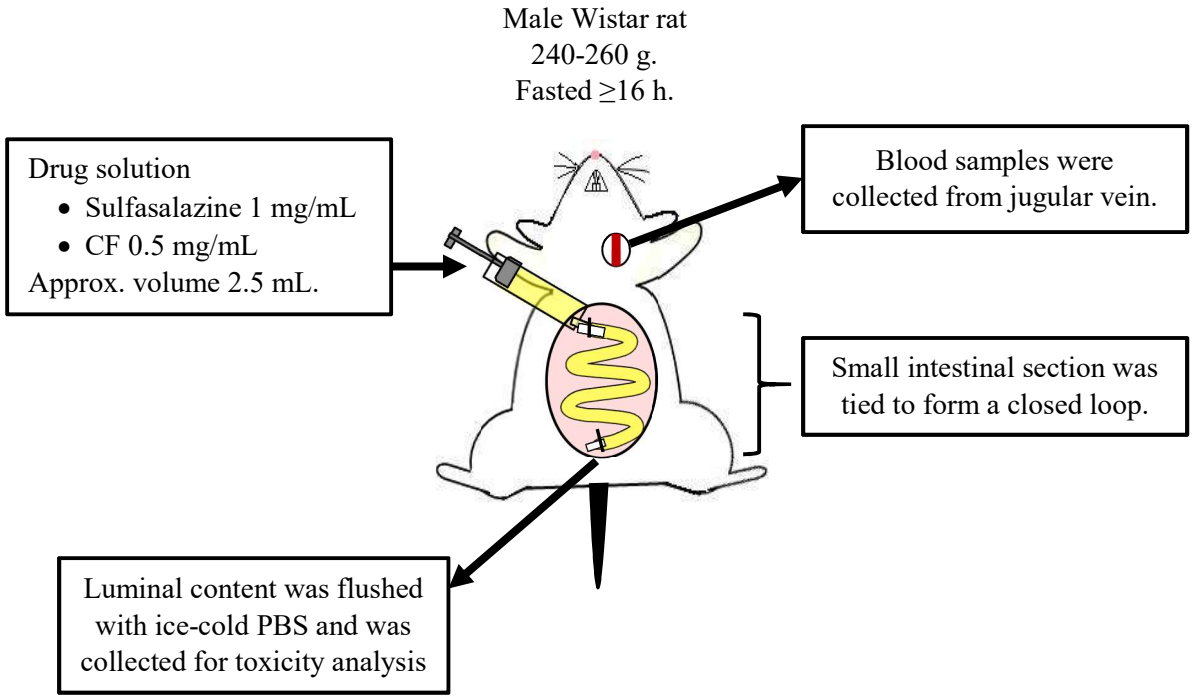


Figure 5. Schematic illustration of an *in situ* closed loop intestinal absorption experiment

1.1.5 Determination of sulfasalazine and 5-ASA by HPLC

Samples were thawed at room temperature if frozen. Protein was removed from the samples by adding three-volume (i.e. 300 µL for 100 µL sample) of ice-cold methanol containing 300 ng/mL piroxicam as internal standard. The samples were vortexed for 30 s. and centrifuged at 13,000 rpm 5°C for 10 min. The clear supernatant was injected into HPLC system consisting of DGU-20A on-line degassing unit, LC-20AD parallel-type double plunger pumps, SIL-20A autosampler, CTO-10A column oven, and SPD-20A UV/VIS detector (Shimadzu Co., Kyoto, Japan). Data collection and integration were carried out by Chromato-PRO version 5.0 (Runtime Instrument Co., Ltd., Japan).

For the analysis of sulfasalazine, isocratic delivery of mobile phase (30 mM pH 3 KH_2PO_4: acetonitrile, 67:33) was used at a flow rate of 1 mL/min. The analytical columns used for plasma and non-plasma sample were Cosmosil 5C18-MS-II 4.6×250 mm and Cosmosil 5C18-MS-II 4.6×150 mm (Nacalai Tesque Inc., Kyoto, Japan), respectively. The absorbance of sulfasalazine and internal standard was observed at 360 nm. Typical runtime of each sample was 10 min for non-plasma sample and 18 min for plasma sample. The lower limit of detection was 15 ng/mL, and the linearity was achieved throughout the analytical range with $r^2 > 0.99$. The concentration of sulfasalazine was calculated from the peak area ratios of sulfasalazine to the internal standard.

For the analysis of 5-ASA in plasma sample, the mobile phase was changed to 10 mM pH 6.5 K_2HPO_4 containing 10 mM tetrabutylammonium hydroxide:acetonitrile, 85:15, flow rate = 1 mL/min. The analytical column was 5C18-MS-II 4.6×250 mm. 5-ASA was monitored using the RF-10AXL fluorescence detector at the excitation and emission wavelength at 360 nm and 465 nm, respectively. The typical runtime of each sample was 15 min. The lower limits of detection and quantification from 100 μL plasma sample were 4.57 and 15.23 ng/mL, respectively.

1.1.6 Determination of CF

For measuring the concentration of CF in non-plasma sample, 100 μL of non-plasma sample was directly transferred to 96-well dark plate. The concentration of CF was determined by a Synergy® HT multi-detection microplate reader (Biotek Instrument Inc., Winooski, VT, USA) with the excitation and emission wavelengths of 485 and 528 nm, respectively. For plasma sample, 100 μL of ice-cold acetonitrile was added and the sample was vortexed for 30 s to precipitate the protein. After centrifugation at 13,000 rpm for 10 min, 100 μL of clear supernatant was transferred to 96-well dark plate and analyzed by microplate reader as described previously.

1.1.7 Calculation and statistical analysis

The apparent permeability coefficient (P_{app}) was calculated from the diffusion chamber experiments by using the following equation.

$$P_{app} = \frac{Flux}{Area \cdot C_0 \cdot 60}$$

P_{app} is the apparent permeability coefficient (cm/s). Flux is the appearance rate of sulfasalazine or CF in the receiver chamber, which was calculated from the linear portion of the cumulative permeated amount versus time curve. Area is the surface area of the permeation membrane (1.78 cm^2) and C_0 is the initial concentration of sulfasalazine and CF (100 μM and 10 μM, respectively). Additionally, the ratio of secretory transport to absorptive transport was illustrated as the efflux ratio (ER). It was calculated by the following equation.

$$Efflux\ ratio\ (ER) = \frac{P_{app\ S \to M}}{P_{app\ M \to S}}$$

$P_{app\ S \to M}$ refers to the apparent permeability coefficient of the secretory (i.e. serosal to mucosal side of the chamber) permeation. $P_{app\ M \to S}$ refers to the apparent permeability coefficient of the absorptive (i.e. mucosal to serosal) permeation.

For the *in situ* closed loop experiment, the area under concentration versus time curves ($AUC_{0\text{-}4\ h}$) values were calculated using the linear trapezoidal rule. The enhancement ratios were calculated by dividing the average value of $AUC_{0\text{-}4\ h}$ from each experiment with the average $AUC_{0\text{-}4\ h}$ of the non-treatment control.

All experiments were performed at least in triplicates. The concentrations of pharmaceutical excipients are stated as percent weight by volume (% w/v). Data are presented as mean ± standard error (S.E.) unless stated otherwise. Statistical differences were evaluated by SPSS™ Statistic version 17.0 using independent-samples *t*-test or 1-way ANOVA followed by Dunnett's t-test. $P < 0.05$ is considered as a minimal level of significance.

1.2 Results and discussions

1.2.1 Bi-directional transport of sulfasalazine across rat intestinal membrane determined by a diffusion chamber method.

The transport of sulfasalazine, a model BCRP substrate was examined by using rat intestinal membrane as the permeation barrier. The cumulative permeated and secreted amounts of sulfasalazine in the presence or absence of various types of pharmaceutical excipients are shown in Fig. 6. The transport parameters of sulfasalazine across rat small intestinal membrane are summarized in Table 2.

Fig. 6 (a)

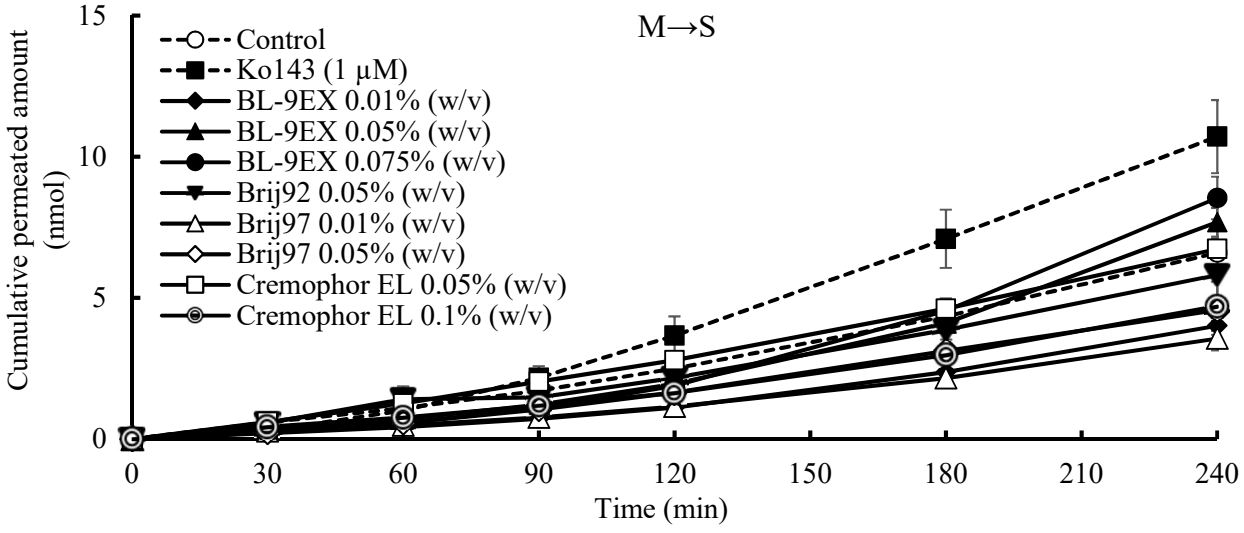


Fig. 6 (b)

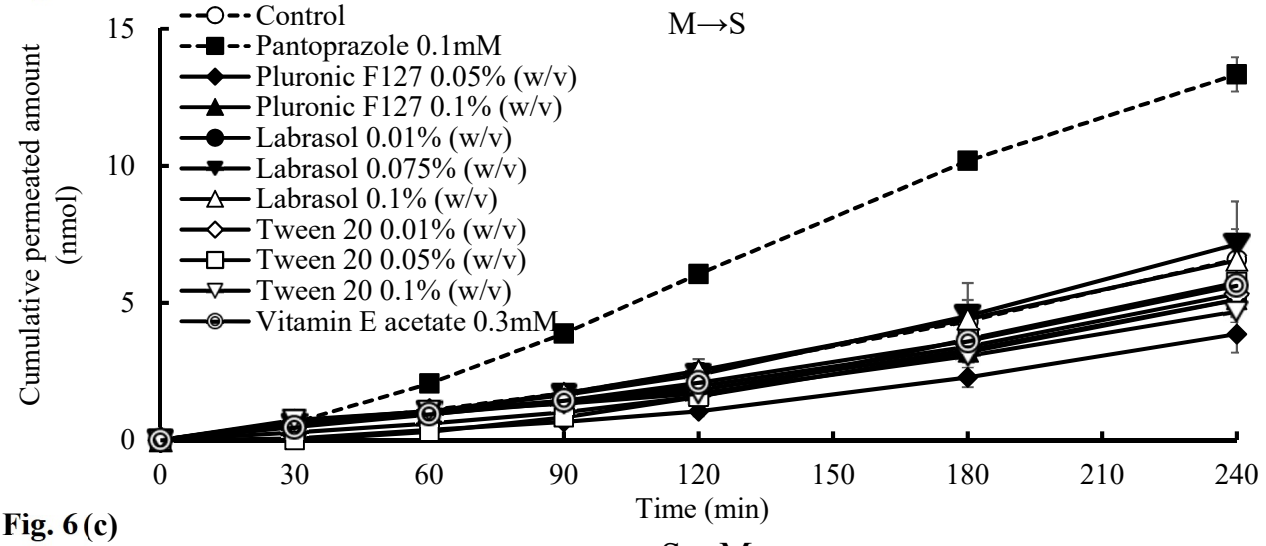


Fig. 6 (c)

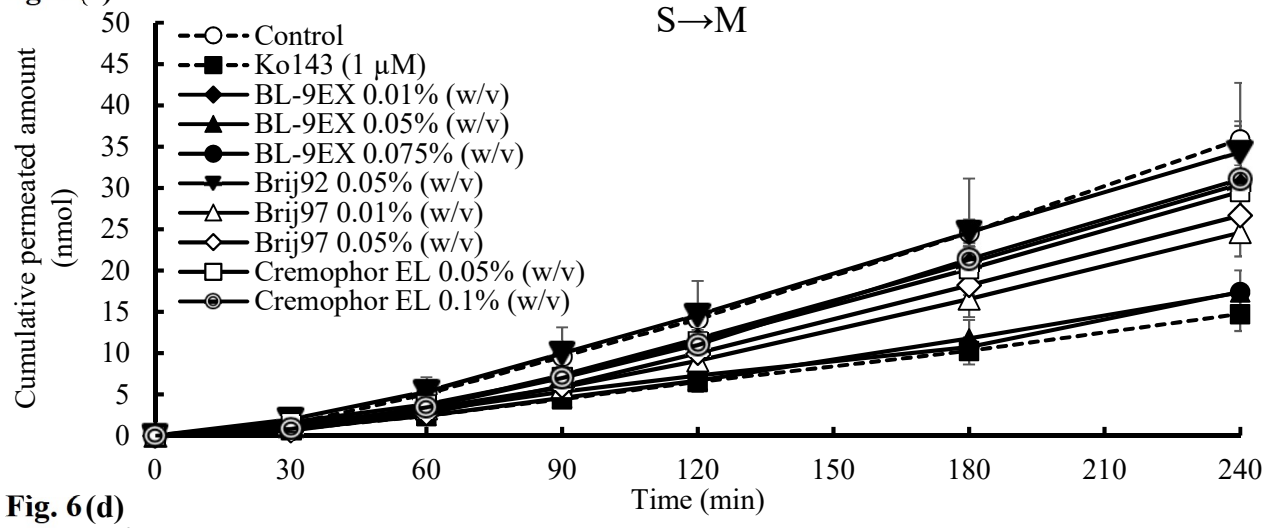


Fig. 6 (d)

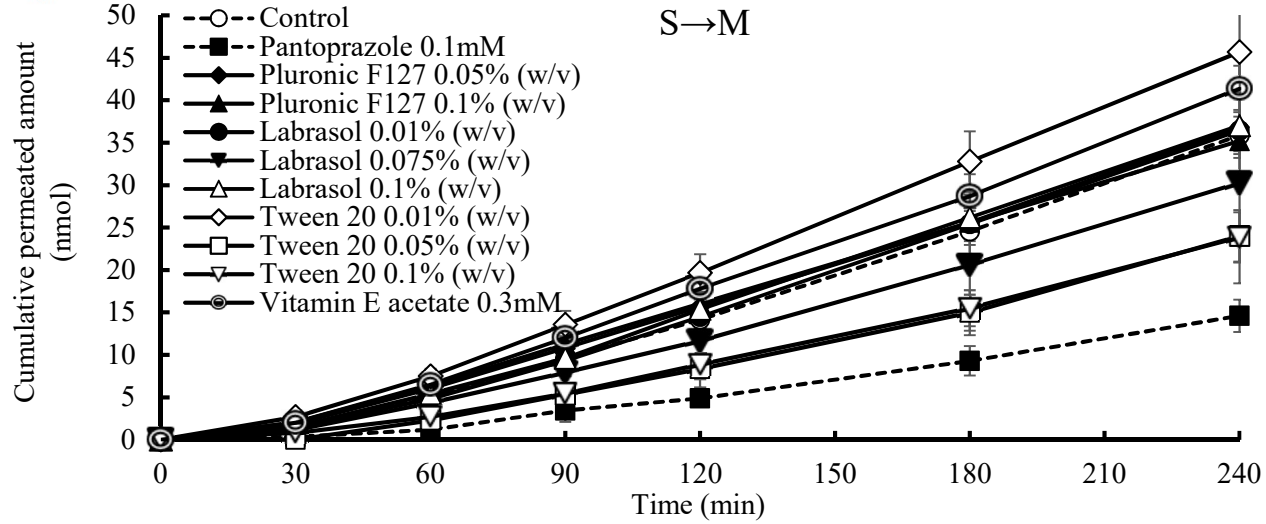


Figure. 6 Time courses of (a, b) absorptive (M→S) and (c, d) secretory (S→M) transport of sulfasalazine in the presence or absence of pharmaceutical excipients across the rat intestinal membrane. Data are expressed as the mean ± S.E. of at least 3 experiments. (*J. Pharm. Sci.,* **107,** 2946-2956 (2018); Figure 1)

Table 2. Intestinal transport of sulfasalazine in the presence of various types of pharmaceutical excipients by an *in vitro* diffusion chamber method

Group	Concentrations (% w/v)	P_{app} ($\times 10^{-6}$ cm/s)		Efflux ratios
		M→S	S→M	
Control		3.20 ± 0.22	16.95 ± 0.95	5.30
BL-9EX	0.01	2.26 ± 0.24	14.63 ± 1.27	6.47
	0.05	4.47 ± 0.27**	8.32 ± 1.05**	1.86
	0.075	5.19 ± 0.44**	7.89 ± 0.45**	1.52
Brij92	0.05	2.84 ± 0.59	15.38 ± 3.39	5.42
Brij97	0.01	1.88 ± 0.20	12.11 ± 1.18*	6.44
	0.05	2.28 ± 0.27	13.01 ± 1.03*	5.70
Cremophor EL	0.05	3.08 ± 0.51	16.32 ± 0.32	5.30
	0.1	2.39 ± 0.49	15.61 ± 3.67	6.53
Pluronic F-127	0.05	2.20 ± 0.32	16.50 ± 0.70	7.50
	0.1	2.75 ± 0.36	14.97 ± 0.65	5.44
Labrasol	0.01	2.57 ± 0.39	17.13 ± 0.83	6.67
	0.075	3.72 ± 0.78	10.95 ± 1.70*	2.84
	0.1	3.14 ± 0.61	16.90 ± 3.10	5.38
Tween 20	0.01	2.63 ± 0.26	20.31 ± 1.98	8.61
	0.05	3.26 ± 0.52	12.23 ± 1.53*	3.75
	0.1	2.33 ± 0.33	11.68 ± 1.27*	5.01
Vitamin E acetate	300 μM	2.77 ± 0.36	18.43 ± 1.53	6.65
Ko143	1 μM	5.49 ± 0.57**	6.43 ± 0.76**	1.17
Pantoprazole	100 μM	5.68 ± 0.74**	7.58 ± 0.78**	1.33

Results are expressed as mean ± S.E. of at least 3 experiments. (**) *p<0.01*, (*) *p<0.05,* compared with the control. (*J. Pharm. Sci.,* **107,** 2946-2956 (2018); Table 2)

As shown in Fig. 6 (a), 6 (c), and Table 2. The secretory transport of sulfasalazine was much greater than its absorptive transport. The P_{app} for the secretory (S→M) and absorptive (M→S) of sulfasalazine alone was 16.95 ± 0.95 and 3.20 ± 0.22 ($\times 10^{-6}$ cm/s), respectively. The efflux ratio was 5.30, indicating that the secretory transport of sulfasalazine across rat intestinal membrane was approximately 5.30-time faster than its absorptive transport. These results are in an accordance to the previous reports.[21,63] After the addition of Ko143, a highly potent and specific BCRP inhibitor, the secretory transport of sulfasalazine was significantly reduced to 6.43 ± 0.76×10^{-6} cm/s, (*p<0.01*). The absorptive transport of sulfasalazine was also significantly increased to 5.49 ± 0.57×10^{-6} cm/s, (*p<0.01*). The efflux ratio was reduced to 1.17 in the presence of 1 μM Ko143. These results suggest that the directional transport was completely neutralized and rodent Bcrp1 is the main mechanism for the efflux transport of sulfasalazine. Additionally,

pantoprazole was used as an alternative BCRP inhibitor. Pantoprazole was recognized as BCRP substrate and was used as a BCRP modulator in several *in vivo* studies.[64,66–68] Results showed that pantoprazole also inhibited Bcrp1 but at much lower potency than Ko143. 100 μM pantoprazole reduced the secretory P_{app} to $7.58 \pm 0.78 \times 10^{-6}$ cm/s, increased the absorptive Papp to $5.68 \pm 0.74 \times 10^{-6}$ cm/s, and the efflux ratio was reduced to 1.33. These results suggested that pantoprazole might inhibit the Bcrp1-mediated sulfasalazine transport by competitively binding to Bcrp1, resulting in the saturation of the transporters and the subsequent reduced binding to sulfasalazine.

Several pharmaceutical excipients demonstrated their ability to reduce the Bcrp1-mediated secretory transport of sulfasalazine. The most promising result was observed in the presence of the polyoxyethylene alkyl ether derivative, BL-9EX. Especially at 0.075%, BL-9EX significantly decreased the secretory P_{app} of sulfasalazine to 7.89 ± 0.45 and increased the absorptive P_{app} to 5.19 ± 0.44 ($\times 10^{-6}$ cm/s), $p<0.01$. The efflux ratio was reduced to 1.52 which also suggested that BL-9EX can neutralize the directional transport of sulfasalazine. After decreasing the concentration to 0.05%, BL-9EX still improved the transport of sulfasalazine by significantly decreasing the secretory P_{app} and increasing the absorptive P_{app}. The other pharmaceutical excipients including Brij97, Labrasol, and Tween 20 demonstrated the ability to modulate Bcrp1 transporter by significantly decreasing the secretory transport of sulfasalazine. However, none of them was able to increase the absorptive P_{app} of sulfasalazine. Interestingly, dose-independent responses were observed in the presence of Labrasol. At 0.01% and 0.1%, Labrasol did not affect the secretory transport of sulfasalazine. However, at 0.075%, the secretory transport of sulfasalazine was significantly reduced. This observation suggested that 0.1% Labrasol might inhibit Bcrp1 but their ability was hindered by some other factors.

Other polyethylene glycol (PEG) containing pharmaceutical excipients including Cremophor EL and Pluronic F127 did not affect the transport of sulfasalazine in this experiment. Cremophor EL was reported to modulate P-gp and BCRP in other *in vitro* experiments.[44,49] However, its potency was weak and might be able to interfere the transport of sulfasalazine, which is the high-affinity substrate of BCRP. Moreover, the difference in the model used in this study might be the other contributing factor.

Pluronic is a long-chain block copolymer consisted of hydrophilic polyethylene oxide (PEO) and hydrophobic polypropylene oxide (PPO) moieties which are arranging in PEO-PPO-PEO fashion. They were well documented that lipophilic Pluronics with immediate length of PPO block from 30-60 units and the hydrophilic lipophilic balance (HLB) value < 20 such as Pluronic P85 (PEO_{26}-PPO_{40}-PEO_{26}) are the most effective for inhibiting P-gp efflux.[69] These lipophilic Pluronics are able to penetrate the cell membrane and reach the mitochondria, where they caused an ATP depletion and a subsequent decrease in P-gp ATPase activities.[45,69,70] Unlike Pluronic P85, Pluronic F127 is hydrophilic because it has much longer

PEO chains. This could prevent Pluronic F127 to penetrate the cell membrane because of this hydrophilic property. Therefore, its ability to modulate Bcrp1-mediated sulfasalazine transport was greatly limited, as observed in this chapter. Additionally, there is a report that Pluronic F127 improved the absorption of rhodamine123 in rat everted gut sacs.[59] However, its effect was minimal and required a relatively higher concentration (1% w/v) to achieve a preferable result.

Vitamin E TPGS is a water-soluble form of vitamin E derivative. It contains a hydrophilic PEG chain and the hydrophobic α-tocopherol head. Vitamin E TPGS was reported to inhibit P-gp by the mechanism affecting the membrane fluidity, conformational flexibility and intracellular ATP levels.[47,48] In this chapter, vitamin E alone did not affect sulfasalazine transport across rat small intestine likely because it lacks the hydrophilic PEG chain. This finding suggested that the PEG moieties are the crucial elements for the pharmaceutical excipients to achieve their inhibitory effects on P-gp or BCRP transporters.

1.2.2 Bi-directional transport of CF across rat intestinal membrane determined by a diffusion chamber method.

The author examined the transport of CF across rat intestinal membrane using the same diffusion chamber method to ensure that the improvement in sulfasalazine permeation was indeed via the transporter inhibition in the transcellular transport but not by the increase in paracellular permeation caused by the leaky tight junctions. The transport parameters of CF across rat small intestinal membrane are summarized in Table 3.

Table 3. Intestinal transport of 5(6)-carboxyfluorescein in the presence of various types of pharmaceutical excipients by an *in vitro* diffusion chamber method

Group	Concentrations (% w/v)	P_{app} ($\times 10^{-6}$ cm/s)		Efflux ratios
		M→S	S→M	
Control		5.29 ± 0.43	6.05 ± 0.79	1.14
Ko143	1 μM	5.13 ± 0.58$^{N.S.}$	5.46 ± 0.26$^{N.S.}$	1.06
BL-9EX	0.05	6.04 ± 0.40$^{N.S.}$	5.58 ± 0.53$^{N.S.}$	0.92
	0.075	6.56 ± 0.17$^{N.S.}$	6.17 ± 0.34$^{N.S.}$	0.94
Brij97	0.01	5.04 ± 0.89$^{N.S.}$	4.55 ± 0.20$^{N.S.}$	0.90
	0.05	5.93 ± 0.43$^{N.S.}$	6.88 ± 0.45$^{N.S.}$	1.16
Labrasol	0.075	5.13 ± 0.75$^{N.S.}$	6.42 ± 0.49$^{N.S.}$	1.25
Tween 20	0.05	5.31 ± 0.79$^{N.S.}$	7.21 ± 0.53$^{N.S.}$	1.36
	0.10	5.33 ± 0.33$^{N.S.}$	5.23 ± 0.49$^{N.S.}$	0.98

Results are expressed as mean ± S.E. of at least 3 experiments. (N.S.) not significantly different, compared with the control. (*J. Pharm. Sci.,* **107,** 2946-2956 (2018); Table 3)

The transport parameters of CF across rat small intestinal membrane showed that the transport of CF was evenly distributed into both directions. The absorptive and secretory P_{app} values of CF were 5.29 ± 0.43, and $6.05 \pm 0.79 \times 10^{-6}$ cm/s, respectively. The efflux ratio was 1.14, suggesting that there was no directional transport. Interestingly, the transport parameters of CF are similar to those of sulfasalazine with the presence of 1 μM Ko143, a positive inhibitor of BCRP transporter. These findings suggested that in the presence of BCRP inhibitor, sulfasalazine slowly diffuses across rat intestinal membrane in the same rate as CF.[71] Additionally, the transport parameters of CF were not changed after the addition of 1 μM Ko143, suggesting that CF is not a substrate of BCRP transporter.

PEG-containing pharmaceutical excipients are known to open the tight-junctions, causing the leakage of intercellular barriers and subsequent increase the paracellular permeation. This mechanism could be applied to improve the paracellular permeation of poorly soluble or poorly absorbable drugs such as curcumin, alendronate, etc.[60–62,72] In general, the concentration needed to open the tight-junctions is significantly higher than that needed to effectively inhibit P-gp or BCRP transporters. BL-9EX, Brij97, Labrasol, and Tween 20 at the concentrations which could improve the permeation of sulfasalazine only slightly changed the permeation parameters of CF. However, the differences are within the margin of errors and none of them was significantly different compared the untreated control. These findings suggested that the pharmaceutical excipients at the evaluated concentrations did not affect the paracellular transport of CF as well as sulfasalazine. The improvement in sulfasalazine permeation as seen in this chapter is likely caused by the inhibition of Bcrp1 transporters by these pharmaceutical excipients.

1.2.3 Intestinal absorption of sulfasalazine determined by an *in situ* closed loop method

BL-9EX, Brij97, Labrasol, and Tween 20 were further examined for their ability to modulate intestinal Bcrp1 transporters using an *in situ* closed loop method in rats. The plasma concentration time curves and pharmacokinetic parameters of sulfasalazine in the presence and absence of various pharmaceutical excipients are shown in Fig. 7 and Table 4.

As seen in Fig. 7 and Table 4, plasma concentration of sulfasalazine after its intestinal administration (1 mg/kg) slowly increased and achieved its maximum concentration (C_{max}) at 393 ± 33 ng/mL. Time to maximum plasma concentration (T_{max}) of sulfasalazine was 120 ± 30 min, and the area under concentration time curve ($AUC_{0\text{-}4\ h}$) was 73.1 ± 5.5 μg·min/mL, suggesting that sulfasalazine was slowly absorbed from the small intestine.

Fig. 7 (a)

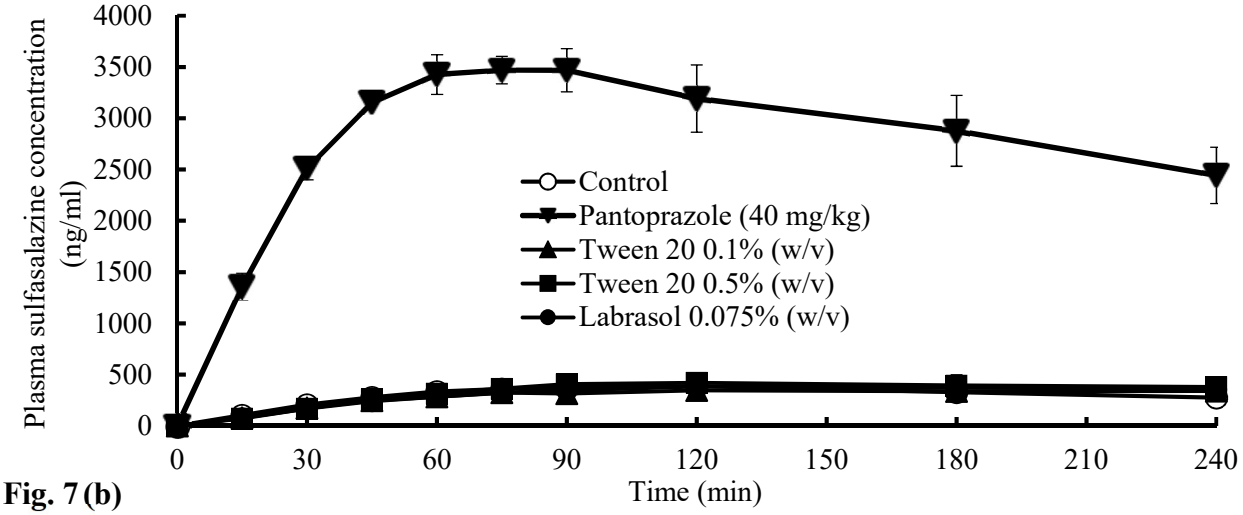


Fig. 7 (b)

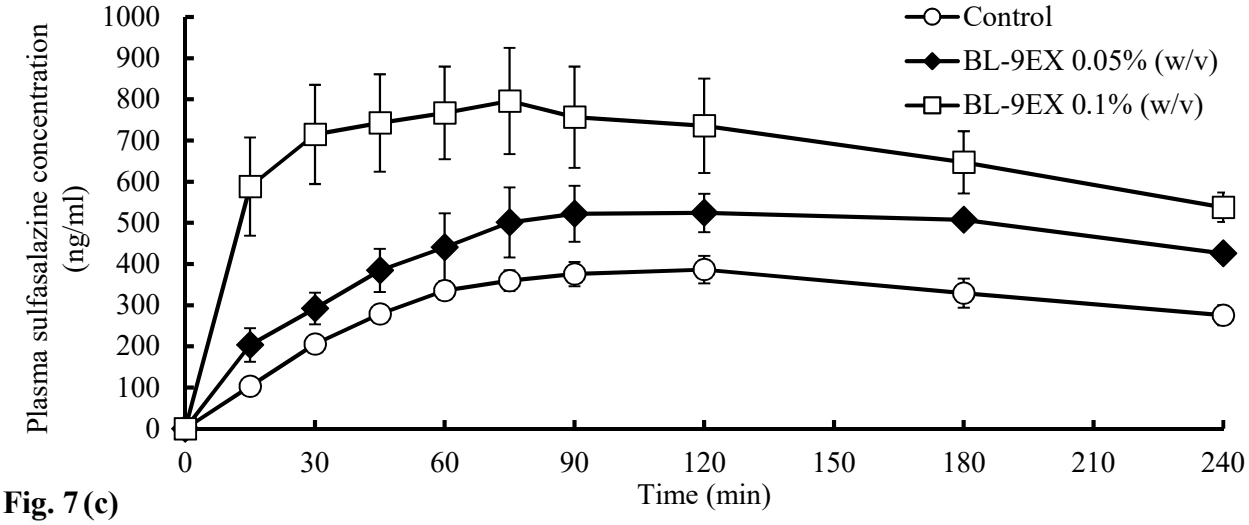


Fig. 7 (c)

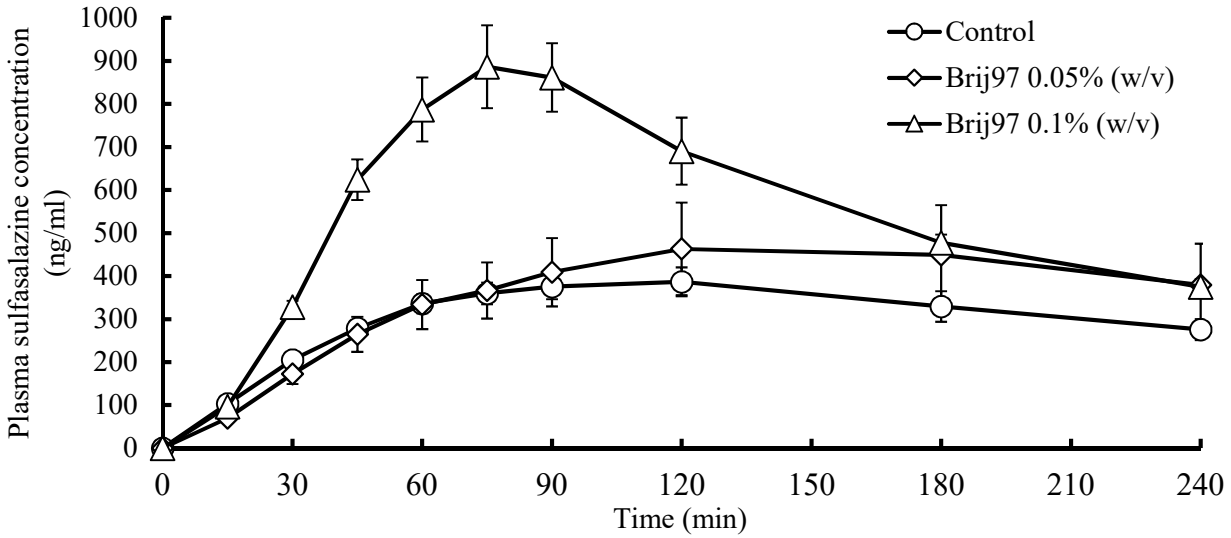


Figure 7. Plasma concentration time curves of sulfasalazine after the intestinal administration of 1 mg/kg sulfasalazine with pantoprazole, Tween 20, or Labrasol (a), BL-9EX (b), and Brij 97 (c). Data are expressed as the mean ± S.E. of at least 3 animals. (*J. Pharm. Sci.,* **107,** 2946-2956 (2018); Figure 2)

Table 4. Pharmacokinetics parameters of sulfasalazine after the intestinal administration in the presence or absence of various types of pharmaceutical excipients measured by an *in-situ* closed loop method.

Experiment	Pharmacokinetic parameters			Enhancement ratio
	C_{max} (ng/ml)	T_{max} (min)	$AUC_{0\text{-}4\ h.}$ (μg·min/ml)	
Control	393 ± 33	120 ± 30	73.1 ± 5.5	-
Pantoprazole (40 mg/kg)	3,517 ± 174**	85 ± 5	676.3 ± 50.7**	9.25
Tween 20 0.1% (w/v)	370 ± 56	125 ± 30	69.8 ± 10.3	0.95
Tween 20 0.5% (w/v)	451 ± 70	130 ± 27	80.7 ± 12.2	1.10
Labrasol 0.075% (w/v)	395 ± 54	180 ± 35	74.5 ± 11.2	1.02
BL-9EX 0.05% (w/v)	553 ± 58*	145 ± 35	105.9 ± 9.1*	1.45
BL-9EX 0.1% (w/v)	843 ± 136*	65 ± 18	159.2 ± 19.6**	2.18
Brij 97 0.05% (w/v)	478 ± 108	130 ± 18	86.4 ± 19.1	1.18
Brij 97 0.1% (w/v)	888 ± 94**	80 ± 5	131.1 ± 10.0**	1.79

Results are expressed as mean ± S.E. of at least 3 animals. (**) $p < 0.01$, (*) $p < 0.05$, compared with the control. (*J. Pharm. Sci.,* **107,** 2946-2956 (2018); Table 5)

Pantoprazole was used as a positive inhibitor of Bcrp1 transporter in this experiment. The inhibitory potency of pantoprazole toward BCRP was low and the dose required to ensure the adequate inhibition of Bcrp1 is significantly greater than its therapeutic dose. The dose of 40 mg/kg was chosen because it was reported to completely inhibit Bcrp1 in rats by other researchers.[66] Results showed that co-administration with pantoprazole greatly increased the plasma concentration of sulfasalazine. The C_{max} of sulfasalazine was significantly increased to 3,517 ± 174 ng/mL, almost 9-fold compared to control. T_{max} was shortened to 85 ± 5 min and over 9-fold increase in $AUC_{0\text{-}4\ h}$ of sulfasalazine was observed during the co-administration with 40 mg/kg pantoprazole (676.3 ± 50.7 μg·min/mL). These results illustrated that pantoprazole could be used as a positive BCRP inhibitor for *in vivo* experiments, suggested that the absorption of sulfasalazine was limited by the presence of Bcrp1 transporters in rats.[67]

The concentrations of pharmaceutical excipients in this experiment were adjusted to be slightly higher than those in the diffusion chamber experiments because the intestinal membrane in the diffusion chamber experiment was more fragile due to more invasive operation during the preparation procedure. Similar to the diffusion chamber experiments, BL-9EX significantly improved the absorption of sulfasalazine. Both C_{max} and $AUC_{0\text{-}4\ h}$ were significantly increased to 843 ± 136 ng/mL ($p < 0.05$) and 159.2

± 19.6 μg·min/mL (*p<0.01*), respectively in the presence of 0.1% BL-9EX. This improvement was still observed after reducing BL-9EX. The C_{max} and $AUC_{0\text{-}4\ h}$ were significantly increased to 553 ± 58 ng/mL (*p<0.05*) and 105.9 ± 9.1 μg·min/mL (*p<0.05*), respectively in the presence of 0.05% BL-9EX. These results suggested that BL-9EX is a potent inhibitor of Bcrp1. Additionally, 0.1% Brij97 significantly improved the intestinal absorption of sulfasalazine by increasing C_{max} and $AUC_{0\text{-}4\ h}$ values of sulfasalazine to 888 ± 94 ng/mL and 131.1 ± 10.0 μg·min/mL, respectively (*p<0.01*). This finding suggested the possibility that Brij97 could be used as the effective Bcrp1 inhibitor.

Other pharmaceutical excipients including Tween 20 and Labrasol did not increase the intestinal absorption of sulfasalazine even at their maximum applicable concentrations. These results are in contrast to other reports[49,58] possibly due to the high-binding affinity of sulfasalazine to Bcrp1 transporters and the weaker potency of Tween 20 and Labrasol compared to BL-9EX or Brij97.

1.2.4 Intestinal absorption of CF determined by an *in situ* closed loop method

BL-9EX and Brij97, which were able to improve sulfasalazine absorption in rats, were further examined with CF absorption using a same experiment procedure. CF was intestinally administered to the rats at 0.5 mg/kg. The plasma concentration time curves and pharmacokinetic parameters of CF in the presence and absence of various pharmaceutical excipients are shown in Fig. 8 and Table 5.

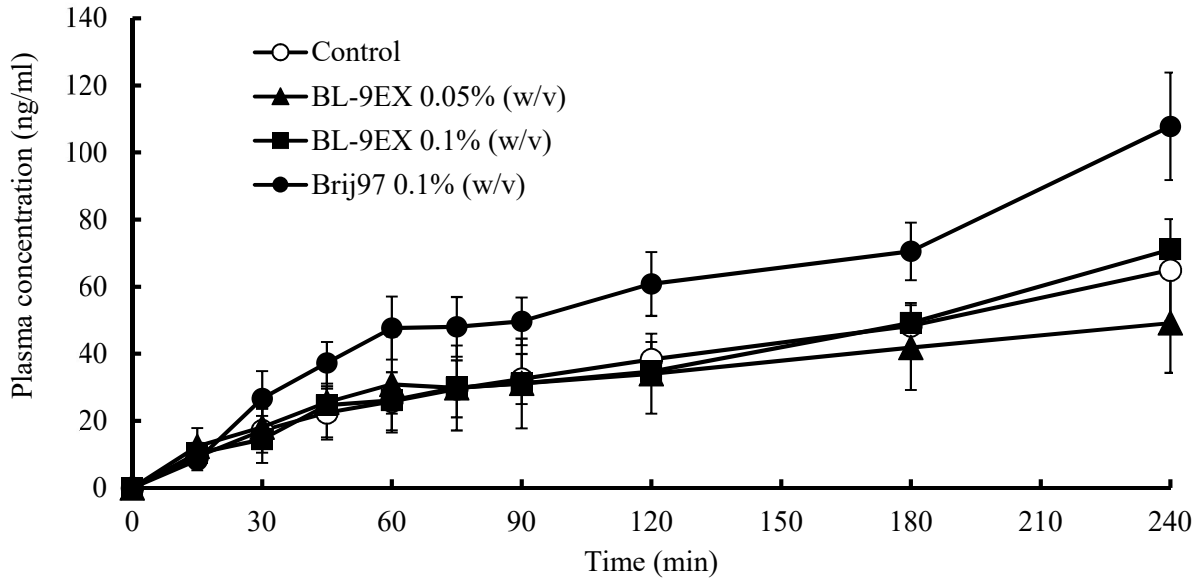


Figure 8. Plasma concentration time curves of 5(6)-carboxyfluorescein after the intestinal administration of 0.5 mg/kg CF with 0.05 %, 0.1 % BL-9EX, and 0.1 % Brij 97. Data are expressed as the mean ± S.E. of at least 3 animals. (*J. Pharm. Sci.,* **107,** 2946-2956 (2018); Figure 4)

Table 5. Pharmacokinetic parameters of 5(6)-carboxyfluorescein after intestinal administration with or without various types of pharmaceutical excipients by an *in-situ* closed loop method

Experiment	Pharmacokinetic parameters			Enhancement ratio
	C_{max} (ng/ml)	T_{max} (min)	$AUC_{0\text{-}4\ h.}$ (μg·min/ml)	
Control	66.8 ± 13.6	190 ± 50	8.80 ± 0.41	-
BL-9EX 0.05% (w/v)	49.2 ± 14.9	240 ± 0	$7.87 \pm 2.72^{N.S.}$	0.89
BL-9EX 0.1% (w/v)	71.1 ± 2.2	240 ± 0	$8.85 \pm 0.49^{N.S.}$	1.01
Brij97 0.1% (w/v)	107.8 ± 16.0	240 ± 0	$13.78 \pm 1.15^{*}$	1.57

Results are expressed as mean ± S.E. of at least 3 animals. (*) *p<0.05,* (N.S.) not significantly different, compared with the control. (*J. Pharm. Sci.,* **107,** 2946-2956 (2018); Table 6)

As shown in Fig. 8 and Table 5, without any excipient, the C_{max} and $AUC_{0\text{-}4\ h}$ values of CF were 66.8 ± 13.6 ng/mL and 8.80 ± 0.41 μg·min/mL, respectively. These values are comparable to the previous report.[60] Considering that the CF was administered at only a half dose of sulfasalazine (0.5 mg/kg vs. 1 mg/kg), the $AUC_{0\text{-}4\ h}$ of CF was approximately 12% compared to that of sulfasalazine. These findings suggested that the intestinal absorption of CF was very poor and even lower than sulfasalazine.

BL-9EX did not increase the intestinal absorption of CF, a paracellular transport marker even at the maximum dose (0.1%). The results suggested that at the concentration up to 0.1%, BL-9EX did not affect the integrity of the tight-junctions and did not increase the paracellular permeation. This supports the hypothesis that the improvement in sulfasalazine absorption was caused by the inhibition of Bcrp1 in the presence of BL-9EX and was not due to the increase in paracellular permeation.

In contrast, the author observed that 0.1% Brij97 significantly increased the absorption of CF. The $AUC_{0\text{-}4\ h}$ value of CF was increased to 13.78 ± 1.15 μg·min/mL (*p<0.05*), suggesting that there was an increase in the paracellular permeation which was likely caused by Brij97. From these findings, it is possible that Brij97 might improve the permeation of sulfasalazine via the paracellular pathway.

1.2.5 Effects of the pharmaceutical excipients on the intestinal metabolism of sulfasalazine

Sulfasalazine was degraded to its metabolites 5-ASA and sulfapyridine by the intestinal bacteria.[21,63,64] There is some possibility that the pharmaceutical excipients might interfere with the intestinal bacteria, limit its ability to metabolize sulfasalazine and the subsequent increase in the intestinal absorption of sulfasalazine. The plasma concentration of 5-ASA should be decreased if there was a

significant inhibition of sulfasalazine metabolism by the excipient. Therefore, the author measured the plasma concentration of 5-ASA after the intestinal administration of sulfasalazine by the *in situ* closed loop method. The plasma concentration versus time curves of 5-ASA after the intestinal administration of 1 mg/kg sulfasalazine are shown in Fig. 9.

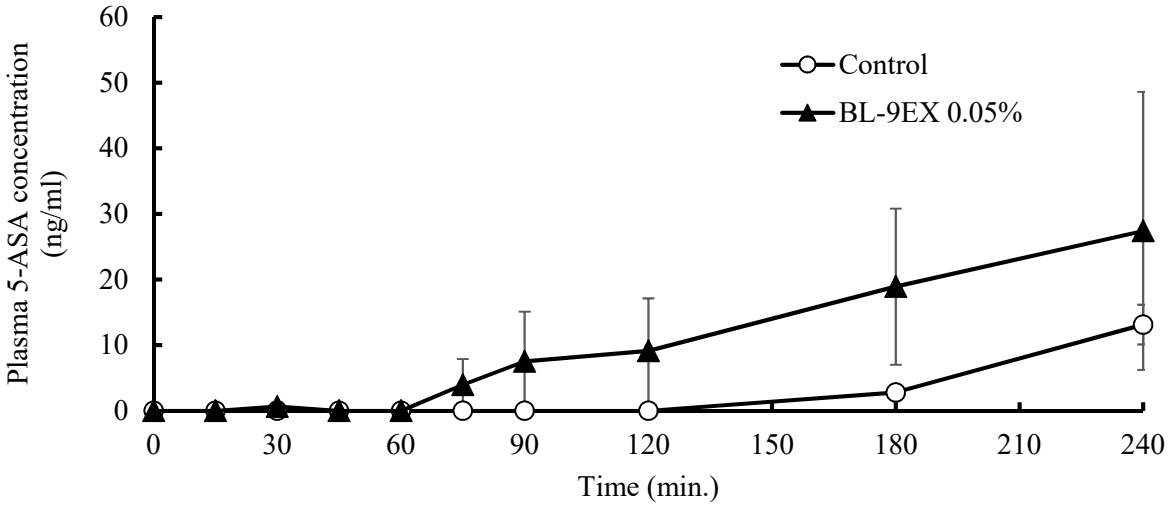


Figure 9. Plasma concentration time curves of 5-ASA after the intestinal administration of 1 mg/kg sulfasalazine with or without 0.05 % BL-9EX. Data are expressed as the mean ± S.E. of at least 3 animals. Keys: (○) Control; (▲) BL-9EX 0.05%.

The plasma concentration of 5-ASA after the intestinal administration of 1 mg/kg sulfasalazine was extremely low. The plasma level of 5-ASA remained below the lower limit of detection (4.56 ng/mL) during most of the experimental period and the $AUC_{0\text{-}4\ h}$ values of 5-ASA could not be accurately determined. On the other hand, a slightly higher plasma concentration of 5-ASA was observed in the BL-9EX 0.05% treated group, suggesting that the presence of 5-ASA in the small intestine might be increased. However, these values are within the margin of errors and the pharmaceutical excipients might not affect the intestinal metabolism of sulfasalazine.

1.3 Conclusions

In this chapter, several issues regarding to the transport of sulfasalazine by BCRP transporter were explored. First of all, the author demonstrated that sulfasalazine transport across rat intestine was mainly into the secretory direction due to the activities of Bcrp1 transporter. This was supported by the results of Bcrp1 inhibitors Ko143 and pantoprazole, both of which could completely neutralized the directional transport of sulfasalazine. In addition, the P_{app} values of sulfasalazine in the presence of Ko143 were similar

to those of the CF, the non-BCRP substrate and paracellular transport marker. These findings suggested that when the Bcrp1 was inhibited, the permeation rate of sulfasalazine across rat intestine was similar to the permeation rate of CF. Next, the intestinal absorption of sulfasalazine in rats examined by the *in situ* closed loop method support the hypothesis that Bcrp1 was behind the limited absorption of sulfasalazine. Sulfasalazine absorption was improved by 9.25-fold after the addition of pantoprazole, a Bcrp1 inhibitor. In addition, 0.1% BL-9EX and Brij97 significantly improved the intestinal absorption of sulfasalazine. BL-9EX and Brij97 were further examined for their effects against the intestinal absorption of CF. Results showed that BL-9EX did not increase the paracellular permeation of CF, indicating that BL-9EX improved the intestinal absorption of sulfasalazine by the inhibition of Bcrp1. On the other hand, Brij97 increased the intestinal absorption of CF, suggesting that Brij97 might increase the intestinal absorption of sulfasalazine via a paracellular permeation. Lastly, the effects of BL-9EX on the plasma concentration of 5-ASA was examined. The results suggested that BL-9EX did not decrease the plasma concentration of 5-ASA. Therefore, the possibility that BL-9EX might inhibit the bacterial metabolism of sulfasalazine was dismissed. In conclusion, the author demonstrated that BL-9EX could be applied as the BCRP modulator to improve the intestinal absorption of BCRP substrates.

Chapter II: Effects of various pharmaceutical excipients on the intestinal transport and absorption of topotecan, an orally active BCRP substrate

In this chapter, the author applied the findings from the first chapter to improve the intestinal absorption of topotecan. Topotecan is a semi-synthetic, water-soluble analog of camptothecin extracted from *Camptotheca acuminata*[73]. It acts by forming a stable covalent complex with the DNA/topoisomerase-I aggregate, the so-called "cleavable complex". This process leads to breaks in the DNA strand resulting in apoptosis and cell death.[74,75] Topotecan is currently approved for the treatment of several cancers such as ovarian cancer[76,77], lung cancers[78], pancreatic cancers[79], leukemia[80], and retinoblastoma[81]. Topotecan is also available in the oral dosage form which is approved for the treatment of a relapsed small cell lung cancer.[82] The oral bioavailability of topotecan was reported to be low (<40%) in humans.[83,84] Furthermore, it was reported to be a substrate of BCRP transporter which plays a major role in the absorption of topotecan in humans.[58,85,86] As previously reported by Sparreboom *et al.* in 2005, in patients with an single nucleotide polymorphism (SNP) variant at nucleotide 421, their BCRP activities were 30% lower and topotecan bioavailability was 1.36-fold greater than patient with wild-type BCRP allele.[87] This report suggests the possibilities that the bioavailability of topotecan could be improved by inhibiting the intestinal BCRP transporter.

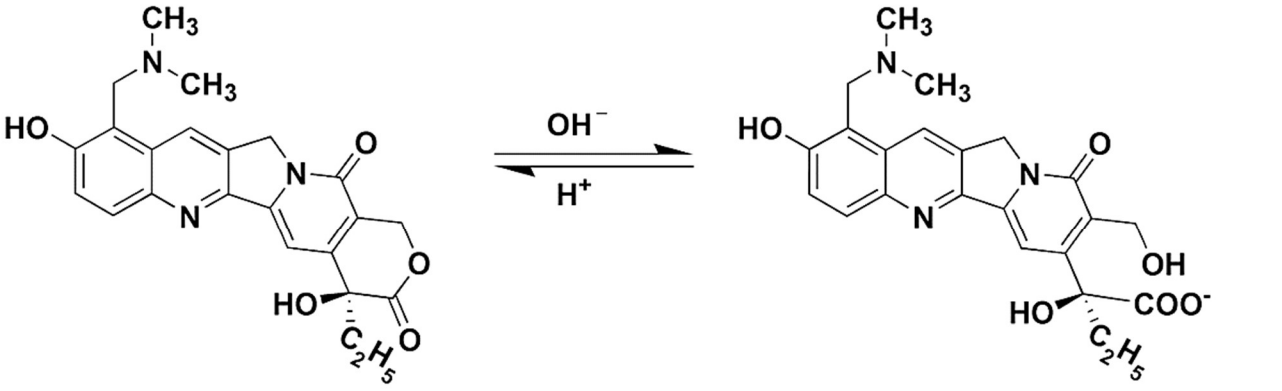


Figure 10. Chemical structures of topotecan lactone (active) form and its carboxylate form.

The bi-directional transport of topotecan was examined using human Caco-2 cell monolayers as a permeation membrane. Before examining the effects of pharmaceutical excipients on the transport of topotecan, the toxicities of these pharmaceutical excipients to Caco-2 cells were evaluated by MTT assay in order to find the maximum applicable concentration for the transport studies. After that, the non-toxic

concentrations of pharmaceutical excipients were used to examine the effects on the bi-directional transport of topotecan across Caco-2 cell monolayers. Furthermore, their effects on the intestinal absorption of topotecan were also evaluated using the *in situ* closed loop method in rats.

Pharmaceutical excipients including BL-9EX, Brij97, Cremophor EL, and Tween 20 which are found to modulate Bcrp1 transporter from the first chapter were applied in this section. Additionally, Pluronic F68 (PEO_{80}-PPO_{27}-PEO_{80}) was included in this chapter because there is a direct evidence to suggest that Pluronic F68 could inhibit BCRP and decrease the biliary excretion of camptothecin, a parent compound of topotecan.[50]

2.1 Materials and methods

2.1.1 Materials

Topotecan hydrochloride was purchased from Toronto Research Chemicals (Ontario, Canada). Brij97 (CAS No.9004-98-2), and BL-9EX (CAS No. 3055-99-0) were obtained from Nikko Chemical Co., Ltd. (Tokyo, Japan). Tween 20, dimethyl sulfoxide (DMSO) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). CF, verapamil hydrochloride, Hank's Balanced Salt Solution (HBSS), and Cremophor EL were purchased from Sigma Aldrich Chemical Co., Ltd. (St. Louis, MO). Labrasol was kindly gifted from Gattefossé (Cedex, France). Pluronic F68 (Kolliphor® P188) was purchased from BASF SE (Ludwigshafen, Germany). MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide), and 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchased from Dōjindo Molecular Technologies, Inc., (Kumamoto, Japan). Ko143 was purchased from Abcam Plc. (Cambridge, UK). HPLC-grade acetonitrile and methanol were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). For Caco-2 cell cultivation, Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), MEM non-essential amino acid solution, trypsin-ethylenediaminetetraacetic acid, 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid, antibiotic-antimycotic mixed solution (10,000 U/mL penicillin, 10 mg/mL streptomycin, 25 mg/mL amphotericin B, and 0.85% saline) were purchased from Life Technologies Corporation (Carlsbad, CA, USA). All other reagents were of laboratory grade.

2.1.2 Animals

All animals were treated in an accordance with 1.1.2.

2.1.3 Cell culture

Caco-2 cells, heterogeneous human epithelial colorectal adenocarcinoma cell line with a passage number of 46, was obtained from Dainippon Sumitomo Pharma Co., Ltd. and used in all experiments involving the Caco-2 cells. The complete growth medium including DMEM, 10% FBS, MEM non-essential amino acid solution and antibiotic antimycotic mixed solution was used. The detailed procedure is available as follows. Initially, the hibernated Caco-2 cells were stored in a sealed-vial inside the liquid nitrogen tank. The re-vitalization of the Caco-2 cells was carried out by rapid thawing the frozen Caco-2 cells in the water bath until approximately half of the frozen content was thawed. After that, the vial was left at the room temperature until completely thawed. Because Caco-2 cells were hibernated in DMSO containing media, this media was removed and replaced with an ice-cold complete growth medium and were seeded into T-75 flask in 5% CO_2, 37°C incubator. The complete growth medium was replaced every 48 h and the Caco-2 cells were allowed to grow until reaching 80%-90% confluence. The cells were cultured for the additional 2-3 passages before use in the MTT assay, transport assay, and other experiments.

2.1.4 Toxicities of pharmaceutical excipients in Caco-2 cells by a MTT assay

The author evaluated the viability of Caco-2 cells after the exposure to various concentrations of pharmaceutical excipients using the methods as described previously,[88,89] with additional modifications. Caco-2 cells from 2.1.3 were seeded into T-75 flask. Upon reaching 80%-90% confluence, cells were trypsinized and were resuspended into the fresh complete growth medium at 1×10^5 cells/mL. This cell suspension was seeded into 96-well flat-bottom cell culture plate. HBSS at pH 7.4 containing 25 mM HEPES was used as a working medium. After the plate was incubated for 60 h, the monolayers were washed with working medium and were incubated with working medium containing various concentrations of pharmaceutical excipients. After 4 h, the incubating medium was removed and quickly replaced with working medium containing 0.5 mg/mL MTT and the cells were incubated for additional 4 h. The MTT solution was subsequently removed and 100 µL DMSO was added into each well to dissolve a formazan crystal. Optical density (OD) was measured at 570 nm by Synergy® HT multi-detection microplate reader (Biotek Instrument Inc., Winooski, VT., USA). The reduction in formazan crystal formation is proportionally related to Caco-2 viability. Caco-2 viability relative to untreated control cells was calculated by dividing the OD from treated samples by the OD from untreated control cells. The schematic illustration of the procedure and the concept of MTT assay are summarized in Fig. 11.

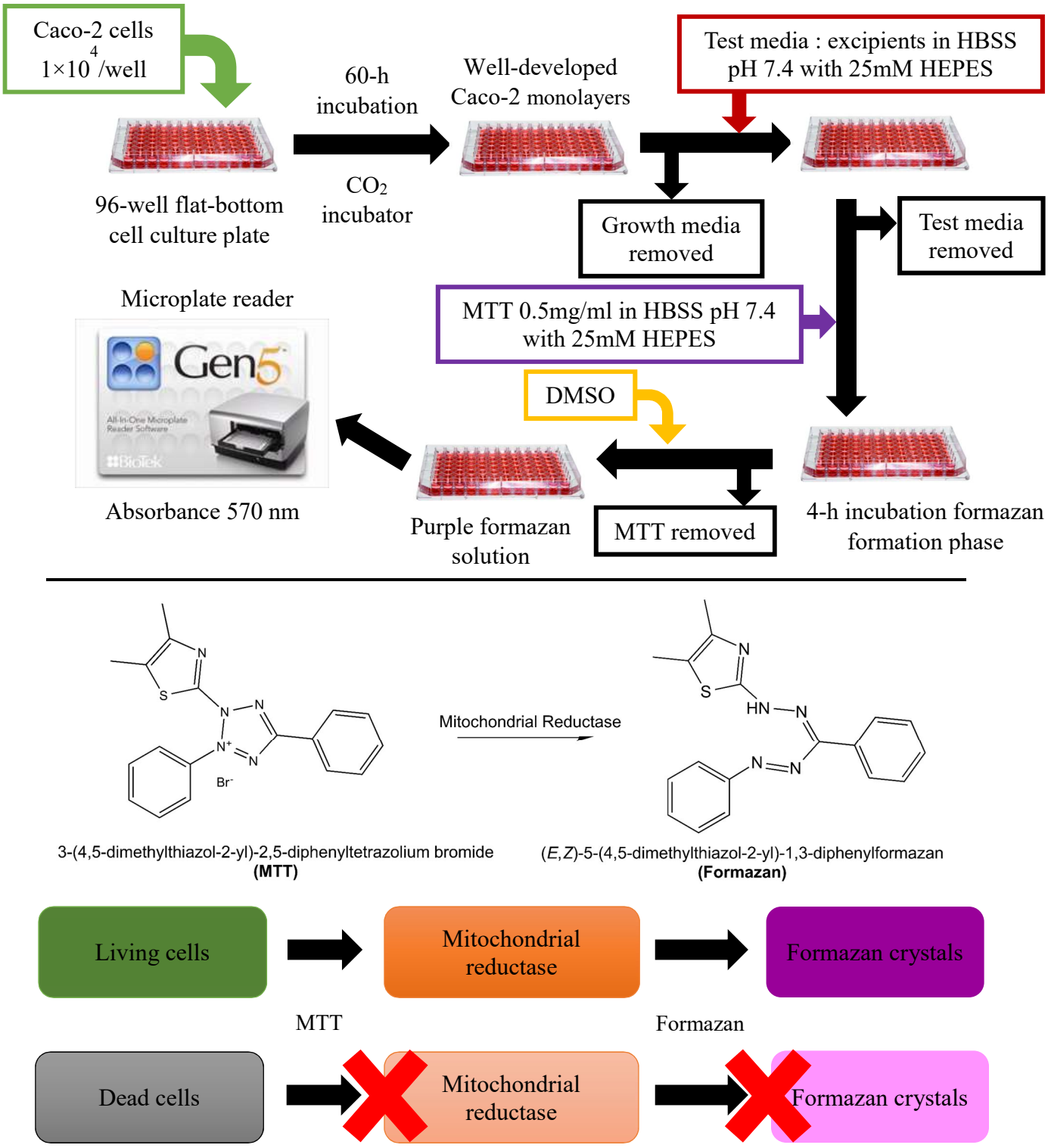


Figure 11. The schematic illustration of the procedure (top) and the concept of MTT assay (bottom)

2.1.5 Bi-directional transport studies of topotecan and CF using Caco-2 cell monolayers

Caco-2 cells, which are known to express BCRP transporter,[90] were used to create a permeation membrane. Caco-2 cell suspension from 2.1.3 contains roughly 3×10^5 cells was seeded into T-75 flask and

was incubated in CO_2 incubator until reaching 90% confluence (usually within 5 days). The cells were trypsinized and resuspended into the growth medium at 2×10^5 cells/mL. Transwell™ cell culture inserts (12-well, 1.12 cm^2, pore size 0.4 µm) (Corning Inc., MA., USA) were used. Each insert was seeded with 0.5 mL cells suspension to the apical side and was allowed to grow in CO_2 incubator for 21-28 days. The growth medium was regularly replaced every 48 h. The integrity of the monolayers was routinely monitored by measuring the transepithelial electrical resistance (TEER) using Millicell® ERS-2 volt-ohm meter (EMD Millipore Corp., MA., USA). Any insert with TEER < 600 $\Omega\cdot cm^2$ was discarded.

The transport studies were carried out by the following procedures. First, the well-developed Transwell inserts were washed and incubated for 1 h in the blank transport medium (HBSS pH 7.4 with 25 mM HEPES) at 37°C in order to stabilize the monolayers. After that, incubating media in donor side of the insert was removed and immediately replaced with drug solution (10 µM topotecan or CF, prepared in transport medium with or without excipient). The incubating media in receiver side of the insert was also removed and replaced with the blank transport medium. TEER was periodically measured and samples were taken from the receiver side and the equal volume of blank transport media was immediately replaced. The schematic illustration of this bi-directional transport is shown in Fig. 12.

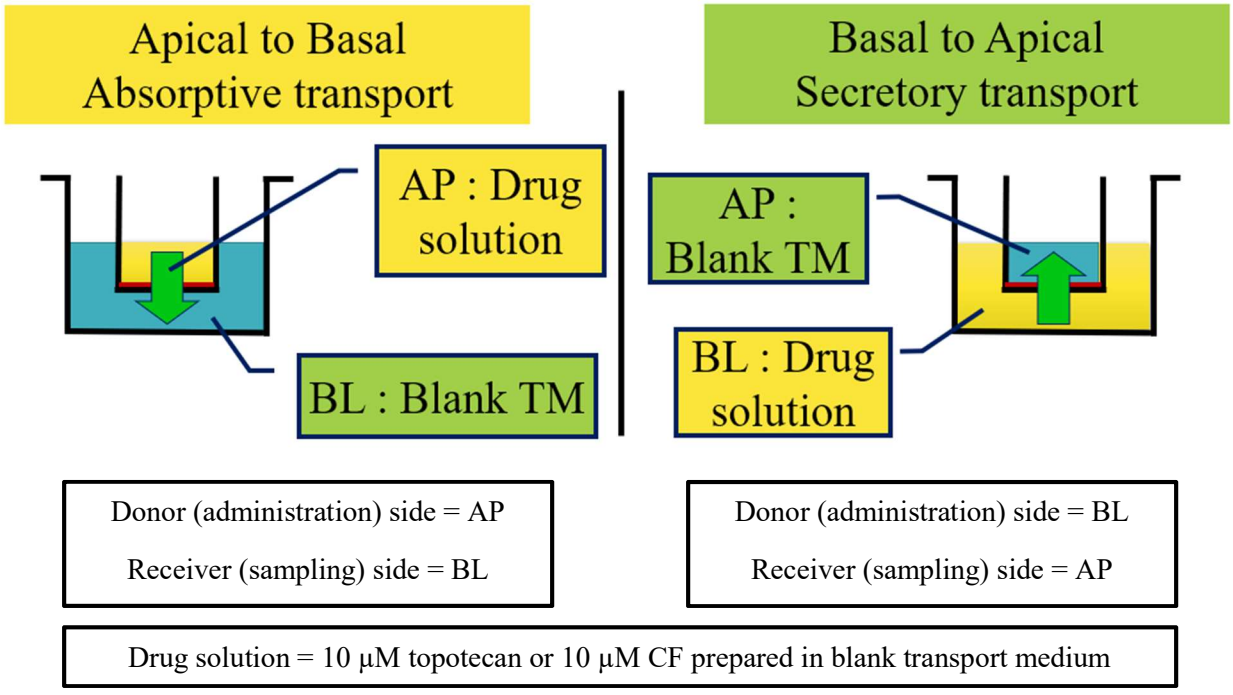


Figure 12. Schematic illustration of the bi-directional transport studies of topotecan and CF using Caco-2 cells monolayers, Caco-2 cells were grown on Transwell® culture inserts (shown in this figure). AP., apical; BL., basolateral; TM., transport medium (HBSS pH 7.4 with 25 mM HEPES), green arrows indicating the direction of the transport.

2.1.6 Intestinal absorption studies by an *in situ* closed loop method

The effects of pharmaceutical excipients on the intestinal absorption of topotecan and CF were evaluated by the *in situ* closed loop method in rats similar to that described in 1.1.4 with some minor modifications according to the update in animal treatment guideline. The total anesthesia was achieved by the intra peritoneal injection of 0.15 mg/kg medetomidine, 2 mg/kg midazolam, and 2.5 mg/kg butorphanol tartrate. The dose of topotecan was 0.5 mg/kg and was prepared in PBS at the concentration of 0.5 mg/mL.

2.1.7 Determination of drugs

The concentrations of topotecan in Caco-2 permeation and *in situ* closed loop studies were determined by HPLC systems equipped with DGU-20A on-line degassing unit, LC-20AT series-type double plunger pumps, SIL-20A autosampler, CTO-10A column oven, and RF-10AXL fluorescence detector (Shimadzu Co., Kyoto, Japan). Data collection and integration were carried out by Chromato-PRO version 5.0 (Runtime Instrument Co., Ltd., Japan). The detailed analytical procedure is available as follows. Samples were thawed at room temperature if frozen. After that, samples were deproteinized and acidified by the addition of a three-volume of 25 mM phosphoric acid in ice-cold methanol. Samples were vortexed for 10 min before centrifugation. A clear supernatant was used for analysis by HPLC. To separate the peak of topotecan, analytical column (5C18-MS-II, 4.6×150 mm, 5µm, Nacalai Tesque, Inc., Japan) and the isocratic flow of 25 mM KH_2PO_4 pH 3.5 and acetonitrile (85:15) at 1 mL/min was used. The clear separation was achieved with the lower limit of detection and quantification were less than 2 ng/mL and 7 ng/mL, respectively. The typical runtime was 8 min for each sample.

The concentrations of CF in Caco-2 cells permeation experiment and *in situ* closed loop experiment were analyzed using the same procedures as described in 1.1.6.

2.1.8 Calculation and statistical analysis

The apparent permeability coefficient (P_{app}) was calculated from the Caco-2 cells permeation experiments by using the equation from 1.1.7 with minor modifications.

$$P_{app} = \frac{Flux}{Area \cdot C_0 \cdot 60}$$

Flux is the rate of appearance of topotecan or CF in the receiver side, which was calculated from the linear portion of the cumulative permeated amount versus time curve. Area is the surface area of the permeation membrane (1.12 cm^2) and C_0 is the initial concentration of topotecan and CF (10 µM). Additionally, efflux ratio (ER) was calculated by the following equation.

$$Efflux\ ratio\ (ER) = \frac{P_{app\ B\rightarrow A}}{P_{app\ A\rightarrow B}}$$

$P_{app\ B\rightarrow A}$ refers to the apparent permeability coefficient of the secretory (i.e. basolateral to apical side of the insert) permeation. $P_{app\ A\rightarrow B}$ refers to the apparent permeability coefficient of the absorptive (i.e. apical to basolateral) permeation.

The calculation of pharmacokinetic parameters by the *in situ* closed loop experiment and the statistical analysis were carried out using the method described in 1.1.7.

2.2 Results and Discussion

2.2.1 MTT cytotoxicity assay

The MTT assay was used to measure the metabolic activities of the living cells. Its mechanism involving the reduction of yellowish MTT solution to purple formazan crystals. This process was driven by the cellular reductant, reduced pyridine nucleotide cofactor (NADH) which was mainly generated from the mitochondrial tricarboxylic acid cycle.[91] The reduction in the formazan formation is proportionally related to the reduction of cell viability. The effects of pharmaceutical excipients on the viability of Caco-2 cells are summarized in Fig. 13.

The relative viabilities of Caco-2 cells after exposure the polyoxyethylene alkyl ether derivatives BL-9EX and Brij97 at 0.00025% and 0.0005% were 65%-75% of the untreated control. This result suggested that BL-9EX and Brij97 caused cellular damage even when applied with a very low concentration. This is likely due to the intrinsic properties of BL-9EX and Brij97. They were reported to have a relatively higher membrane solubilization capacity compared to other pharmaceutical excipients.[50,92–94] Additionally, because Caco-2 cell monolayers did not express a protective mucous layer[95], they are more susceptible to the high membrane solubilizing activities of BL-9EX and Brij97. On the other hand, Cremophor EL, Labrasol, Pluronic F68, and Tween 20 did not affect the viability of Caco-2 cells because they had less membrane solubilization capacities. Therefore, they were all qualified for the next bi-directional transport studies.

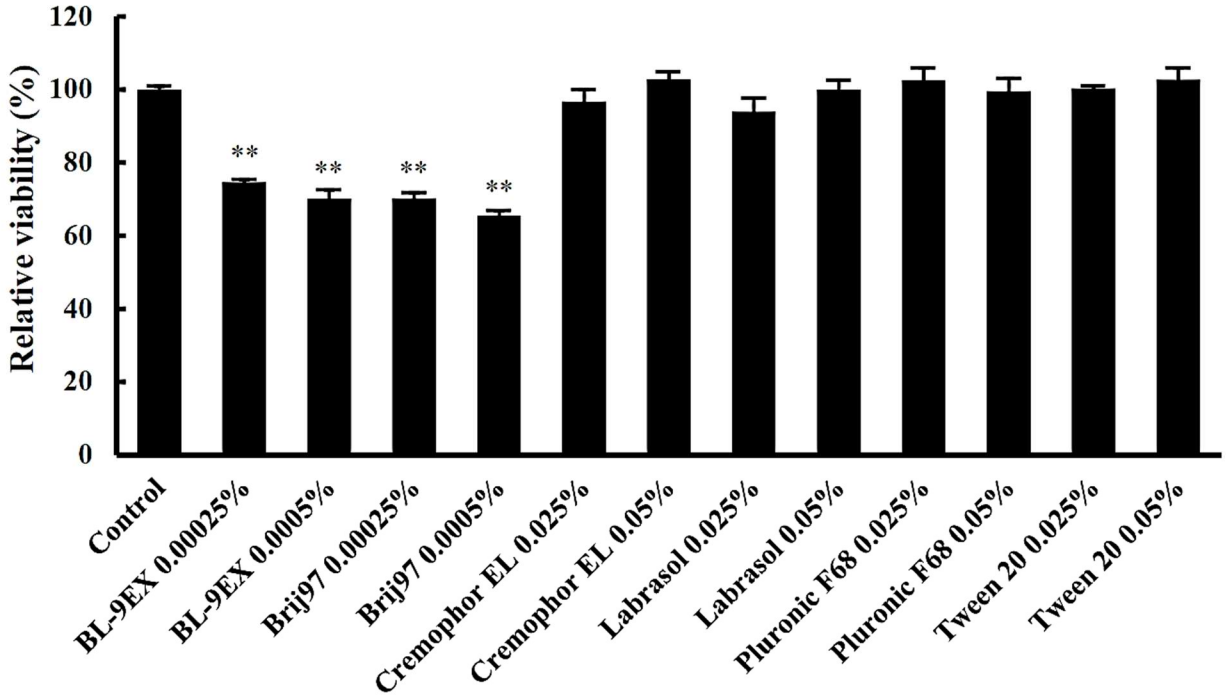


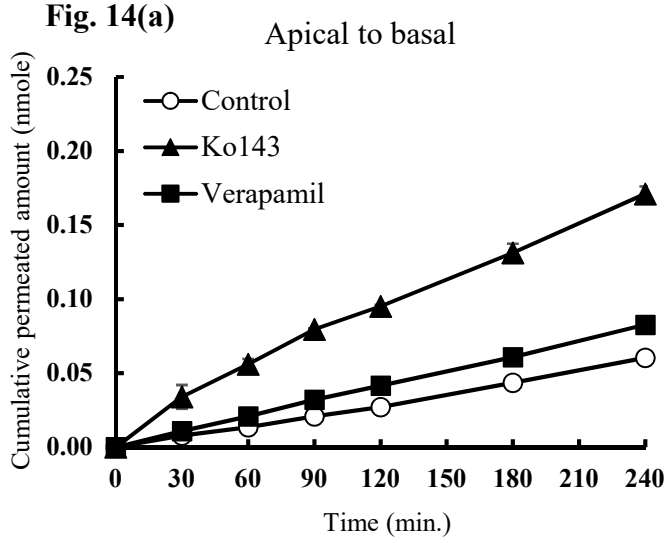
Figure 13. Relative viability of Caco-2 cells after exposure to various pharmaceutical excipients at the indicated concentrations. Results are expressed as mean ± SE of 3–6 experiments. (**) denotes significantly different results from the control group ($p<0.01$). (*J. Pharm. Sci*., **108,** 1315-1325 (2019); Figure 1)

2.2.2 Effects of pharmaceutical excipients on the transport of topotecan across Caco-2 cell monolayers

By using the same passage of Caco-2 cells for the whole experiment, the author was able to avoid the possible fluctuation in BCRP expression level which was reported to vary from passage to passage.[90] The transport of topotecan was measured up to 4 h. Additionally, the TEER was routinely measured during the experimental period to evaluate the integrity of the monolayers.

As seen in Fig. 14 and Table 6, the secretory and absorptive P_{app} values of topotecan across Caco-2 cell monolayer were 5.60 ± 0.32 and 0.35 ± 0.01 ($\times 10^{-6}$ cm/s, respectively). The ER value of topotecan alone was 16.00, suggesting that topotecan was preferentially transported into the secretory direction. However, there are several conflict reports about which transporter is actually driving the secretory transport of topotecan. Some reports suggested that topotecan is a dual P-gp/BCRP substrate. For example, Li et al.[85] reported that the secretory flux of topotecan across Caco-2 cell monolayers was dramatically reduced in the presence of 100 μM verapamil (a P-gp substrate/inhibitor) or 40 μM fumitremorgin C (a BCRP inhibitor). A previous report by de Vries et al.[96] demonstrated that AUC values of topotecan in Bcrp1$^{(-/-)}$ or Mdr1a/b$^{(-/-)}$ knockout mice were 1.5 fold higher than those of wild type mice, while the AUC values of topotecan in dual Mdr1a/b$^{(-/-)}$Bcrp1$^{(-/-)}$ knockout mice were 12-fold higher than in wild type mice.

Fig. 14(a)



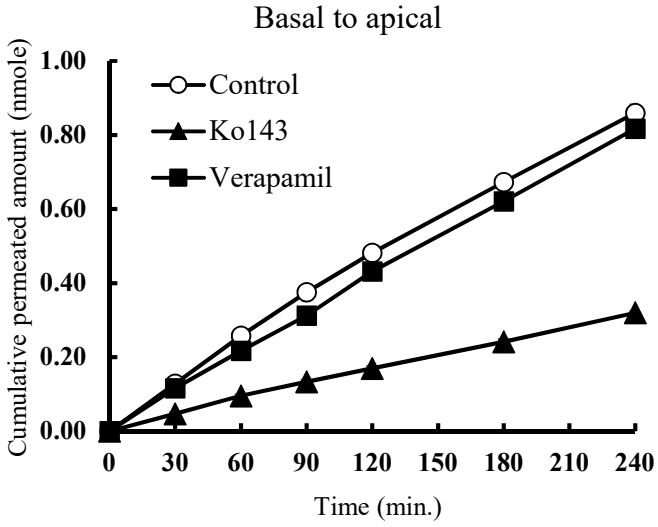
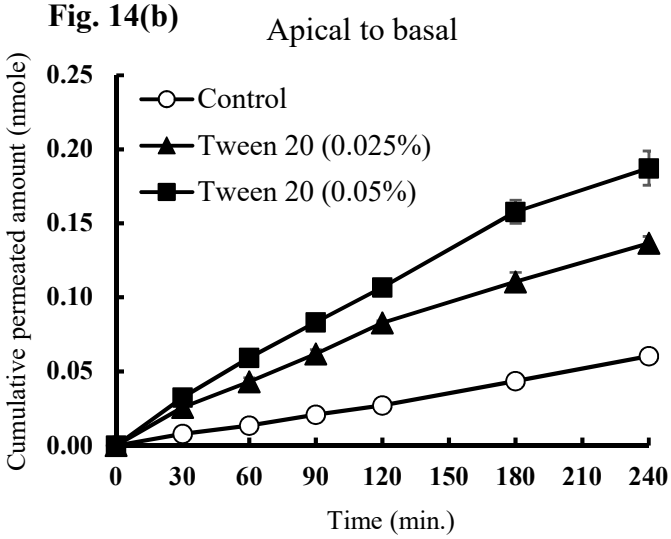


Fig. 14(b)



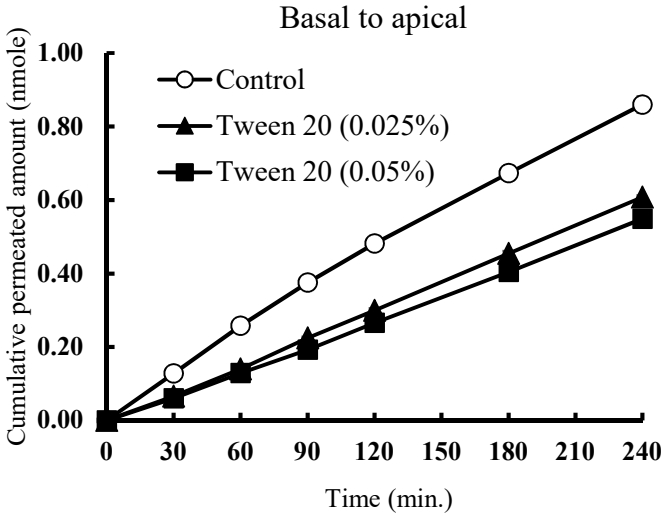
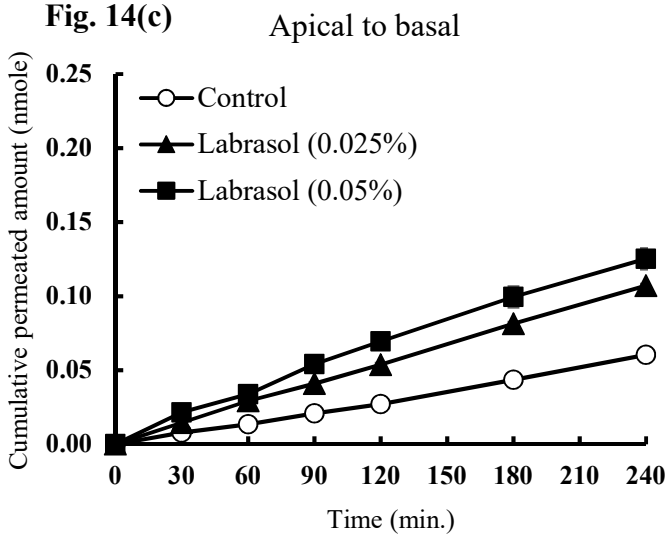


Fig. 14(c)



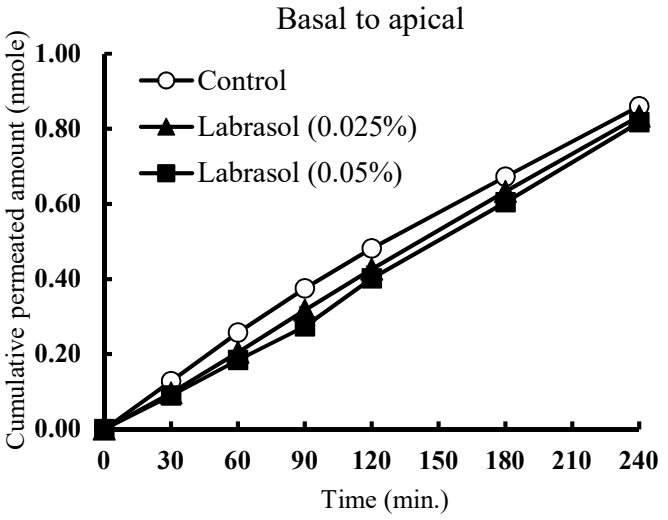


Fig. 14(d)

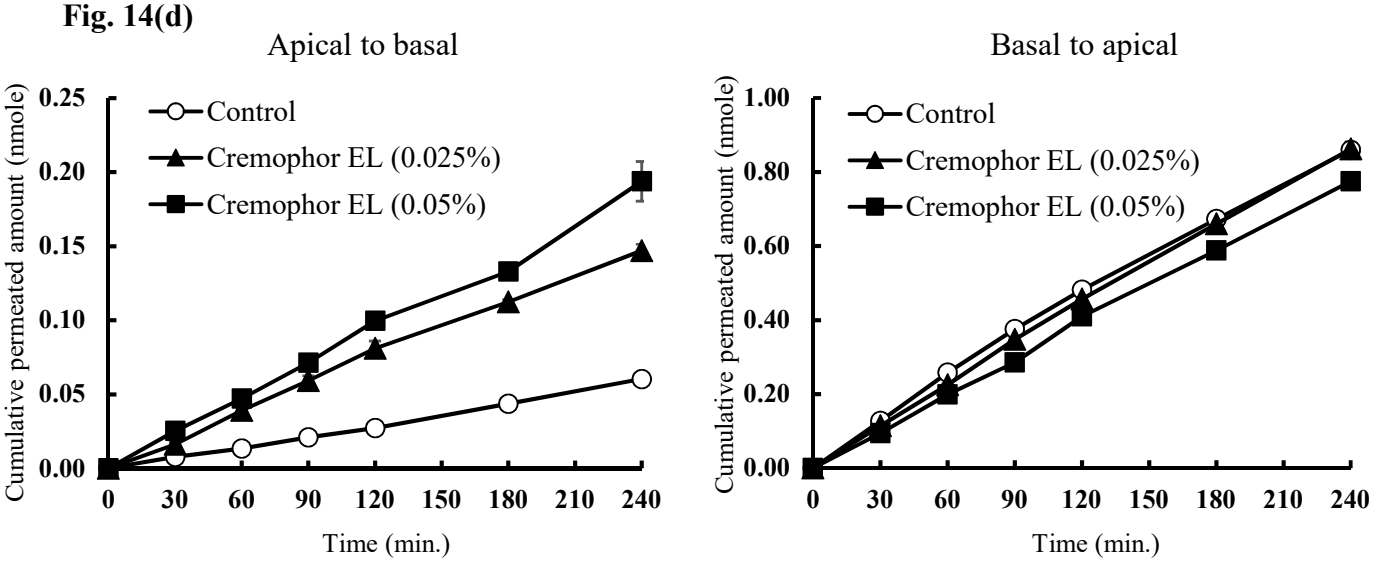


Fig. 14(e)

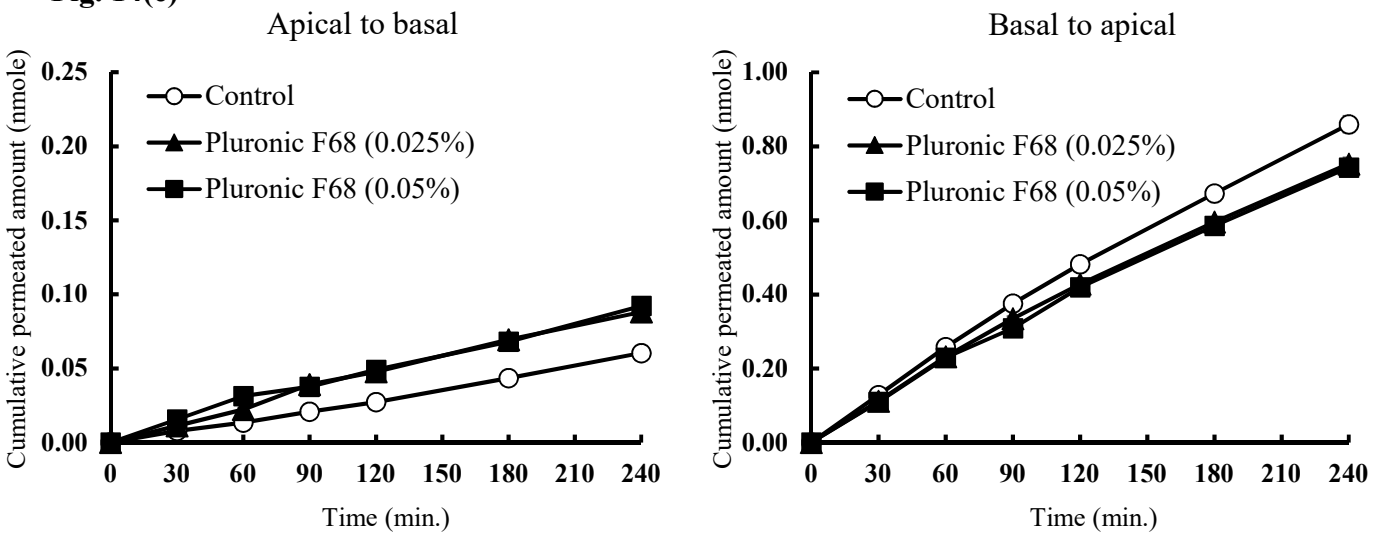


Figure 14. Cumulative permeated amount versus time curve of topotecan across Caco-2 cell monolayers in the presence of 1 μM Ko143 or 50 μM verapamil (a), Tween 20 (b), Labrasol (c), Cremophor EL, (d) and Pluronic F68 (e). Absorptive and secretory transport of topotecan is shown in the left and right panels, respectively. (*J. Pharm. Sci*., **108,** 1315-1325 (2019); Figure 2)

Topotecan was also reported to be primarily transported by BCRP. For example, the AUC values of topotecan in Bcrp1$^{(-/-)}$ mice after oral administration of 1 mg/kg topotecan were 3.6-fold higher than those in wild type mice.[58] Jonker et al.[86] reported that the oral bioavailability of topotecan co-administered with GF120918, a BCRP inhibitor in P-gp deficient mice was more than 6-fold greater than that in the absence of the inhibitor GF120918.

Table 6. Apparent permeability coefficient values of topotecan in the presence of various pharmaceutical excipients determined by Caco-2 cell permeability

Experiment	Concentration	Apparent permeability coefficient ($\times 10^{-6}$ cm/s)		Efflux ratio
		$P_{app\ A\rightarrow B}$	$P_{app\ B\rightarrow A}$	
Control	-	0.35 ± 0.01	5.60 ± 0.32	16.00
Ko143	1 μM	1.08 ± 0.06**	1.82 ± 0.02**	1.69
Verapamil	50 μM	0.46 ± 0.06	5.09 ± 0.03	11.07
Tween 20	0.025%	0.72 ± 0.07**	4.10 ± 0.03**	5.69
	0.05%	1.07 ± 0.13**	3.77 ± 0.09**	3.52
Labrasol	0.025%	0.72 ± 0.03**	5.51 ± 0.27	7.65
	0.05%	0.73 ± 0.03**	5.48 ± 0.22	7.46
Cremophor EL	0.025%	0.85 ± 0.11**	5.25 ± 0.09	6.18
	0.05%	1.22 ± 0.22**	4.74 ± 0.06**	3.89
Pluronic F68	0.025%	0.48 ± 0.01	4.52 ± 0.11**	9.42
	0.05%	0.54 ± 0.05	4.70 ± 0.01**	8.70

Results are expressed as mean ± SE of at least 3 experiments. (**) *p<0.01* compared with the control. (*J. Pharm. Sci*., **108,** 1315-1325 (2019); Table 2)

In order to clarify the role of transporters on the secretory transport of topotecan, verapamil, a P-gp inhibitor and Ko143, a BCRP inhibitor were used. The dose of Ko143 was set to 1 μM according to the report that it could completely neutralize the BCRP-mediated vectorial transport of [^{3}H]estrone-3-sulfate, a BCRP substrate, in Caco-2 cells.[90] Additionally, the sensitivity of Ko143 to P-gp was >300-fold lower than that to BCRP. The effective concentration of P-gp inhibitor GF120918 for reversal of 50% of the resistance in P-gp mediated paclitaxel resistance cells (EC_{50}) was 0.0030 ± 0.0004 μM, while EC_{50} of Ko143 was 1.0 ± 0.3 μM. Moreover, 0.5 μM Ko143 was reported to completely reverse BCRP-mediated drug resistance but had very little effect on the resistance of MDCKII/MRP2 cells to vincristine, a MRP2 substrate (IC_{50} =12.8 ± 0.3 nM and 13.5 ± 1.3 nM, for with and without 0.5 μM Ko143, respectively).[97] In the presence of 1 μM Ko143, the secretory P_{app} value of topotecan was significantly reduced to 1.82±0.02 $\times 10^{-6}$ cm/s (*p<0.01*) and the absorptive P_{app} was significantly increased to 1.08 ± 0.06 $\times 10^{-6}$ cm/s (*p<0.01*). The efflux ratio was reduced to 1.69, almost 10-fold reduction, suggesting that Ko143 at 1 μM which did not interfere with P-gp[40], could effectively inhibit BCRP transporter in Caco-2 cells and neutralized the directional transport of topotecan. On the other hand, verapamil was used at 50 μM. Verapamil is a highly potent and specific P-gp inhibitor with IC_{50} values in Caco-2 cells of 8.44 μM for fexofenadine[96] or 32.2 μM for digoxin.[98] However, verapamil loses specificity toward P-gp, and likely inhibits BCRP at higher

concentrations.[99] Therefore, 50 μM was considered adequate for verapamil to inhibit P-gp without interfering with BCRP. As shown in Fig. 14 (a) and Table 6, 50 μM verapamil had no effect on topotecan permeation across Caco-2 monolayers in our bi-directional transport studies, suggesting that topotecan is not a substrate of P-gp and the directional transport of topotecan was mainly driven by a BCRP transporter. These findings are also supported by the other literature.[100]

Tween 20 and Cremophor EL could significantly increase the transport of topotecan across Caco-2 cells. At 0.05%, Tween 20 significantly increased the absorptive P_{app} value of topotecan to $1.07 \pm 0.07 \times 10^{-6}$ cm/s ($p<0.01$). This is similar to the P_{app} values from the group treated with Ko143 ($1.08 \pm 0.06 \times 10^{-6}$ cm/s). The secretory P_{app} value was significantly reduced to $3.77 \pm 0.09 \times 10^{-6}$ cm/s ($p<0.01$) and ER was reduced to 3.52. These results suggested that Tween 20 could be used as a BCRP modulator to improve the intestinal absorption of topotecan. Cremophor EL at 0.05% also gave promising results by significantly increasing the absorptive P_{app} value of topotecan by almost 3.5-fold ($1.22 \pm 0.22 \times 10^{-6}$ cm/s, $p<0.01$). The secretory P_{app} and ER values of topotecan were reduced to $4.74 \pm 0.06 \times 10^{-6}$ cm/s and 3.89, respectively, in the presence of 0.05% Cremophor EL. These results also suggested that Cremophor EL might be used as a BCRP modulator to improve the intestinal absorption of topotecan.

Interestingly, while both Tween 20 and Cremophor EL increased the absorptive transport and simultaneously decreased the secretory transport of topotecan, Pluronic F68 only decreased the secretory P_{app} and Labrasol only increased the absorptive P_{app} of topotecan. These results are comparable to those reported by Li *et al.*[56] They reported that 0.01% Pluronic F68 reduced the secretory transport of scutellarin, a substrate of MRP2, but did not increase the absorptive transport of the same substrate. The same report also suggested that Labrasol could only improve the absorptive transport of scutellarin with limit effect on its secretory transport.[56] Because BCRP and MRP2 are co-localized on the apical membrane of Caco-2 cells, the transporter location and the ability to penetrate the membrane of the pharmaceutical excipients might be involved. However, the exact mechanism is unclear.

The effects of pharmaceutical excipients on the tightness of tight junction in Caco-2 cell monolayers were evaluated by measuring the TEER. The results are summarized in Fig. 15. There was no significant reduction in TEER value, suggesting that these pharmaceutical excipients at the tested concentration did not loosen the tight junction of Caco-2 cell monolayers. It is worth noting that in the Pluronic F68-treated Caco-2 cell monolayers, TEER values remained constant throughout the duration of the experiment (Fig. 15 (a)). This is possibly due to the intrinsic cytoprotective properties of Pluronic F68 which was reported to effectively restores damaged cells after electroporation, heat shock, multiple freeze-thaw cycles, or intense radiation.[45,101]

Fig. 15(a)

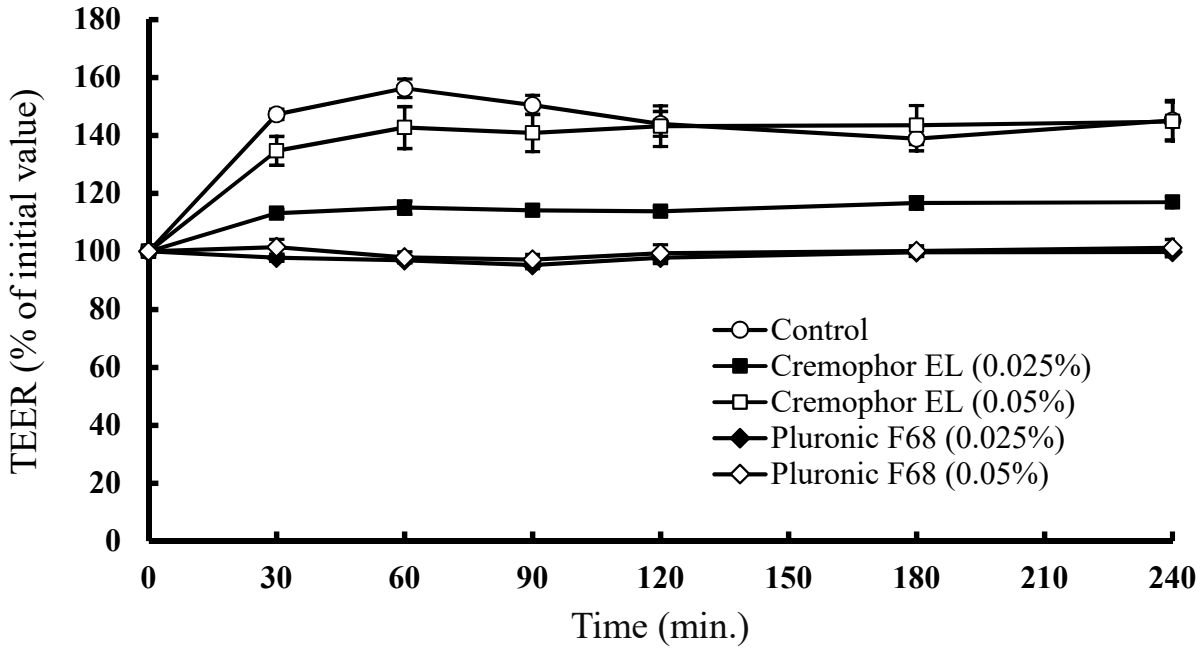


Fig. 15(b)

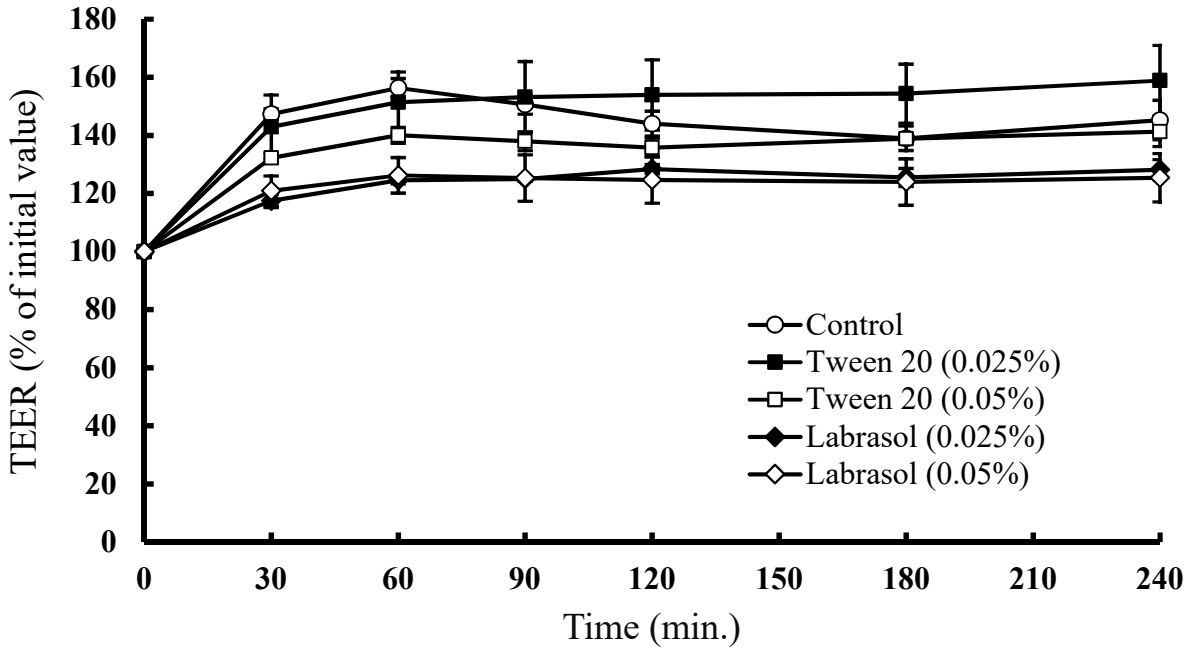


Figure 15. Effects of various pharmaceutical excipients on transepithelial electrical resistance (TEER) during the bi-directional transport of topotecan across Caco-2 cells monolayers. Results are expressed as mean ± SE of 3–6 experiments. (*J. Pharm. Sci*., **108,** 1315-1325 (2019); Figure 3)

2.2.3 Bi-directional transport of CF across Caco-2 cell monolayers

Table 7. Apparent permeability coefficients of CF in the presence of various pharmaceutical excipients determined by a Caco-2 cell permeability experiment

Experiment	Concentration	Apparent permeability coefficient ($\times 10^{-8}$ cm/s)		Efflux ratio
		$P_{app\ A\rightarrow B}$	$P_{app\ B\rightarrow A}$	
Control	-	5.36 ± 0.21	5.93 ± 0.34	1.11
Tween 20	0.025%	5.89 ± 1.25 N.S.	7.23 ± 0.73 N.S.	1.29
	0.05%	5.06 ± 0.42 N.S.	7.28 ± 0.47 N.S.	1.19
Labrasol	0.025%	5.44 ± 0.41 N.S.	6.98 ± 0.10 N.S.	1.28
	0.05%	4.90 ± 0.67 N.S.	6.95 ± 0.67 N.S.	1.42
Cremophor EL	0.025%	5.62 ± 0.67 N.S.	7.22 ± 0.73 N.S.	1.29
	0.05%	6.11 ± 1.19 N.S.	7.28 ± 0.47 N.S.	1.19
Pluronic F68	0.05%	5.82 ± 0.57 N.S.	6.94 ± 0.57 N.S.	1.19

Results are expressed as mean±SE of at least 3 experiments. (N.S.) no significant difference compared with the control. (*J. Pharm. Sci*., **108,** 1315-1325 (2019); Table 3)

The effects of pharmaceutical excipients on the bi-directional transport of CF across Caco-2 cell monolayers are summarized in Table 7. In the absence of excipient, absorptive and secretory P_{app} value of CF were 5.36 ± 0.21, $5.93 \pm 0.34 \times 10^{-8}$ (cm/s, respectively). These values are several magnitudes lower than those from the diffusion chambers using rat intestinal membrane in 1.2.2. This is likely caused by the stronger tight-junction in adenocarcinoma cell lines such as Caco-2 cell than in normal cells.[102] The addition of Tween 20, Labrasol, Cremophor EL, and Pluronic F68 led to modest changes in P_{app} values that did not differ significantly from control values. The ERs of CF in the presence of pharmaceutical excipients ranged from 1.19 to 1.42 relative to control. From these results, it was concluded that the improvement in topotecan permeation as observed in 2.2.2 was caused by the inhibition of BCRP transporters and the increase in transcellular permeation of topotecan.

2.2.4 Intestinal absorption of topotecan by an *in situ* closed loop method

The rodent Bcrp1 gene is a 657 amino acids protein, sharing 86% sequence homology to human BCRP.[23] Bcrp1 was reported to play a major role on oral topotecan absorption in rats.[103] Therefore, the author hypothesized that rat Bcrp1 is primarily responsible for topotecan secretory transport, and may respond to pharmaceutical excipients similar to human BCRP. To further clarify the hypothesis, Tween 20, Labrasol, Cremophor EL, and Pluronic F68 were further examined whether they can improve the intestinal absorption of topotecan in rats. The same concentration (0.05%) of these pharmaceutical excipients was

used for this study. The plasma concentration versus time curves and the pharmacokinetic parameters after the intestinal administration of 0.5 mg/kg topotecan in rats are summarized in Fig. 16 and Table 8.

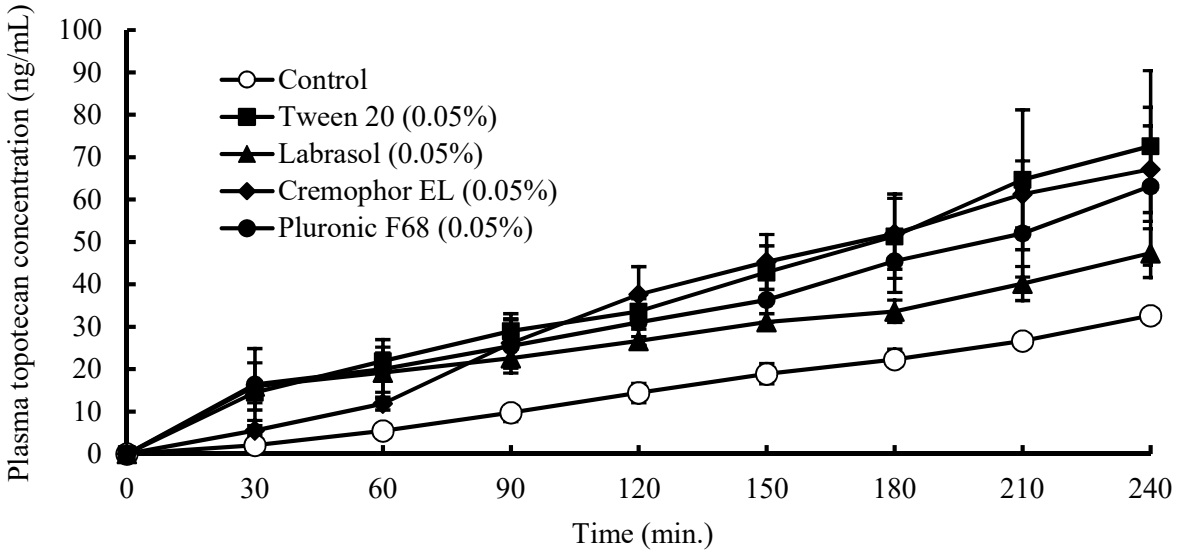


Figure 16. Plasma-concentration time profiles of topotecan (0.5 mg/kg) after intestinal administration by an *in situ* closed loop method. Results are expressed as mean ± SE of 3–6 animals. (*J. Pharm. Sci*., **108,** 1315-1325 (2019); Figure 4)

Table 8. Pharmacokinetic parameters of topotecan after intestinal administration of 0.5 mg/kg topotecan with or without pharmaceutical excipients determined by an *in situ* closed loop method

Group	$AUC_{0\text{-}4\ h.}$ (μg·min/mL)	Enhancement ratio[a]
Control	3.48 ± 0.24	-
Tween 20 (0.05%)	8.83 ± 1.58*	2.54
Labrasol (0.05%)	6.39 ± 0.47	1.83
Cremophor EL (0.05%)	8.19 ± 1.17*	2.35
Pluronic F68 (0.05%)	7.75 ± 1.34	2.23

Results are expressed as mean±SE of at least 3 experiments. (*) $p<0.05$ compared with the control.
[a] Enhancement ratios were calculated by dividing AUC values of each experiment group with the AUC value from the control group. (*J. Pharm. Sci*., **108,** 1315-1325 (2019); Table 4)

As seen in Fig. 16 and Table 8, the intestinal absorption profiles of topotecan with or without pharmaceutical excipients were in the similar fashion. Unfortunately, because the plasma concentration of topotecan was gradually increasing until the end of experiment (4 h), the precise and accurate calculation of C_{max} and T_{max} values was not possible. These results are in contrast to the previous report by Yamagata *et al.*[58] likely due to the different in the administration method. Without any excipient, topotecan was

slowly absorbed and the plasma concentration of topotecan 4 h after administration was 32.7 ± 0.7 ng/mL with the $AUC_{0\text{-}4\ h}$ value of 3.48 ± 0.24 μg·min/mL. After the addition of 0.05% Tween 20, the intestinal absorption of topotecan was significantly improved. The plasma topotecan concentration at 4 h after administration was 72.6 ± 17.8 ng/mL, and the $AUC_{0\text{-}4\ h}$ value was significantly improved to 8.83 ± 1.58 μg·min/mL ($p<0.05$), a 2.54-fold increase over the control. These findings suggested that Tween 20 could be applied as a potentially effective adjuvant to improve the intestinal absorption of topotecan. In addition, 0.05% Cremophor EL also significantly improved the intestinal absorption of topotecan. The $AUC_{0\text{-}4\ h}$ values of topotecan was increased to 8.19 ± 1.17 μg·min/mL ($p<0.05$) and the plasma concentration of topotecan at 4 h after the administration was 67.4 ± 9.9 ng/mL. These findings are in accordance to the previous report[49], suggesting that in addition to Tween 20, Cremophor EL is also an effective adjuvant to improve the intestinal absorption of topotecan.

The intestinal absorption of topotecan was slightly improved in the presence of 0.05% Labrasol and Pluronic F68. The $AUC_{0\text{-}4\ h}$ values of topotecan in the presence of 0.05% Labrasol or Pluronic F68 were increased to 6.39±0.47 and 7.75±1.34 μg·min/mL, respectively. Unfortunately, these values are not statistically different from the control. These results are different from in 2.2.2 possibly because their inhibitory effects against rodent Bcrp1 were weaker than with human BCRP. Additionally, because Caco-2 cell is a non-mucus secreting cell while there was an observable mucous barrier in the *in situ* closed loop experiment[60,61], Labrasol and Pluronic F68 might not be able to penetrate this barrier which might be related to the inferior effects with *in situ* experiment than with Caco-2 cells.

2.2.5 Intestinal absorption of CF by an *in situ* closed loop method

The effects of Tween 20 and Cremophor EL on the paracellular permeation of CF were additionally examined in order to clarify the absorption enhancing pathway of drugs by these pharmaceutical excipients. The plasma concentration versus time curves and the pharmacokinetic parameters of CF are summarized in Fig. 17 and Table 9.

As seen in Fig. 17, after the intestinal administration of 0.5 mg/kg of CF, it was slowly absorbed and reached its C_{max} of 19.68 ± 2.62 ng/mL after 160 ± 40 min. The plasma concentrations of CF after the addition of 0.05% Tween 20 or Cremophor EL were similar to those of the control. The maximum plasma concentrations of CF were 53.24 ± 13.03 and 23.51 ± 7.08 ng/mL in the presence of 0.05% Tween 20 and Cremophor EL, respectively. These values are in the margin of errors and not statistically different from the control.

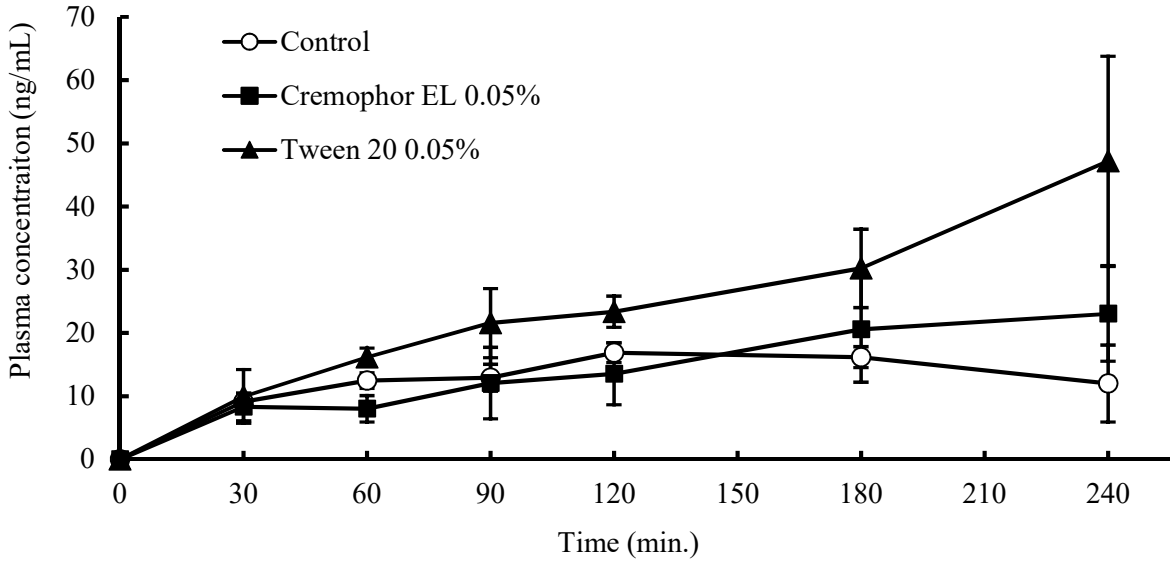


Figure 17. Plasma-concentration time profiles of 5(6)-carboxyfluorescein (CF) (0.5 mg/kg) after intestinal administration by an *in situ* closed loop method. Results are expressed as mean ± SE of 3–6 animals. (*J. Pharm. Sci*., **108,** 1315-1325 (2019); Figure 6)

Table 9. Pharmacokinetic parameters of CF after intestinal administration of 0.5 mg/kg CF with or without pharmaceutical excipients determined by an *in situ* closed loop method

Group	$AUC_{0\text{-}240min}$ (μg·min/mL)	Enhancement ratio[a]
Control	3.12 ± 0.34	-
Cremophor EL (0.05%)	3.39 ± 1.09 N.S.	1.09
Tween 20 (0.05%)	5.71 ± 0.67 N.S.	1.83

Results are expressed as mean±SE of at least 3 experiments. (N.S.) no significant difference compared with the control.

[a] Enhancement ratios were calculated by dividing AUC values of each experiment group with the AUC value from the control group. (*J. Pharm. Sci*., **108,** 1315-1325 (2019); Table 5)

The $AUC_{0\text{-}4\ h}$ of CF after its intestinal administration was 3.12 ± 0.34 μg·min/mL. After the addition of 0.05% Cremophor EL and Tween 20, the $AUC_{0\text{-}4\ h}$ values were increased to 3.39 ± 1.09 and 5.71 ± 0.67 μg·min/mL, respectively. However, these differences were not statistically different from the control. These findings suggested that the paracellular permeation of CF was not altered by the pharmaceutical excipients and the improvement in topotecan absorption might be due to the inhibition of Bcrp1 function by Cremophor EL and Tween 20.

2.3 Conclusions

In this chapter, the pharmaceutical excipients were applied to improve the intestinal absorption of topotecan, the orally-active topoisomerase-I inhibitor anticancer drug. The oral bioavailability of topotecan was limited due to the efflux capability of intestinal BCRP transporter. First, the pharmaceutical excipients from chapter I were further examined with MTT cytotoxicity assays. The results showed that BL-9EX and Brij97 strongly interacted with Caco-2 cells, resulting in the significant reduction in cell viability. In contrast, other pharmaceutical excipients including Cremophor EL, Labrasol, Pluronic F68, and Tween 20 did not affect the viability of Caco-2 cells. These findings suggested that the stronger membrane solubilization capability of BL-9EX and Brij97 as well as the lack of the mucus layer in Caco-2 cells might be the main factors contributing the toxicity to Caco-2 cells. Next, the bi-directional transport of topotecan across Caco-2 cell monolayer experiments showed that most pharmaceutical excipients improved the transport of topotecan. Tween 20 and Cremophor EL improved both secretory and absorptive transport of topotecan, while Labrasol and Pluronic F68 only improved the absorptive and secretory transport, respectively. After comparing the transport parameters of CF across Caco-2 cell monolayers, it is concluded that the paracellular transport was not affected by the addition of these pharmaceutical excipients. TEER values measured during the transport experiments showed that these pharmaceutical excipients might not loosen the tight junction of Caco-2 cells. In addition, the TEER values from Caco-2 cells treated with Pluronic F68 remained constant throughout the experiment. This phenomenon might be caused by its intrinsic cytoprotective properties of Pluronic F68.[45,101] Next, the pharmaceutical excipients were further examined using the *in situ* closed loop method. Results showed that Cremophor EL and Tween 20 significantly improved the intestinal absorption of topotecan. Pluronic F68 and Labrasol also slightly improved the absorption but unfortunately, those increases in $AUC_{0\text{-}4\ h}$ values are not statistically different, suggesting that their Bcrp1 inhibitory effects in *in situ* were weaker than in *in vitro* Caco-2 cell experiments. Lastly, the effects of Tween 20 and Cremophor EL on the intestinal transport of CF showed that the paracellular permeation of CF was not affected by these pharmaceutical excipients. From these findings, the author concluded that Cremophor EL and Tween 20 are the effective BCRP modulators and could be used to improve the intestinal absorption of topotecan.

Chapter III: Possible inhibitory mechanisms of BCRP transporter by the pharmaceutical excipients and their safety

There are several possible mechanisms of BCRP inhibition by the pharmaceutical excipients. According to their chemical formula shown in Table 1, the pharmaceutical excipients (excluding vitamin E acetate) in this study have an amphipathic characteristic (consisted of both hydrophilic and lipophilic moieties in the molecule). They are likely to form a micelle at the concentration above their CMC and the interaction of the drugs to the micelles was directly related to the ability to improve the absorption of the drugs.[104] Therefore, it is important to evaluate the interaction of drugs with the pharmaceutical excipients. The author measured the micellar interaction between model drugs and the pharmaceutical excipients by using an equilibrium dialysis method.

It was reported that the pharmaceutical excipients containing PEG chains, fatty acid or fatty alcohol with intermediate chain length and the intermediate HLB values ranging from 10 to 17 are likely to be the most effective against P-gp-mediated efflux transport of epirubicin across Caco-2 cells.[105] This is likely because they could disturb the lipid bilayers properties which control membrane partitioning, binding, and transport of P-gp substrates.[106] In addition, the changes in the lipid bilayers properties were reported to reduce the activities of P-gp due to the inhibition of P-gp ATPase activities.[54,107,108] Moreover, particular types of Pluronic copolymers were reported to affect the fluidity of the lipid bilayers which could also affect the activities of P-gp transporters.[69,107] These changes in membrane microenvironment might affect the activity of BCRP in the similar manner as was the case of P-gp. In order to evaluate the effects of pharmaceutical excipients on the lipid bilayer, the author measured the changes in membrane fluidity after the exposure to pharmaceutical excipients. Two fluorescent probes including 1,6-diphenyl-1,3,5-hexatriene (DPH) and N,N,N-trimethyl-4-(6-phenyl-1,3,5-hexatrien-1-yl)phenylammonium p-toluene sulfonate (tma-DPH) were used to measure the changes in membrane fluidity of Caco-2 cells. In addition, there is a possibility that pharmaceutical excipients might inhibit BCRP and P-gp in the transcription level.[109] Therefore, the mRNA expression level of BCRP in Caco-2 cells after the exposure to pharmaceutical excipients was also evaluated using a reverse-transcription polymerase chain reaction (RT-PCR) method.

Lastly, because the pharmaceutical excipients in this study are considered as surfactants which can rupture the cell membrane, causing the damage and affect the viability of the intestinal epithelial cells.[52,110] The author examined the toxicities of these pharmaceutical excipients on the intestinal epithelial cells by measuring the leakage of protein and lactate dehydrogenase enzyme (LDH) into the luminal space during the absorption experiments in rats.

3.1 Materials and methods

3.1.1 Materials

All materials are purchased or obtained from the suppliers as already stated in 1.1.1 and 2.1.1 with the following additions. The dialysis cellulose membrane with 1kDa molecular weight cut-off (MWCO) (Spectra/Por® 6 Dialysis Membrane, Pre-wetted RC tubing) was purchased from Spectrum Laboratories Inc., (Rancho Dominguez, CA., USA). Triton X-100, bovine serum albumin (BSA), DPH, and Coomassie Brilliant Blue G-250 (Brilliant Blue G) were purchased from Sigma Aldrich Chemical Co., Ltd. (St. Louis, MO, USA). tma-DPH was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Benzyl alcohol and N,N-dimethylformamide were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Cholesterol and ethinyl estradiol (E2) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). LDH reference from chicken heart was purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). Cytotoxic detection kits (LDH) were provided by Roche Diagnostics GmbH (Mannheim, Germany) and Dōjindo Molecular Technologies, Inc., (Kumamoto, Japan). Tetrahydrofuran was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan).

3.1.2 Animals

All animals were treated using the same procedure as described in 2.1.2.

3.1.3 Equilibrium dialysis experiments

The micellar interaction between pharmaceutical excipients and model drugs was examined using the same principle as described in the previous reports.[65,104] Drug solutions were first prepared in PBS at pH 7.4 and the concentrations were adjusted to match the concentrations in their respective bi-directional transport studies (100 μM for sulfasalazine and 10 μM for topotecan). The pharmaceutical excipients stock solutions were prepared by dissolving the pharmaceutical excipients with the drug solution. After that, stock solutions were sequentially diluted with the excipient-free drug solution to obtain 1% to 0.01% solutions. The prepared solutions (4 mL) were subsequently filled into the dialysis bags and were placed in a beaker containing 90 mL PBS as the outer fluid. The dialysis system was kept under 37°C with constant stirring. The samples were taken from the outer fluid at the predetermined interval up to 24 h and were immediately replaced with the same amount of PBS. The concentration of sulfasalazine and topotecan in the samples were analyzed using the method previously described in 1.1.5 and 2.1.7, respectively. The concept of the equilibrium dialysis experiment is illustrated in Fig. 18.

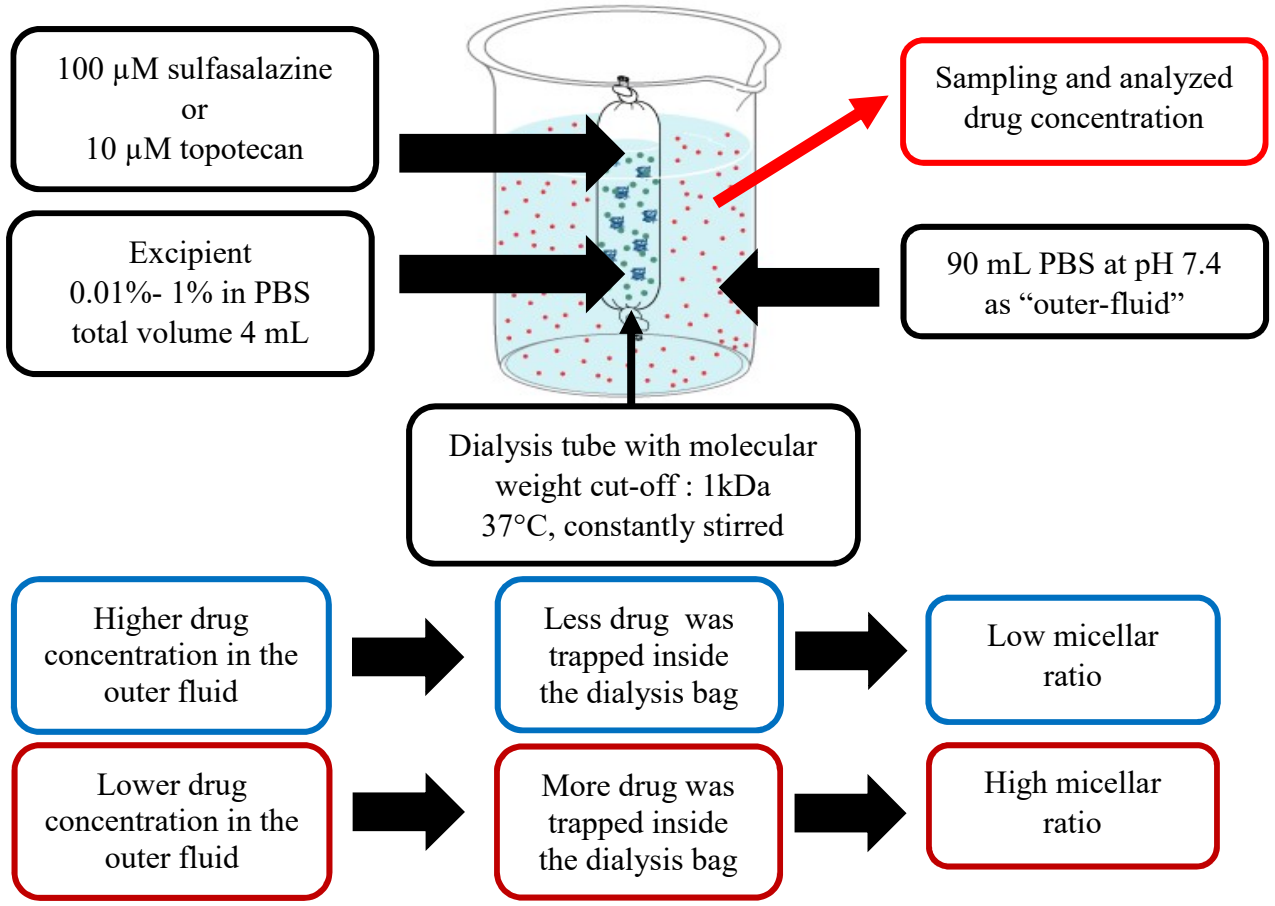


Figure 18. Schematic illustration and the concept of the equilibrium dialysis experiment

The entrapment degree of drugs into micelles at equilibrium was expressed by the micellar ratios, which were calculated by using the following equation.

$$\text{Micellar ratio (\%)} = \frac{(\text{Conc. of outer fluid } \textit{without} \text{ excipient}) - (\text{Conc. of outer fluid } \textit{with} \text{ excipient})}{\text{Conc. of outer fluid } \textit{without} \text{ excipient}} \times 100$$

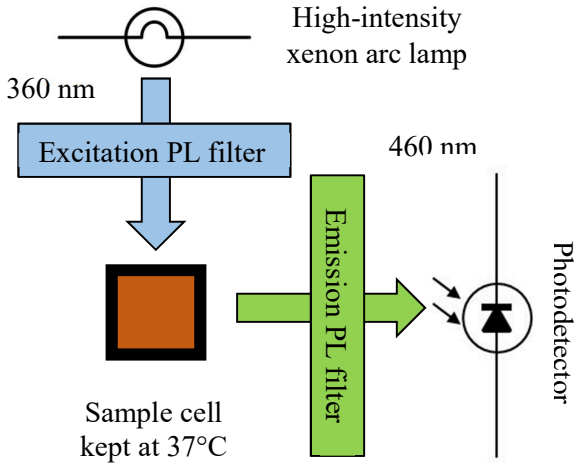
3.1.4 Effects of pharmaceutical excipients on the membrane fluidity of Caco-2 cells

The author measured the change in membrane fluidity in Caco-2 cells by the method as described previously,[111] with minor modifications. The detailed process of the experiment is described as follows. The Caco-2 cells were handled using the procedure as already described in 2.1.3 and were re-suspended in 25 mM HEPES-HBSS pH 7.4 at 2×10^5 cells/mL. The fluorescent probes tma-DPH and DPH were prepared in N,N-dimethylformamide and tetrahydrofuran, respectively at 2 mM. The Caco-2 cells were labeled with either tma-DPH or DPH by incubating with 2 µM solution of tma-DPH or DPH for 5 min or 60 min, respectively. After the labeling, Caco-2 cells were washed and re-suspended to 2×10^5 cells/mL in 25 mM HEPES-HBSS pH 7.4. The labelled Caco-2 cells were incubated with various types and concentrations of pharmaceutical excipients for the additional 30 min. The fluorescence anisotropy of tma-DPH and DPH

labelled Caco-2 cells was monitored using the F-2700 fluorescence spectrophotometer (Hitachi High-Technologies Corp., Tokyo, Japan) equipped with polarization (PL) filters in the parallel and perpendicular orientations to the excitation light (λ_{ex}= 360 nm, λ_{em} =430 nm). Fluorescence anisotropy (r) was calculated using the following equation.

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}}$$

$I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities measured in parallel and perpendicular, respectively, to the excitation light. The schematic illustration of the experiment is shown in Fig. 19.



Measuring parameters	Excitation PL filter angle	Emission PL filter angle
$i_{\perp}$	90°	0°
i_{ll}	90°	90°
$I_{\perp}$	0°	90°
I_{ll}	0°	0°

Figure 19. Schematic illustration of the tma-DPH and DPH fluorescence anisotropy measurement

3.1.5 Analysis of reverse-transcription polymerase chain reaction (RT-PCR)

RT-PCR is commonly used in molecular biology for mRNA analysis, including detection and quantitation, by the use of fluorescent probes.[112] The author examined the effects of pharmaceutical excipients on the BCRP mRNA expression in Caco-2 cells using the following procedure. First, Caco-2 cells were obtained from the process described in 2.1.3 and were seeded into 24-well plate (Falcon™ Polystyrene Microplates, Thermo Fisher Scientific, MS., USA.) at 1.7×10^5 cell/well. The cells were allowed to grow in the 5% CO_2 atmosphere until reaching confluence after 72 h. After that, the monolayers were washed with HBSS and the growth media containing the pharmaceutical excipient with various concentrations was added. The monolayers were incubated for the additional 6 h before the isolation of total RNA.

Total RNA from the Caco-2 cells was extracted with Sepasol-RNA I Super G total RNA isolation reagent (Nacalai Tesque, Inc. (Kyoto, Japan) by using the manufacturer provided protocol. The isolated total RNA was quantified by microplate reader (Biotek Instrument Inc., Winooski, VT, USA), and was

later diluted to 50 ng in 6 µL by double-distilled water before the reverse transcription. The construction of complementary DNA (cDNA) was performed by ReverTra Ace® qPCR RT master mix with gDNA Remover (Toyobo Co., Ltd. Osaka, Japan) using the manufacturer provided protocol.

The author performed a PCR assays using the TB Green methodology by TB Green™ Premix Ex Taq™ II RT-PCR kit (Takara Bio Inc., Shiga, Japan). The BCRP ABCG2 gene was PCR-amplified with the specific primers (forward primer 5′-GGTCTGTTGGTCAATCTCACA-3′, reverse primer 5′-TCCATA TCGTGGAATGCTGAAG-3′).[13,16] Human glyceraldehyde-3-phosphate dehydrogenase (hGAPDH) was used as a reference and was PCR-amplified with the specific primers (forward primer 5′-TGTTGCCA TCAATGACCCCTT-3′, reverse primer 5′-CTCCACGACGTACTCAGCG-3′).[113] All primers were provided by Griner Bio-One Co., Ltd. (Tokyo, Japan). LightCycler® Nano (Roche Molecular Systems Inc., Basel, Switzerland) was used to control and monitor the reactions. The PCR process was initiated by the initial denaturation at 95°C for 30 s and followed by 36 cycles of 3-stage amplification (annealing: 56°C, 30 s; extension: 72°C 60 s; denaturation 95°C 10 s). The expression levels of BCRP mRNA were expressed as the ratios to hGAPDH mRNA.

3.1.6 Evaluation of intestinal membrane damage in the *in situ* closed loop experiments by Bradford and LDH assays

The toxicities of pharmaceutical excipients were determined by measuring the leakage of protein and LDH enzymes into the luminal fluid which was collected after the *in situ* closed loop experiments (1.1.4 and 2.1.6). Any solid matter in this luminal fluid was removed by centrifugation at 200 G. for 7 min. The clear supernatant was stored at 4°C until analysis.

The amount of protein was determined by the method of Bradford which observing the reaction of the protein with Coomassie Brilliant Blue (CBB). The CBB reagent was prepared according to the following procedure. First, 5 mg of CBB was dissolved in 95% ethanol and subsequently acidified by the addition of 5 mL 85% phosphoric acid. The final volume of CBB reagent was adjusted to 50 mL by distilled water and was kept in light-sealed container until use. 20 µL of luminal fluid samples were allowed to react with 200 µL CBB reagent in a dark container. Protein concentration was measured by monitoring the absorbance at 595 nm using microplate reader and BSA was used as a reference standard.

The activity of LDH enzymes in the luminal fluid was also evaluated to assess the damage of the intestinal epithelial membrane. The cytotoxic detection kit (LDH) and the manufacturer's protocols were used. The absorbance was monitored at 490 nm using the microplate reader and LDH from chicken heart was used as a reference.

3.2 Results and Discussion

3.2.1 Micellar interactions between pharmaceutical excipients and the model drugs

When the author considering the effective concentrations of pharmaceutical excipients which can inhibit the function of BCRP, micellar interaction is one of the important limiting factors. Because several substrates of ABC transporters are usually lipophilic or amphiphilic, they are likely to be entrapped into the lipophilic core of the micelle.[114] The micellar entrapment could heavily affect the intestinal absorption of drugs. For example, 1,440 mg Cremophor EL was reported to significantly increase the oral bioavailability of fexofenadine, a P-gp substrate, in humans. The entrapped ratio of fexofenadine into the micelle of Cremophor EL was 24%. In contrast, the same dose of Cremophor EL adversely decreased the oral bioavailability of saquinavir, an another P-gp substrate. Further examination revealed that most of saquinavir (92.8%) was entrapped into the micelles of Cremophor EL.[104] These findings suggested that the drugs with strong micellar interaction are less likely to gain the benefit from the existence of the pharmaceutical excipients. In addition, saquinavir (as mesylate) has the log P value of 4.4,[115] suggesting that it is a low-solubility compound. On the other hand, fexofenadine (as hydrochloride) is more soluble and has the log P value of 0.49.[116] Therefore, the micellar entrapment ratios might be directly related to the solubility of the drug and the water solubility of drugs decreased, the entrapping percent of drugs into the micelles was increased.

Table 10. Micellar interactions between sulfasalazine and various types of pharmaceutical excipients

Excipients conc. (w/v)	Micellar ratio (%)[a]						
	BL-9EX	Brij92	Brij97	Cremophor EL	Pluronic F127	Labrasol	Tween 20
0.01%	1.31	2.16	3.43	0.00	0.11	0.00	0.00
0.05%	4.56	2.52	5.02	2.36	1.67	1.17	0.00
0.1%	6.84	7.16	9.19	3.43	3.32	3.08	2.49
0.5%	24.60	22.58	34.25	10.60	10.70	3.18	9.81
1%	58.46	34.89	50.51	19.29	16.00	17.79	21.06

[a] The percentages of sulfasalazine entrapped into micelles (micellar ratios) were calculated from the following equation

$$\text{Micellar ratio (\%)} = \frac{\text{(Conc. of outer fluid without excipient)-(Conc. of outer fluid with excipient)}}{\text{Conc. of outer fluid without excipient}} \times 100$$

(*J. Pharm. Sci.,* **107,** 2946-2956 (2018); Table 4)

Sulfasalazine has the log P value of 3.88, suggesting that it is a low water soluble drug.[64] The equilibrium was achieved after 24 h and the micellar ratios of entrapped sulfasalazine in the presence of various types of pharmaceutical excipients are summarized in Table 10. The micellar ratios less than 10% are considered to be preferable and should not interfere with the permeation of the drugs.[65] At 1% concentration, all pharmaceutical excipients had >10% micellar ratios, and a strong micellar interaction with sulfasalazine was observed. The micellar ratio decreased as the excipient concentration decreased. At 0.1% concentration, all pharmaceutical excipients had less than 10% micellar ratio, suggesting the micellar interaction was low and the absorption of sulfasalazine should have benefit by the addition of pharmaceutical excipients with the concentration not greater than 0.1%.

In contrast to sulfasalazine, topotecan (hydrochloride) has the log P value of 0.8,[117] suggesting that it is far more water soluble than sulfasalazine. As initially expected, the equilibrium of topotecan was achieved within just 4 h. The micellar ratios even at the maximum concentration of the pharmaceutical excipients (1%) were extremely low (0%-2%). There was also no observable difference between the types of pharmaceutical excipients. These results suggested that topotecan was less likely to be entrapped inside the micelles and the maximum applicable concentration of the pharmaceutical excipients to improve the intestinal absorption of topotecan might be greater than 0.1%.

3.2.2 Effects of pharmaceutical excipients on the membrane fluidity of Caco-2 cells

Because ABC transporters resides on the cell membrane, all important steps of their efflux cycle were reported to take place inside or within the vicinity of the lipid bilayers. For example, it is believed that the substrates bind to the their binding sites at the hydrophobic region of the lipid bilayers.[6,106] Therefore, the change in the lipid microenvironment of the membrane could affect the binding of the substrate to the transporter and consequently decrease its efflux capability.[114,118] Moreover, ABC transporters including BCRP operated via a “flip-flop” mechanism which invokes ATP-alternating access. The binding of ATP to the NBD triggered the conformation shift from inward-facing to the outward-facing conformation.[6,119] This mechanism required a precise condition of the lipid bilayer as well as a specific annular-lipid surround the transporter protein for the transporter to function optimally.[6,119,120]

The author used two fluorescent probes to measure the change in membrane fluidity caused by the pharmaceutical excipients. The changes in membrane fluidity of Caco-2 cells could be observed by measuring the changes in the fluorescence anisotropy of DPH and tma-DPH-labelled Caco-2 cells.[44,121] The increases in the fluorescence anisotropy indicated the decrease in membrane fluidity (more rigidity) and the decreases in fluorescence anisotropy indicated the increase in membrane fluidity (less rigidity).[111] Because DPH is more lipophilic than tma-DPH by nature, it is mainly attached to the hydrophobic inner region of

the lipid bilayers. In contrast, tma-DPH is more hydrophilic and is mainly attached on the hydrophilic outer surface of the lipid bilayers.[121] Therefore, the changes in the DPH and tma-DPH fluorescence anisotropy indicated the changes in membrane fluidity of the inner region or outer region (respectively) of the lipid bilayers. Benzyl alcohol, a known membrane fluidizer and cholesterol, a known membrane rigidizer, were used as positive controls for the increase and decrease in membrane fluidization, respectively.[44,54,111,121] The effects of pharmaceutical excipients on the membrane fluidity of the Caco-2 cells are summarized in Fig. 20.

In Fig. 20 (a), the fluorescence anisotropy of DPH was significantly increased in the presence of 200 μM cholesterol, a typical membrane rigidizer. In contrast, DPH fluorescence anisotropy was significantly decreased in the presence of 30 mM benzyl alcohol, a known membrane fluidizer. Interestingly, the tested pharmaceutical excipients (excluding Pluronic F68) significantly decreased DPH fluorescence anisotropy, suggesting that most pharmaceutical excipients increased membrane fluidity of the inner hydrophobic region of the Caco-2 cell membranes. The fluorescence anisotropy of tma-DPH-labelled Caco-2 cells is shown in Fig. 20 (b). Similar to Fig. 20 (a), cholesterol and benzyl alcohol increased and decreased the fluorescence anisotropies of tma-DPH, suggested that both positive controls also modified the fluidity of the outer region of the Caco-2 cell membrane. Interestingly, a significant increase in tma-DHP fluorescence anisotropy could only be observed in the presence of 0.05% Pluronic F68. These results suggested that only Pluronic F68 increased membrane rigidity in the outer hydrophilic region of the Caco-2 cell membrane. In contrast, other pharmaceutical excipients specifically increased the membrane fluidity of the inner hydrophobic region of the Caco-2 cell membrane. These results are comparable with the previous reports.[44,69] An *in silico* simulation revealed that due to the distinctive PEO-PPO-PEO arrangement and its longer PEO chains relative to PPO region of Pluronic F68, it is suggested that these long PEO chains are likely to adhere to the hydrophilic heads while the hydrophobic PPO region partially insert into the inner lipophilic leaflet of the bilayers.[107] In this particular condition, Pluronic F68 is likely to decrease the lateral mobility and cause a solidification of the outer cellular surface.[69] Therefore, the increase in tma-DPH fluorescence anisotropy was observed. In addition, the alteration of the membrane fluidity seems to be well correlated to the inhibition of BCRP and the changes in transport parameters of topotecan across Caco-2 cell monolayers as described in 2.2.2 and 2.2.3. Therefore, the author believed that the main BCRP inhibitory mechanism of the pharmaceutical excipients was by alternating the fluidity of the lipid bilayers which caused the disruption of the transporter activities and ultimately, decreased the efflux transport of its substrates.

Fig. 20 (a)

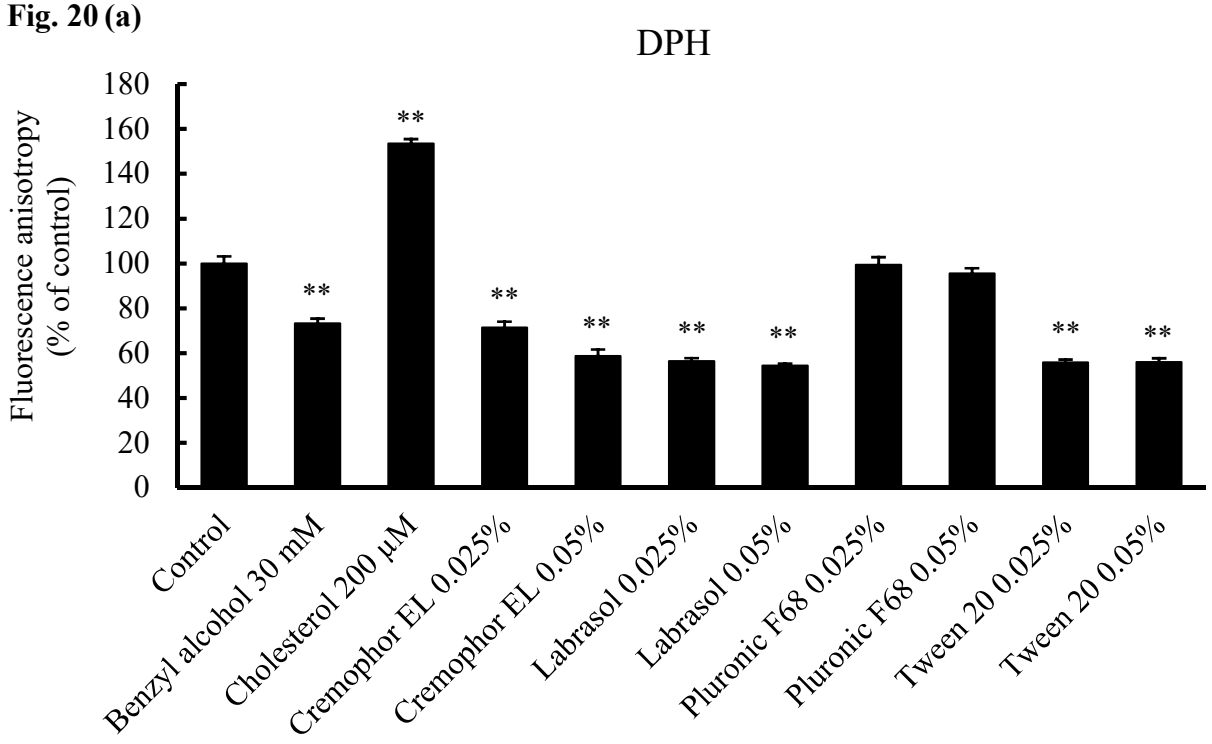


Fig. 20 (b)

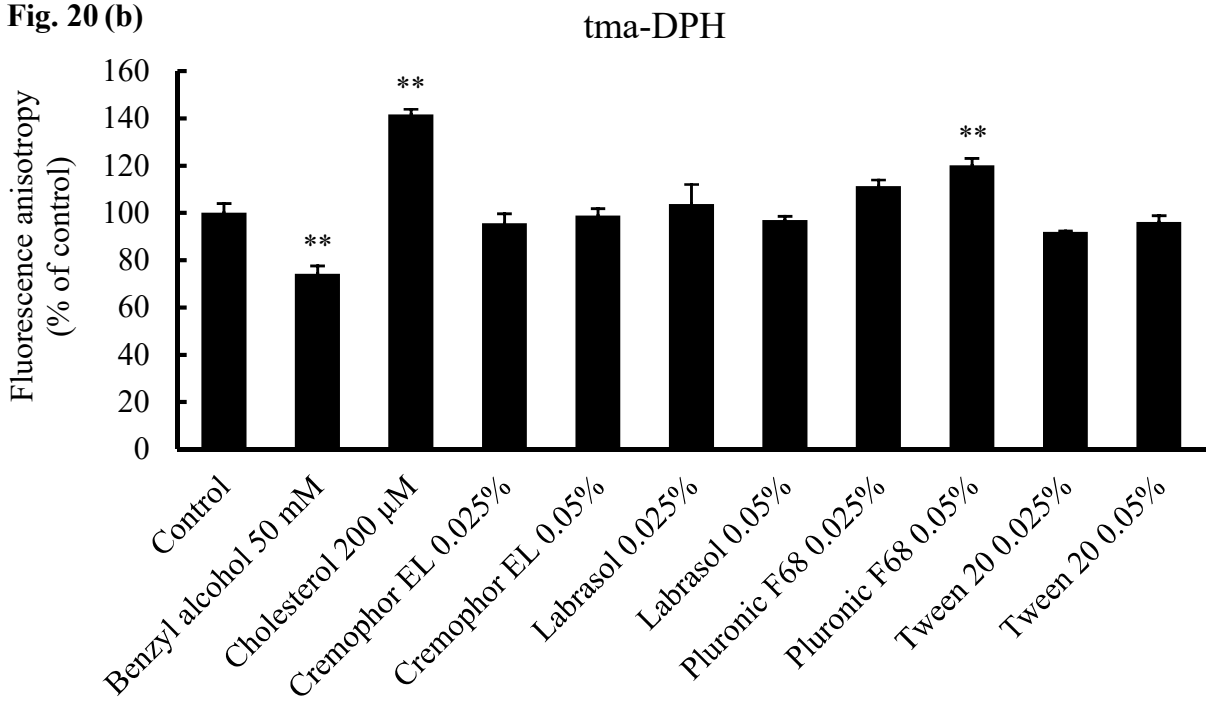


Figure 20. Effects of pharmaceutical excipients on the membrane fluidity of Caco-2 cells. Fluorescence anisotropy was measured from Caco-2 cells labeled with (a) DPH or (b) tma-DPH as probes. Results are expressed as mean ± SE of 3–5 experiments. (**) $p < 0.01$, (*) $p < 0.05$, compared with the corresponding control. (*J. Pharm. Sci*., **108,** 1315-1325 (2019); Figure 7)

3.2.3 Effects of pharmaceutical excipients on BCRP mRNA expression in Caco-2 cells

In addition to the direct effect of these pharmaceutical excipients on the BCRP transporter itself, several PEG-containing pharmaceutical excipients are known to inhibit P-gp and BCRP efflux pumps by the downregulation of MDR1 and ABCG2.[109,122] To further evaluate this possible inhibitory mechanism, the expression level of BCRP mRNA in Caco-2 cells after the exposure to the pharmaceutical excipients was examined by a RT-PCR method.

Caco-2 cells were exposed to the pharmaceutical excipients at various concentrations for 6 h. Ethinyl estradiol (E2) was used as a positive control in this study because it was reported to downregulate ABCG2 mRNA expression in human placental BeWo cells.[123] The results are summarized in Fig. 21.

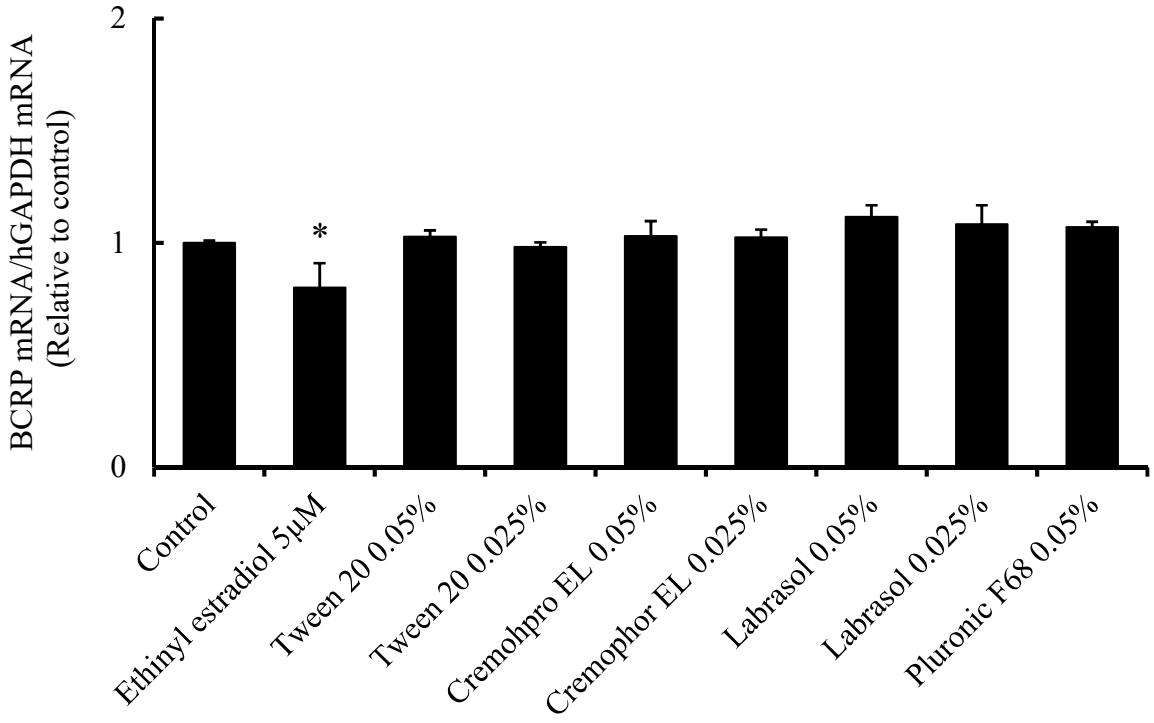


Figure 21. Effects of pharmaceutical excipients on the BCRP mRNA expression in Caco-2 cells. BCRP mRNA expression level was normalized to hGAPDH. Data are expressed as mean ± S.E. of 3-6 experiments. (*) $p<0.05$, compared with the control.

Differences in the membrane transport of xenobiotics and endogenous compounds caused by different levels of sexual hormones such as testosterone and estradiol have been previously described in several studies.[13,16] As shown in Fig. 21, 5 μM E2 significantly decreased the expression level of BCRP mRNA. This result was comparable to the previous report.[123] In contrast, none of the evaluated pharmaceutical excipients affect the expression level of BCRP mRNA. This result suggested that at the tested concentration, the inhibitory mechanism of BCRP transporter by the pharmaceutical excipients was

not related to the downregulation of BCRP mRNA. However, since the expression level of BCRP mRNA is not always correlated to the BCRP protein expression level as well as its activity,[4,13] the protein expression of BCRP should be evaluated in the further studies.

3.2.4 Toxicity of pharmaceutical excipients on the small intestinal membrane

An *in vivo* study of a pharmaceutical excipient must take into account its intestinal membrane toxicity, because any damage to the intestinal membrane can disrupt the barrier function in the intestine and subsequently increases the intestinal permeability of drugs.[124,125] The damage to the intestinal membrane could be evaluated by the increase in the LDH or protein being released into the intestinal lumen. LDH is a cytosolic enzyme which is released during the tissue damage. It was widely considered as a marker of cell injury.[110,126] In addition, the protein is also released from the cytosol of the damaged epithelial cells into the intestinal lumen which could be determined by the method of Bradford.[71,127] The LDH activities and protein amount are increased when the intestinal membrane is damaged. Effects of the pharmaceutical excipients on the toxicities to the intestinal epithelial membrane after the administration of topotecan and sulfasalazine are summarized in Fig. 22 and 23, respectively.

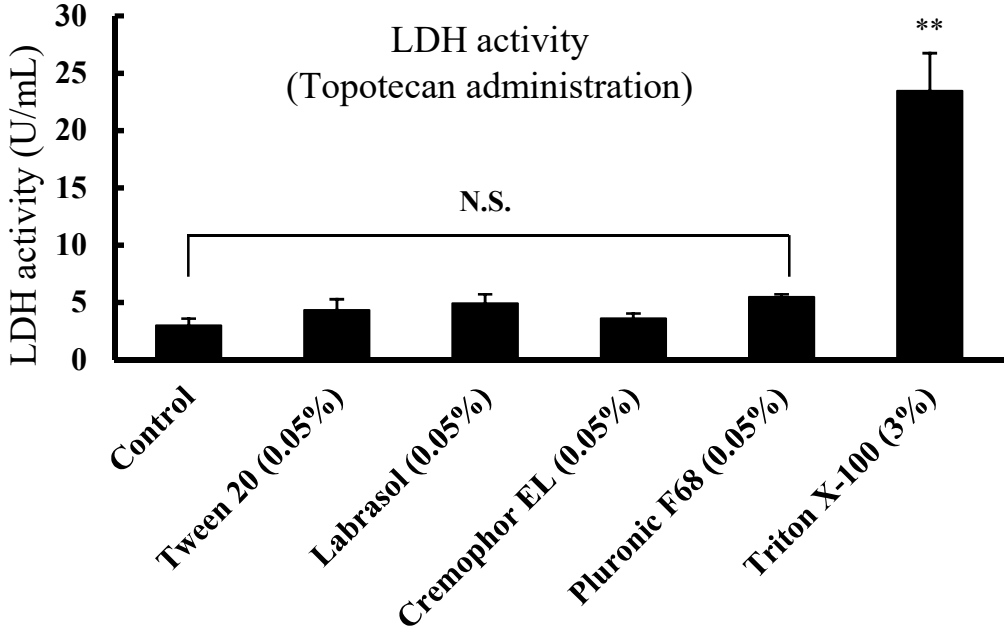


Figure 22. Intestinal membrane toxicity of pharmaceutical excipients at 4 h after the intestinal administration of 0.5 mg/kg topotecan by the *in situ* closed loop method. Intestinal membrane damage was determined by measuring LDH activity. Results are expressed as mean ± SE of at least 3 animals. (**) $p<0.01$, (N.S.) no significant difference, compared with control. (*J. Pharm. Sci*., **108,** 1315-1325 (2019); Figure 5)

Fig. 23 (a)

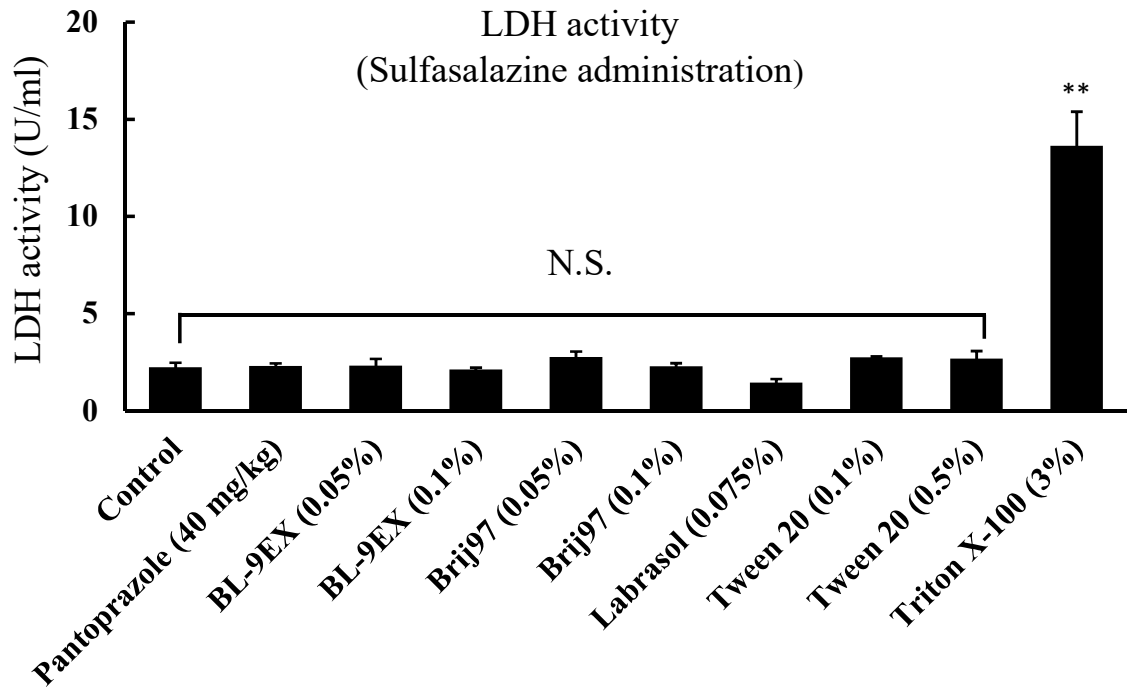


Fig. 23 (b)

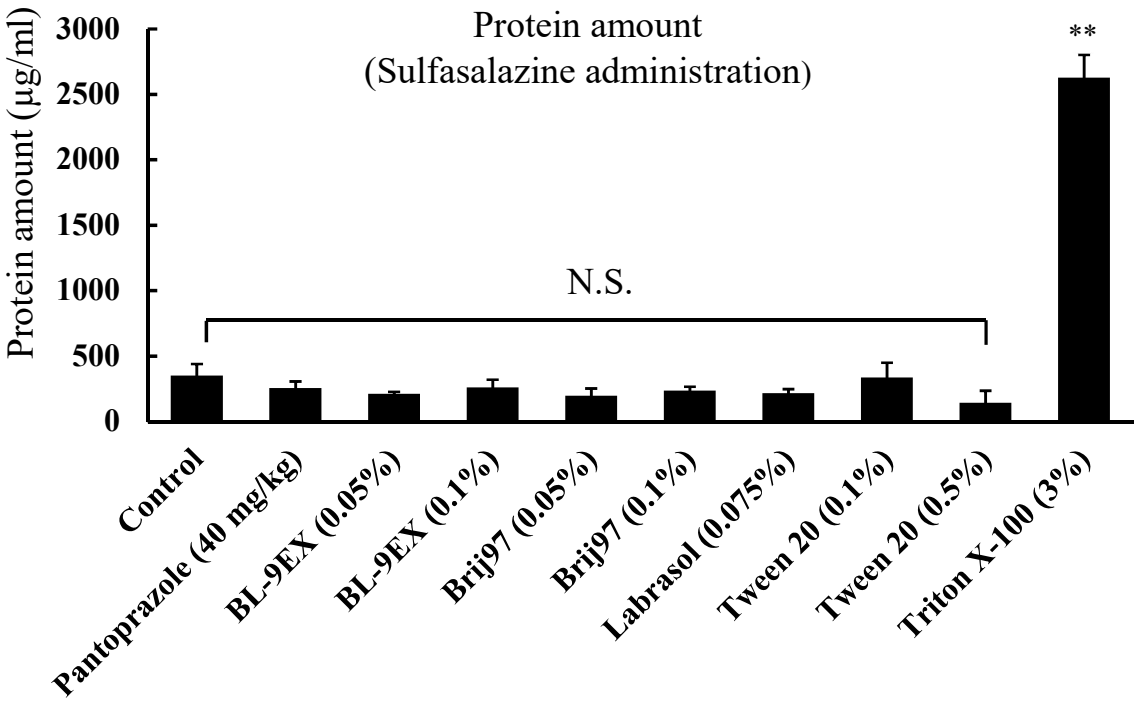


Figure 23. The activities of LDH (a) and the amount of released protein (b) at 4 h after the intestinal administration of 1 mg/kg sulfasalazine with various pharmaceutical excipients. Data are expressed as the mean ± S.E. of at least 3 animals. (N.S.) not significantly different compared with the control. (*J. Pharm. Sci.,* **107,** 2946-2956 (2018); Figure 3)

Non-ionic surfactant Triton X-100 at 3% is known to cause severe damage to the small intestinal epithelial cells which can be visually observed as well as by the toxicity probes (LDH and protein).[60,62,72] Therefore, it is used as a positive control for intestinal membrane damage. As seen in Fig. 22, compared to the topotecan alone, 3% Triton X-100 significantly enhanced the LDH activities in the luminal fluid collected at 4 h after the intestinal administration with topotecan. This result suggested that 3% Triton-X 100 caused damage to the intestinal epithelial membrane. In contrast, the LDH activities in the presence of 0.05% Tween 20, Cremophor EL, Labrasol, and Pluronic F68 were not statistically different compared with the control. These results suggested that at 0.05%, these pharmaceutical excipients did not cause any significant damage to the small intestinal membrane. The similar results were also observed after the intestinal administration of 1 mg/kg sulfasalazine (Fig. 23). Triton X-100 at 3% significantly increased LDH activities compared to its respective control, while other excipients including BL-9EX, Brij97, Labrasol, and Tween 20 at up to 0.1%, did not increase theses toxicological markers. These results suggested that the tested pharmaceutical excipients did not cause serious damage and the intestinal membrane was well tolerated. In addition, the author observed that protein level in the luminal fluid after the 4 h exposure showed similar results as with the LDH activities. These results further support that there was no significant damage to the intestinal membrane.

The *in situ* toxicities parameter of BL-9EX and Brij97 as observed by LDH activities and protein release in this study are in accordance with the previous reports by Zhao *et al.*[53,54], suggesting that BL-9EX and Brij97 did not cause serious damage to the small intestinal epithelial membrane and are safe for *in vivo* applications. Interestingly, these results are different from those observed from the MTT assays using Caco-2 cells as shown in Fig. 13. The author believed that there are 2 hypotheses regarding to this discrepancy as already discussed in 2.2.1. First, polyoxyethylene alkyl ether derivatives were reported to have a relatively higher membrane solubilization capacity compared to other pharmaceutical excipients.[50,92–94] Second, because Caco-2 cell monolayers do not express a protective mucous layer[95], while in rats small intestine, the thick mucus layers are clearly visible.[60,61,72] Therefore, Caco-2 cells is more susceptible to the high membrane solubilizing activities of BL-9EX and Brij97, while in the small intestine, the epithelial cells are protected by the mucous layers. The toxicity results from this study supported these hypotheses and suggested that BL-9EX, Tween 20, and Cremophor EL might be safe as BCRP modulators for the application to improve the intestinal absorption of its substrates.

3.3 Conclusions

In this chapter, several issues regarding to the possible inhibitory mechanisms of BCRP transporters and the important factors affecting the effectiveness of these pharmaceutical excipients were discussed. First of all, the drugs with greater lipophilicity are more likely to be entrapped in to the micelles than the drugs which are more hydrophilic. The author suggested the pharmaceutical excipients which are amphiphilic should be examined for their micellar interaction with the drugs. As observed in 3.2.1, the micellar ratios of sulfasalazine (log P = 3.88) were far greater than the micellar ratios of topotecan (log P = 0.8), suggesting that sulfasalazine was more likely to be entrapped inside the micelle than topotecan. Micellar ratios of sulfasalazine considerably decreased, as the concentration of pharmaceutical excipients was reduced to 0.1%. Therefore, the author suggested that for sulfasalazine as a compound of interest, 0.1% is the maximum applicable concentration for the pharmaceutical excipients. On the other hand, topotecan might benefit from the addition of the pharmaceutical excipients with greater than 0.1% concentration.

Next, the membrane fluidity measurement revealed that Cremophor EL, Labrasol, and Tween 20 specifically increased the fluidity of the inner lipid layer of Caco-2 cells membrane. Pluronic F68 decreased the fluidity of the outer hydrophilic layer of Caco-2 cells membrane. These changes in membrane fluidity are directly related to the changes in transport parameters of topotecan across Caco-2 cell monolayers. These findings suggested that the pharmaceutical excipients might inhibit BCRP transporters by altering the membrane fluidity and might directly affect the function of BCRP transporters. Especially, the increase in membrane fluidity of the inner hydrophobic region of the lipid bilayers caused by these pharmaceutical excipients is considered to be the most important inhibitory mechanism of BCRP transporter by the pharmaceutical excipients. In addition, the BCRP mRNA expression after the exposure to the pharmaceutical excipients showed that pharmaceutical excipients did not inhibit the expression level of BCRP at its transcription level. Therefore, the pharmaceutical excipients might not affect the level of BCRP protein expression. However, BCRP protein expression should be evaluated to further clarify this hypothesis.

Lastly, toxicity parameters including LDH activities and protein release amount did not increase after the addition of up to 0.1% of Brij97, BL-9EX Cremophor EL, Labrasol, or Tween 20. These results suggested that they did not cause significant damage to the small intestinal epithelial membrane. Therefore, the author believe that they might be the promising candidates for further *in vivo* application.

Summary

In chapter I, the author demonstrated that the transport of sulfasalazine across the rat intestine was mainly into the secretory direction due to the activities of Bcrp1 transporter. This was supported by the results of Ko143 and pantoprazole, typical BCRP inhibitors. Next, the result of the intestinal absorption of sulfasalazine might support the hypothesis that Bcrp1 was behind the limited absorption of sulfasalazine. Sulfasalazine absorption was improved by 9.25-fold after the addition of pantoprazole, a Bcrp1 inhibitor. In addition, 0.1% BL-9EX and Brij97 significantly improved the intestinal absorption of sulfasalazine, suggesting that they are promising Bcrp1 modulators. Additionally, the effects of BL-9EX and Brij97 on the intestinal absorption of CF were examined. Results indicated that BL-9EX did not increase the paracellular permeation of CF, suggesting that BL-9EX might improve the intestinal absorption of sulfasalazine by the inhibition of Bcrp1. On the other hand, Brij97 increased the intestinal absorption of CF, suggesting that Brij97 might increase the intestinal absorption of sulfasalazine via a paracellular permeation pathway. Lastly, the author demonstrated that BL-9EX did not decrease the plasma concentration of 5-ASA, an active metabolite of sulfasalazine. Therefore, it is not possible that BL-9EX might inhibit the intestinal metabolism of sulfasalazine. In conclusion, the author demonstrated that BL-9EX could be applied as a BCRP modulator to improve the intestinal absorption of BCRP substrates.

In chapter II, the pharmaceutical excipients were applied to improve the intestinal absorption of topotecan. First, the toxicity of pharmaceutical excipients from chapter I was further examined by MTT cytotoxicity assays. The results showed that BL-9EX and Brij97 reduced the viability of Caco-2 cells. In contrast, other pharmaceutical excipients including Cremophor EL, Labrasol, Pluronic F68, and Tween 20 did not affect the viability of Caco-2 cells. Next, the bi-directional transport of topotecan across Caco-2 cell monolayer experiments showed that most pharmaceutical excipients could improve the transport of topotecan. Tween 20 and Cremophor EL could improve both secretory and absorptive transport of topotecan, while Labrasol and Pluronic F68 only improved the absorptive and secretory transport, respectively. After comparing the transport parameters of CF across Caco-2 cell monolayers, it is concluded that the paracellular transport was not affected by the addition of these pharmaceutical excipients. TEER values measured during the transport experiments showed that these pharmaceutical excipients might not loosen the tight junction of the Caco-2 cells. Next, *in situ* closed loop experiments revealed that Cremophor EL and Tween 20 significantly improved the intestinal absorption of topotecan. Lastly, the effects of Tween 20 and Cremophor EL on the intestinal transport of CF showed that the paracellular permeation of CF was not affected by these pharmaceutical excipients. From these findings, the author concluded that Cremophor EL and Tween 20 are the effective BCRP modulators and could be used to improve the intestinal absorption of topotecan.

The author also demonstrated that the micellar ratios of sulfasalazine were far greater than the micellar ratios of topotecan, suggesting that sulfasalazine was more likely to be entrapped inside the micelle than topotecan. Micellar ratios of sulfasalazine decreased considerably as the concentration of pharmaceutical excipients was reduced to 0.1%. Therefore, the author suggested that for sulfasalazine as model a compound, 0.1% is the maximum applicable concentration for the pharmaceutical excipients. On the other hand, topotecan might have the benefits from the addition of the pharmaceutical excipients with greater than 0.1% concentration. Next, the membrane fluidity measurement revealed that Cremophor EL, Labrasol, Tween 20 specifically increased the fluidity of the inner lipid layer of Caco-2 cells membrane. Pluronic F68 decreased the fluidity of the outer hydrophilic layer of Caco-2 cells membrane. The changes in membrane fluidity are directly related to the changes in transport parameters of topotecan across Caco-2 cell monolayers. These findings suggested that the pharmaceutical excipients inhibited the function of BCRP transporter by altering the membrane fluidity, and the increase in membrane fluidity caused by the pharmaceutical excipients was considered to be the most important inhibitory mechanism of BCRP transporter. In addition, the BCRP mRNA expression after the exposure to the pharmaceutical excipients showed that pharmaceutical excipients did not inhibit the expression level of BCRP mRNA at its transcription level. Lastly, toxicity parameters including LDH activities and protein release amount did not increase after the addition of up to 0.1% of Brij97, BL-9EX Cremophor EL, Labrasol, or Tween 20. These results suggested that they did not cause significant damage to the small intestinal epithelial membrane.

From all these experiments, the author can conclude that the pharmaceutical excipients BL-9EX, Cremophor EL, and Tween 20 are safe and might be the useful BCRP modulators to improve the intestinal absorption of sulfasalazine, topotecan, and other substrates of BCRP transporters.

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References

1. Doyle, L. a *et al.* A multidrug resistance transporter from human MCF-7 breast cancer cells. *Proc. Natl. Acad. Sci. U. S. A.* **95,** 15665–15670 (1998).

2. Hollenstein, K., Dawson, R. J. & Locher, K. P. Structure and mechanism of ABC transporter proteins. *Curr. Opin. Struct. Biol.* **17,** 412–418 (2007).

3. Sosnik, A. Reversal of multidrug resistance by the inhibition of ATP-binding cassette pumps employing 'Generally Recognized As Safe' (GRAS) nanopharmaceuticals: A review. *Adv. Drug Deliv. Rev.* **65,** 1828–1851 (2013).

4. Jani, M. *et al.* Structure and function of BCRP, a broad specificity transporter of xenobiotics and endobiotics. *Arch. Toxicol.* **88,** 1205–1248 (2014).

5. Doyle, L. A. & Ross, D. D. Multidrug resistance mediated by the breast cancer resistance protein BCRP (ABCG2). *Oncogene* **22,** 7340–7358 (2003).

6. Taylor, N. M. I. *et al.* Structure of the human multidrug transporter ABCG2. *Nature* **546,** 504–509 (2017).

7. Ferreira, R. J., Bonito, C. A., Cordeiro, M. N. D. S., Ferreira, M. J. U. & Dos Santos, D. J. V. A. Structure-function relationships in ABCG2: Insights from molecular dynamics simulations and molecular docking studies. *Sci. Rep.* **7,** 1–17 (2017).

8. Wang, H. *et al.* Membrane topology of the human breast cancer resistance protein (BCRP/ABCG2) determined by epitope insertion and immunofluorescence. *Biochemistry* **47,** 13778–13787 (2008).

9. Xu, Y. *et al.* Allocrite sensing and binding by the breast cancer resistance protein (ABCG2) and P-glycoprotein (ABCB1). *Biochemistry* **54,** 6195–6206 (2015).

10. Kim, Y. & Chen, J. Molecular structure of human P-glycoprotein in the ATP-bound, outward-facing conformation. *Science* **359,** 915–919 (2018).

11. Johnson, Z. L. & Chen, J. ATP binding enables substrate release from multidrug resistance protein 1. *Cell* **172,** 81–89 (2018).

12. Giri, N. *et al.* Substrate-dependent breast cancer resistance protein (Bcrp1/Abcg2)-mediated interactions: consideration of multiple binding sites in *in vitro* assay design. *Drug Metab. Dispos.* **37,** 560–570 (2009).

13. Gutmann, H., Hruz, P., Zimmermann, C., Beglinger, C. & Drewe, J. Distribution of breast cancer

resistance protein (BCRP/ABCG2) mRNA expression along the human GI tract. *Biochem. Pharmacol.* **70,** 695–699 (2005).

14. Maliepaard, M. *et al.* Subcellular localization and distribution of the breast cancer resistance protein transporter in normal human tissues. *Cancer Res.* **61,** 3458–3464 (2001).

15. Huls, M. *et al.* The breast cancer resistance protein transporter ABCG2 is expressed in the human kidney proximal tubule apical membrane. *Kidney Int.* **73,** 220–225 (2008).

16. Enokizono, J., Kusuhara, H. & Sugiyama, Y. Effect of breast cancer resistance protein (Bcrp/Abcg2) on the disposition of phytoestrogens. *Mol. Pharmacol.* **72,** 967–975 (2007).

17. Jonker, J. W. *et al.* The breast cancer resistance protein BCRP (ABCG2) concentrates drugs and carcinogenic xenotoxins into milk. *Nat. Med.* **11,** 127–129 (2005).

18. Van Herwaarden, A. E. & Schinkel, A. H. The function of breast cancer resistance protein in epithelial barriers, stem cells and milk secretion of drugs and xenotoxins. *Trends Pharmacol. Sci.* **27,** 10–16 (2006).

19. Robey, R. W., Polgar, O., Deeken, J., To, K. W. & Bates, S. E. ABCG2: Determining its relevance in clinical drug resistance. *Cancer Metastasis Rev.* **26,** 39–57 (2007).

20. Kashihara, Y. *et al.* Small-dosing clinical study: pharmacokinetic, pharmacogenomic (SLCO2B1 and ABCG2), and interaction (atorvastatin and grapefruit juice) profiles of 5 probes for OATP2B1 and BCRP. *J. Pharm. Sci.* **106,** 2688–2694 (2017).

21. Tomaru, A. *et al.* Studies on the intestinal absorption characteristics of sulfasalazine, a breast cancer resistance protein (BCRP) substrate. *Drug Metab. Pharmacokinet.* **28,** 71–74 (2013).

22. Lee, C. A. *et al.* Breast cancer resistance protein (ABCG2) in clinical pharmacokinetics and drug interactions: practical recommendations for clinical victim and perpetrator drug-drug interaction study design. *Drug Metab. Dispos.* **43,** 490–509 (2015).

23. Allen, J. D., Brinkhuis, R. F., Wijnholds, J. & Schinkel, A. H. The mouse Bcrp1/Mxr/Abcp gene: amplification and overexpression in cell lines selected for resistance to topotecan, mitoxantrone, or doxorubicin. *Cancer Res.* **59,** 4237–4241 (1999).

24. Gandhi, Y. A. & Morris, M. E. Structure-activity relationships and quantitative structure-activity relationships for breast cancer resistance protein (ABCG2). *AAPS J.* **11,** 541–552 (2009).

25. Robey, R. W. *et al.* ABCG2: a perspective. *Adv. Drug Deliv. Rev.* **61,** 3–13 (2009).

26. Poirier, A. *et al.* The need for human breast cancer resistance protein substrate and inhibition evaluation in drug discovery and development: why, when, and how? *Drug Metab. Dispos.* **42,** 1466–1477 (2014).

27. Juliano, R. L. & Ling, V. A surface glycoprotein modulating drug permeability in chinese hamster ovary cell mutants. *Biochim. Biophys. Acta* **455,** 152–162 (1976).

28. Hennessy, M. & Spiers, J. P. A primer on the mechanics of P-glycoprotein the multidrug transporter. *Pharmacol. Res.* **55,** 1–15 (2007).

29. Tsuruo, T., Iida, H., Tsukagoshi, S. & Sakurai, Y. Overcoming of vincristine resistance in P388 leukemia *in vivo* and *in vitro* through enhanced cytotoxicity of vincristine and vinblastine by verapamil. *Cancer Res.* **41,** 1967–1972 (1981).

30. Ogihara, T. *et al.* What kinds of substrates show P-glycoprotein-dependent intestinal absorption? Comparison of verapamil with vinblastine. *Drug Metab. Pharmacokinet.* **21,** 238–244 (2006).

31. Gottesman, M. M., Fojo, T. & Bates, S. E. Multidrug resistance in cancer: role of ATP-dependent transporters. *Nat. Rev. Cancer* **2,** 48–58 (2002).

32. Krishna, R. & Mayer, L. D. Multidrug resistance (MDR) in cancer. Mechanisms, reversal using modulators of MDR and the role of MDR modulators in influencing the pharmacokinetics of anticancer drugs. *Eur. J. Pharm. Sci.* **11,** 265–283 (2000).

33. Durmus, S., Hendrikx, J. J. M. A. & Schinkel, A. H. Apical ABC transporters and cancer chemotherapeutic drug disposition. *Adv. Cancer Res.* **125,** 1–41 (2015).

34. Rabindran, S. K. *et al.* Reversal of a novel multidrug resistance mechanism in human colon carcinoma cells by fumitremorgin C. *Cancer Res.* **58,** 5850–5858 (1998).

35. Rabindran, S. K., Ross, D. D., Doyle, L. A., Yang, W. & Greenberger, L. M. Fumitremorgin C reverses multidrug resistance in cells transfected with the breast cancer resistance protein. *Cancer Res.* **60,** 47–50 (2000).

36. Yamazaki, M., Fujimoto, H. & Kawasaki, T. Chemistry of tremorogenic metabolites. I. Fumitremorgin A from aspergillus fumigatus. *Chem. Pharm. Bull. (Tokyo).* **28,** 245–254 (1980).

37. Nishiyama, M. & Kuga, T. Central effects of the neurotropic mycotoxin fumitremorgin A in the rabbit (I). Effects on the spinal cord. *Jpn. J. Pharmacol.* **50,** 167–173 (1989).

38. Allen, J. D. *et al.* Potent and specific inhibition of the breast cancer resistance protein multidrug

transporter *in vitro* and in mouse intestine by a novel analogue of fumitremorgin C. *Mol. Cancer Ther.* **1,** 417–425 (2002).

39. Liu, K. *et al.* Metabolism of Ko143, an ABCG2 inhibitor. *Drug Metab. Pharmacokinet.* **32,** 193–200 (2017).

40. Weidner, L. D. *et al.* The inhibitor Ko143 is not specific for ABCG2. *J. Pharmacol. Exp. Ther.* **354,** 384–393 (2015).

41. Polgar, O., Robey, R. W. & Bates, S. E. ABCG2: structure, function and role in drug response. *Expert Opin. Drug Metab. Toxicol.* **4,** 1–15 (2008).

42. Riehm, H. & Biedler, J. L. Potentiation of drug effect by Tween 80 in Chinese hamster cells resistant to actinomycin D and daunomycin. *Cancer Res.* **32,** 1195–1200 (1972).

43. Friche, E., Jensen, P. B., Sehested, M., Demant, E. J. & Nissen, N. N. The solvents Cremophor EL and Tween 80 modulate daunorubicin resistance in the multidrug resistant Ehrlich ascites tumor. *Cancer Commun.* **2,** 297–303 (1990).

44. Rege, B. D., Kao, J. P. . & Polli, J. E. Effects of nonionic surfactants on membrane transporters in Caco-2 cell monolayers. *Eur. J. Pharm. Sci.* **16,** 237–246 (2002).

45. Alakhova, D. Y. & Kabanov, A. V. Pluronics and MDR reversal: an update. *Mol. Pharm.* **11,** 2566–2578 (2014).

46. Kaur, V., Garg, T., Rath, G. & Goyal, A. K. Therapeutic potential of nanocarrier for overcoming to P-glycoprotein. *J. Drug Target.* **22,** 859–870 (2014).

47. Collnot, E. M. *et al.* Vitamin E TPGS P-glycoprotein inhibition mechanism: influence on conformational flexibility, intracellular ATP levels, and role of time and site of access. *Mol. Pharm.* **7,** 642–651 (2010).

48. Collnot, E. M. *et al.* Mechanism of inhibition of P-glycoprotein mediated efflux by vitamin E TPGS: influence on ATPase activity and membrane fluidity. *Mol. Pharm.* **4,** 465–474 (2007).

49. Yamagata, T. *et al.* Effect of excipients on breast cancer resistance protein substrate uptake activity. *J. Control. Release* **124,** 1–5 (2007).

50. Zhang, X., Pan, W., Gan, L., Zhu, C. & Gan, Y. Effects of breast cancer resistance protein inhibitors and pharmaceutical excipients on decreasing gastrointestinal toxicity of camptothecin analogs. *Acta Pharmacol. Sin.* **29,** 1391–1398 (2008).

51. Lin, Y. *et al.* Effects of Labrasol and other pharmaceutical excipients on the intestinal transport and absorption of rhodamine123, a P-glycoprotein substrate, in rats. *Biol. Pharm. Bull.* **30,** 1301–1307 (2007).

52. Goole, J. *et al.* The effects of excipients on transporter mediated absorption. *Int. J. Pharm.* **393,** 17–31 (2010).

53. Zhao, W. *et al.* Effects of polyoxyethylene alkyl ethers on the intestinal transport and absorption of rhodamine 123: a P-glycoprotein substrate by *in vitro* and *in vivo* studies. *J. Pharm. Sci.* **105,** 1526–1534 (2016).

54. Zhao, W. *et al.* Effects of 2 polyoxyethylene alkyl ethers on the function of intestinal P-glycoprotein and their inhibitory mechanisms. *J. Pharm. Sci.* **105,** 3668–3679 (2016).

55. Alvarez, A. I. *et al.* Modulation of the activity of ABC transporters (P-glycoprotein, MRP2, BCRP) by flavonoids and drug response. *J. Pharm. Sci.* **99,** 598–617 (2010).

56. Li, L., Yi, T. & Lam, C. W. K. Inhibition of human efflux transporter ABCC2 (MRP2) by self-emulsifying drug delivery system: influences of concentration and combination of excipients. *J. Pharm. Pharm. Sci.* **17,** 447–460 (2014).

57. Ma, L., Wei, Y., Zhou, Y., Ma, X. & Wu, X. Effects of Pluronic F68 and Labrasol on the intestinal absorption and pharmacokinetics of rifampicin in rats. *Arch. Pharm. Res.* **34,** 1939–1943 (2011).

58. Yamagata, T. *et al.* Improvement of the oral drug absorption of topotecan through the inhibition of intestinal xenobiotic efflux transporter, breast cancer resistance protein, by excipients. *Drug Metab. Dispos.* **35,** 1142–1148 (2007).

59. Guan, Y. *et al.* Effect of pluronic P123 and F127 block copolymer on P-glycoprotein transport and CYP3A metabolism. *Arch. Pharm. Res.* **34,** 1719–1728 (2011).

60. Alama, T., Kusamori, K., Katsumi, H., Sakane, T. & Yamamoto, A. Absorption-enhancing effects of gemini surfactant on the intestinal absorption of poorly absorbed hydrophilic drugs including peptide and protein drugs in rats. *Int. J. Pharm.* **499,** 58–66 (2016).

61. Li, X. *et al.* Improvement of intestinal absorption of curcumin by cyclodextrins and the mechanisms underlying absorption enhancement. *Int. J. Pharm.* **535,** 340–349 (2018).

62. Nakaya, Y. *et al.* Enhanced oral delivery of bisphosphonate by novel absorption enhancers: improvement of intestinal absorption of alendronate by N-acyl amino acids and N-acyl taurates and

their absorption-enhancing mechanisms. *J. Pharm. Sci.* **105,** 3680–3690 (2016).

63. Mols, R., Deferme, S. & Augustijns, P. Sulfasalazine transport in *in-vitro*, ex-vivo and *in-vivo* absorption models: contribution of efflux carriers and their modulation by co-administration of synthetic nature-identical fruit extracts. *J. Pharm. Pharmacol.* **57,** 1565–1573 (2005).

64. Dahan, A. & Amidon, G. L. Small intestinal efflux mediated by MRP2 and BCRP shifts sulfasalazine intestinal permeability from high to low, enabling its colonic targeting. *Am. J. Physiol. Liver Physiol.* **297,** G371–G377 (2009).

65. Shono, Y. *et al.* Modulation of intestinal P-glycoprotein function by Cremophor EL and other surfactants by an *in vitro* diffusion chamber method using the isolated rat intestinal membranes. *J. Pharm. Sci.* **93,** 877–885 (2004).

66. Kunimatsu, S., Mizuno, T., Fukudo, M. & Katsura, T. Effect of P-glycoprotein and breast cancer resistance protein inhibition on the pharmacokinetics of sunitinib in rats. *Drug Metab. Dispos.* **41,** 1592–1597 (2013).

67. Karibe, T., Imaoka, T., Abe, K. & Ando, O. Curcumin as an *in vivo* selective intestinal breast cancer resistance protein inhibitor in cynomolgus monkeys. *Drug Metab. Dispos.* **46,** 667–679 (2018).

68. Breedveld, P., Beijnen, J. H. & Schellens, J. H. M. Use of P-glycoprotein and BCRP inhibitors to improve oral bioavailability and CNS penetration of anticancer drugs. *Trends Pharmacol. Sci.* **27,** 17–24 (2006).

69. Batrakova, E. V, Li, S., Alakhov, V. Y., Miller, D. W. & Kabanov, A. V. Optimal structure requirements for pluronic block copolymers in modifying P-glycoprotein drug efflux transporter activity in bovine brain microvessel endothelial cells. *J. Pharmacol. Exp. Ther.* **304,** 845–854 (2003).

70. Alakhova, D. Y. *et al.* Differential metabolic responses to pluronic in MDR and non-MDR cells: a novel pathway for chemosensitization of drug resistant cancers. *J. Control. Release* **142,** 89–100 (2010).

71. Hamid, K. A., Katsumi, H., Sakane, T. & Yamamoto, A. The effects of common solubilizing agents on the intestinal membrane barrier functions and membrane toxicity in rats. *Int. J. Pharm.* **379,** 100–108 (2009).

72. Alama, T. *et al.* Enhanced oral delivery of alendronate by sucrose fatty acids esters in rats and their absorption-enhancing mechanisms. *Int. J. Pharm.* **515,** 476–489 (2016).

73. Burris, H. A. *et al.* Activity of topotecan, a new topoisomerase I inhibitor, against human tumor colony-forming units *in vitro*. *J. Natl. Cancer Inst.* **84,** 1816–1820 (1992).

74. Sharom, F. J. ABC multidrug transporters: structure, function and role in chemoresistance. *Pharmacogenomics* **9,** 105–127 (2008).

75. Pommier, Y., Leo, E., Zhang, H. & Marchand, C. DNA topoisomerases and their poisoning by anticancer and antibacterial drugs. *Chem. Biol.* **17,** 421–433 (2010).

76. Hoskins, P. *et al.* Randomized phase II study of two schedules of topotecan in previously treated patients with ovarian cancer: a National Cancer Institute of Canada Clinical Trials Group study. *J. Clin. Oncol.* **16,** 2233–2237 (1998).

77. Bookman, M. A. *et al.* Topotecan for the treatment of advanced epithelial ovarian cancer: an open-label phase II study in patients treated after prior chemotherapy that contained cisplatin or carboplatin and paclitaxel. *J. Clin. Oncol.* **16,** 3345–3352 (1998).

78. von Pawel, J. *et al.* Topotecan versus cyclophosphamide, doxorubicin, and vincristine for the treatment of recurrent small-cell lung cancer. *J. Clin. Oncol.* **17,** 658–667 (1999).

79. Stevenson, J. P. *et al.* Phase II trial of topotecan as a 21-day continuous infusion in patients with advanced or metastatic adenocarcinoma of the pancreas. *Eur. J. Cancer* **34,** 1358–1362 (1998).

80. Kantarjian, H. New developments in the treatment of acute myeloid leukemia: focus on topotecan. *Semin. Hematol.* **36,** 16–25 (1999).

81. Nitschke, R. *et al.* Topotecan in pediatric patients with recurrent and progressive solid tumors: a pediatric oncology group phase II study. *J. Pediatr. Hematol. Oncol.* **20,** 315–318 (1998).

82. Horita, N. *et al.* Topotecan for relapsed small-cell lung cancer: systematic review and meta-analysis of 1347 patients. *Sci. Rep.* **5,** 15437–15444 (2015).

83. Schellens, J. H. *et al.* Bioavailability and pharmacokinetics of oral topotecan: a new topoisomerase I inhibitor. *Br. J. Cancer* **73,** 1268–1271 (1996).

84. Herben, V. M. *et al.* Oral topotecan: bioavailablity and effect of food co-administration. *Br. J. Cancer* **80,** 1380–1386 (1999).

85. Li, H. *et al.* Involvement of P-glycoprotein, multidrug resistance protein 2 and breast cancer resistance protein in the transport of belotecan and topotecan in Caco-2 and MDCKII cells. *Pharm. Res.* **25,** 2601–2612 (2008).

86. Jonker, J. W. *et al.* Role of breast cancer resistance protein in the bioavailability and fetal penetration of topotecan. *J. Natl. Cancer Inst.* **92,** 1651–1656 (2000).

87. Sparreboom, A. *et al.* Effect of ABCG2 genotype on the oral bioavailability of topotecan. *Cancer Biol. Ther.* **4,** 650–658 (2005).

88. Fu, Q. *et al.* The effect of phytic acid on tight junctions in the human intestinal Caco-2 cell line and its mechanism. *Eur. J. Pharm. Sci.* **80,** 1–8 (2015).

89. Jaisamut, P., Wiwattanawongsa, K., Graidist, P., Sangsen, Y. & Wiwattanapatapee, R. Enhanced oral bioavailability of curcumin using a supersaturatable self-microemulsifying system incorporating a hydrophilic polymer; *in vitro* and *in vivo* investigations. *AAPS PharmSciTech* **19,** 730–740 (2018).

90. Xia, C. Q., Liu, N., Yang, D., Miwa, G. & Gan, L. S. Expression, localization, and functional characteristics of breast cancer resistance protein in Caco-2 cells. *Drug Metab. Dispos.* **33,** 637–643 (2005).

91. Berridge, M. V, Herst, P. M. & Tan, A. S. Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction. *Biotechnol. Annu. Rev.* **11,** 127–152 (2005).

92. Gould, L. A., Lansley, A. B., Brown, M. B., Forbes, B. & Martin, G. P. Mitigation of surfactant erythrocyte toxicity by egg phosphatidylcholine. *J. Pharm. Pharmacol.* **52,** 1203–1209 (2000).

93. Kaur, G., Mehta, S. K., Kumar, S., Bhanjana, G. & Dilbaghi, N. Coencapsulation of hydrophobic and hydrophilic antituberculosis drugs in synergistic brij 96 microemulsions: a biophysical characterization. *J. Pharm. Sci.* **104,** 2203–2212 (2015).

94. Matsuda, S., Hisama, M., Shibayama, H., Itou, N. & Iwaki, M. Application of the reconstructed rabbit corneal epithelium model to assess the *in-vitro* eye irritant test of chemicals. *Yakugaku Zasshi* **129,** 1113–1120 (2009).

95. Gagnon, M., Zihler Berner, A., Chervet, N., Chassard, C. & Lacroix, C. Comparison of the Caco-2, HT-29 and the mucus-secreting HT29-MTX intestinal cell models to investigate Salmonella adhesion and invasion. *J. Microbiol. Methods* **94,** 274–279 (2013).

96. de Vries, N. A. *et al.* P-glycoprotein and breast cancer resistance protein: two dominant transporters working together in limiting the brain penetration of topotecan. *Clin. Cancer Res.* **13,** 6440–6449 (2007).

97. Wahajuddin, K. S. R., Singh, S. P. & Taneja, I. Investigation of the functional role of P-glycoprotein in limiting the oral bioavailability of lumefantrine. *Antimicrob. Agents Chemother.* **58,** 489–494 (2014).

98. Petri, N., Tannergren, C., Rungstad, D. & Lennernäs, H. Transport characteristics of fexofenadine in the Caco-2 cell model. *Pharm. Res.* **21,** 1398–1404 (2004).

99. Matsson, P. *et al.* A global drug inhibition pattern for the human ATP-binding cassette transporter breast cancer resistance protein (ABCG2). *J. Pharmacol. Exp. Ther.* **323,** 19–30 (2007).

100. Tiwari, A. K., Zhang, R. & Gallo, J. M. Overlapping functions of ABC transporters in topotecan disposition as determined in gene knockout mouse models. *Mol. Cancer Ther.* **12,** 1343–1355 (2013).

101. Karleta, V. *et al.* Poloxamer 188 supplemented culture medium increases the vitality of Caco-2 cells after subcultivation and freeze/thaw cycles. *ALTEX* **27,** 191–197 (2010).

102. Artursson, P., Palm, K. & Luthman, K. Caco-2 monolayers in experimental and theoretical predictions of drug transport. *Adv. Drug Deliv. Rev.* **46,** 27–43 (2001).

103. Matsuda, Y. *et al. In vivo* assessment of the impact of efflux transporter on oral drug absorption using portal vein-cannulated rats. *Drug Metab. Dispos.* **41,** 1514–1521 (2013).

104. Tomaru, A. *et al.* Effects of cremophor EL on the absorption of orally administered saquinavir and fexofenadine in healthy subjects. *Drug Metab. Pharmacokinet.* **30,** 221–226 (2015).

105. Lo, Y. Relationships between the hydrophilic-lipophilic balance values of pharmaceutical excipients and their multidrug resistance modulating effect in Caco-2 cells and rat intestines. *J. Control. Release* **90,** 37–48 (2003).

106. Clay, A. T. & Sharom, F. J. Lipid bilayer properties control membrane partitioning, binding, and transport of P-glycoprotein substrates. *Biochemistry* **52,** 343–354 (2013).

107. Hezaveh, S., Samanta, S., De Nicola, A., Milano, G. & Roccatano, D. Understanding the interaction of block copolymers with DMPC lipid bilayer using coarse-grained molecular dynamics simulations. *J. Phys. Chem. B* **116,** 14333–14345 (2012).

108. Regev, R., Assaraf, Y. G. & Eytan, G. D. Membrane fluidization by ether, other anesthetics, and certain agents abolishes P-glycoprotein ATPase activity and modulates efflux from multidrug-resistant cells. *Eur. J. Biochem.* **259,** 18–24 (1999).

109. Hegedüs, C., Telbisz, Á., Hegedűs, T., Sarkadi, B. & Özvegy-Laczka, C. Lipid regulation of the ABCB1 and ABCG2 multidrug transporters. *Adv. Cancer Res.* **125,** 97–137 (2015).

110. Ujhelyi, Z. *et al.* Evaluation of cytotoxicity of surfactants used in self-micro emulsifying drug delivery systems and their effects on paracellular transport in Caco-2 cell monolayer. *Eur. J. Pharm. Sci.* **47,** 564–573 (2012).

111. Barta, C. A., Sachs-Barrable, K., Feng, F. & Wasan, K. M. Effects of monoglycerides on P-glycoprotein: modulation of the activity and expression in Caco-2 cell monolayers. *Mol. Pharm.* **5,** 863–875 (2008).

112. Gillet, J. P. & Gottesman, M. M. Advances in the molecular detection of ABC transporters involved in multidrug resistance in cancer. *Curr. Pharm. Biotechnol.* **12,** 686–692 (2011).

113. Gao, Y., Chen, Y., Xu, D., Wang, J. & Yu, G. Differential expression of ANXA1 in benign human gastrointestinal tissues and cancers. *BMC Cancer* **14,** 520–511 (2014).

114. Koga, K. *et al.* Enhancing mechanism of Labrasol on intestinal membrane permeability of the hydrophilic drug gentamicin sulfate. *Eur. J. Pharm. Biopharm.* **64,** 82–91 (2006).

115. Lin, J. Human immunodeficiency virus protease inhibitors. From drug design to clinical studies. *Adv. Drug Deliv. Rev.* **27,** 215–233 (1997).

116. Lin, H. *et al.* Transport of anti-allergic drugs across the passage cultured human nasal epithelial cell monolayer. *Eur. J. Pharm. Sci.* **26,** 203–210 (2005).

117. Abeylath, S. C. & Amiji, M. M. 'Click' synthesis of dextran macrostructures for combinatorial-designed self-assembled nanoparticles encapsulating diverse anticancer therapeutics. *Bioorg. Med. Chem.* **19,** 6167–6173 (2011).

118. Li, M. *et al.* Excipients enhance intestinal absorption of ganciclovir by P-gp inhibition: Assessed *in vitro* by everted gut sac and *in situ* by improved intestinal perfusion. *Int. J. Pharm.* **403,** 37–45 (2011).

119. Bechara, C. *et al.* A subset of annular lipids is linked to the flippase activity of an ABC transporter. *Nat. Chem.* **7,** 255–262 (2015).

120. Higgins, C. F. Multiple molecular mechanisms for multidrug resistance transporters. *Nature* **446,** 749–757 (2007).

121. Wu, S. J. & Robinson, J. R. Transcellular and lipophilic complex-enhanced intestinal absorption of

human growth hormone. *Pharm. Res.* **16,** 1266–1272 (1999).

122. al-Rubeai, M., Emery, A. N., Chalder, S. & Goldman, M. H. A flow cytometric study of hydrodynamic damage to mammalian cells. *J. Biotechnol.* **31,** 161–177 (1993).

123. Wang, H. *et al.* Regulation of BCRP/ABCG2 expression by progesterone and 17β-estradiol in human placental BeWo cells. *Am. J. Physiol. Metab.* **290,** E798–E807 (2006).

124. Yamamoto, A., Uchiyama, T., Nishikawa, R., Fujita, T. & Muranishi, S. Effectiveness and toxicity screening of various absorption enhancers in the rat small intestine: effects of absorption enhancers on the intestinal absorption of phenol red and the release of protein and phospholipids from the intestinal membrane. *J. Pharm. Pharmacol.* **48,** 1285–1289 (1996).

125. Maher, S., Leonard, T. W., Jacobsen, J. & Brayden, D. J. Safety and efficacy of sodium caprate in promoting oral drug absorption: from *in vitro* to the clinic. *Adv. Drug Deliv. Rev.* **61,** 1427–1449 (2009).

126. Korzeniewski, C. & Callewaert, D. M. An enzyme-release assay for natural cytotoxicity. *J. Immunol. Methods* **64,** 313–320 (1983).

127. Gao, Y. *et al.* Improvement of intestinal absorption of insulin and water-soluble macromolecular compounds by chitosan oligomers in rats. *Int. J. Pharm.* **359,** 70–78 (2008).

Publications and presentations

Publications

1. Li X, Uehara S, Sawangrat K, Morishita M, Kusamori K, Katsumi H, Sakane T, Yamamoto A. Improvement of intestinal absorption of curcumin by cyclodextrins and the mechanisms underlying absorption enhancement. *Int J Pharm.* **535,** 330-349 (2018).

2. Sawangrat K, Morishita M, Kusamori K, Katsumi H, Sakane T, Yamamoto A. Effects of various pharmaceutical excipients on the intestinal transport and absorption of sulfasalazine, a typical substrate of breast cancer resistance protein transporter. *J Pharm Sci.* **107,** 2946-2956 (2018).

3. Sawangrat K, Yamashita S, Tanaka A, Morishita M, Kusamori K, Katsumi H, Sakane T, Yamamoto A. Modulation of intestinal transport and absorption of topotecan, a BCRP substrate by various pharmaceutical excipients and their inhibitory mechanisms of BCRP transporter. *J Pharm Sci*. **108,** 1315-1325 (2019).

Academic presentations

1. The 136th annual meeting of Pharmaceutical Society of Japan (PSJ), Mar 2016, Yokohama, Japan – Poster presentation

2. The 32nd annual meeting of the Japan Society of Drug Delivery System (DDS), Jun 2016, Shizuoka, Japan – Oral presentation

3. The 1st Academy of Pharmaceutical Science and Technology, Japan (APSTJ) Global Education Seminar, Sep 2016, Kyoto, Japan – Invited speaker

4. The 32nd annual meeting of the Academy of Pharmaceutical Science and Technology, Japan (APSTJ), Jun 2017, Saitama, Japan – Oral presentation

5. Kyoto Pharmaceutical University Integral Seminar in Pharmaceutical Sciences 2017, Aug 2017, Kyoto, Japan – Oral presentation

6. The 34th annual meeting of the Japan Society of Drug Delivery System (DDS), Jun 2018, Nagasaki, Japan – Oral presentation