Improvement of intestinal absorption of poorly absorbed dugs by gemini surfactant and sucrose fatty acid esters and their absorption enhancing mechanisms

ジェミニ型界面活性剤及びショ糖脂肪酸エステルによる難吸収性薬物の消化管吸収改善ならびにその吸収促進機構

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Preface

Oral route is preferred for the administration of therapeutic compounds because needle-borne infections and the pain associated with injections are avoided, in addition to patient compliance.

My research revolves around the theme of oral delivery of poorly absorbed drugs with various molecular weights. During my studying years in the department of Biopharmaceutics at Kyoto Pharmaceutical University, I have studied and evaluated many strategies for enhancing the intestinal absorption of variety of model and real drugs. They include CF, FD4, FD10, and FD70, calcitonin, insulin, alendronate and amphotericin B (Please see the section of model drugs). The strategies I used included using absorption enhancers (the gemini surfactant SLG-30, sucrose fatty acid esters and the non-ionic surfactant, IGEPAL), formulating self-emulsifying drug delivery systems, formulating cubosomes and formulating nanoparticles. I succeeded at enhancing the intestinal absorption of the drugs I mentioned above without serious damages to the intestinal membrane. However, this Ph. D thesis contains only the data I have already published. Unfortunately, because the lack of time that I had until writing theses lines, the data of effects of IGEPAL on the improving the intestinal absorption of drugs, and the data of enhancing the intestinal absorption of amphotericin B were not published yet. Therefore, I will not include them in this Ph. D thesis.

In this thesis, I started with an introduction about the biopharmaceutics. Although the introduction is long, but believe me, it is just a brief one. If you have knowledge in biopharmaceutics and do not want to read it, please skip it.

Chapter I talks about a new type of absorption enhancers called gemini surfactants. It talks about the effects of gemini surfactants on the intestinal absorption of hydrophilic model drugs which vary in the molecular weight and physiochemical characteristics and the possible mechanisms in which the gemini surfactant affect the intestinal membrane.

Chapter II talks about enhancing the intestinal absorption of a bisphosphonate drug, alendronate, by using sucrose fatty acids esters. It talks about the effects of sucrose fatty acids esters on the intestinal absorption of alendronate and the suggested mechanisms in which the sucrose fatty acids esters exert their effects.

Abbreviations

AAC	area above the curve
ALN	alendronate
AUC	area under the curve
BA	bioavailability
BBMVs	brush border membrane vesicles
BCA	bicinchoninic acid
BCS	biopharmaceutical classification system
CF	5(6)-carboxyfluorescein
C_{max}	peak concentrations
D%	decrement in plasma calcium or glucose levels
DNS-Cl	dansyl chloride
DMEM	Dulbecco's modified Eagle medium
DPH	1,6-diphenyl-1,3,5-hexatriene
EGTA	ethylenebis (oxyethylenenitrilo) tetraacetic acid
ER	enhancement ratio
FBS	fetal bovine serum
FDs	fluorescein isothiocyanate dextrans
Fig.	figure
GI	gastrointestinal
HBSS	Hank's Balanced Salt solution
HEPES	2-(4-(2-hydroxyethyl)-1-piperazinyl)ethanesulfonic acid
HLB	hydrophilic lipophilic balance
i.v	intravenous
JAM	junction adhesion molecules
LDH	lactate dehydrogenase
mM	millimolar
µM	micromolar
MW	molecular weight
NaGC	sodium glycocholate
PA%	pharmacological availability

P_{app}	apparent permeability coefficient
PBS	phosphate-buffered saline
PVDF	polyvinylidene difluoride
SDS-Page	sodium dodecyl sulfate polyacrylamide gel electrophoresis
S.E.	standard error
SLG-30	sodium dilauramidoglutamide lysine
TBS	tris-buffered saline
TTBS	tween 20 - tris-buffered saline
TEER	transepithelial electrical resistance
TJs	tight junctions
tma-DPH	1-(4-(trimethylamino)phenyl)-6-phenylhexa-1,3,5-hexatriene-p-toluenesulfonate
T_{max}	time to reach the peak concentrations
Tris	tris (hydroxymethyl) aminomethane
v/v	volume/volume
w/v	weight/volume

Abstract

Oral administration represents the most convenient, safest, and least expensive way to deliver a drug and it is the route most often used. The lack of pain and discomfort associated with injections, and the elimination of possible infections caused by inappropriate use or reuse of needles are some of the advantages offered by the oral route. Moreover, most patients prefer swallowing medicines to other routes of administration. However, many obstacles have to be overcome in the gastrointestinal (GI) tract for better absorption of the target drugs. The physiological barriers, mainly represented by the presence of tight junctions (TJs), the biochemical barriers and the physico-chemical characteristics of the target drug, are the major challenges in enhancing the GI absorption of drugs. Various strategies have been evaluated to improve the intestinal absorption of such drugs, including the use of additives like absorption enhancers. Absorption enhancers, which help to achieve the efficiency-safety balance, are considered one of the most promising agents for the improvement the intestinal absorption of drugs.

Chapter 1. Absorption-enhancing effects and mechanisms of gemini surfactant on the intestinal absorption of poorly absorbed hydrophilic drugs including peptide and protein drugs in rats

Gemini surfactants, consisting of two monomeric surfactants and linked through a spacer, are an important class of functionalized amphiphilic molecules. These surfactants are superior to the single-chain conventional surfactants, owing to better wetting properties due to their greater surface activities. The intestinal absorption of drugs, with or without sodium dilauramidoglutamide lysine (SLG-30), a gemini surfactant, was examined by an *in situ* closed-loop method in rats. The intestinal absorption of 5(6)-carboxyfluorescein (CF) and fluorescein isothiocyanate-dextrans (FD4, FD10 and FD70) was significantly enhanced in the presence of SLG-30 at a concentration of 0.5% (v/v), with 16, 13, 6 and 3 times higher than the control groups, respectively. Such effect being reversible. Moreover, the plasma glucose levels significantly decreased when insulin was co-administered with SLG-30 at a concentration of 0.5% (v/v) into the large intestines (59% decrease of the initial value), suggestive of the increased intestinal absorption of insulin from the large intestines. Furthermore, the plasma calcium levels significantly decreased when calcitonin was co-administered with SLG-30 at a concentration of 0.5% (v/v) into small (29% decrease of the initial value) and large (46% decrease of the initial value) intestines, suggestive of the increased intestinal absorption of calcitonin from both small and large intestines. Comparison studies with conventional absorption enhancers including sodium laurate and sodium glycocholate suggested that SLG-30 was superior, effective as a whole compound and stable in the small intestines. In addition, no significant increase in the lactate dehydrogenase (LDH) activity or in protein release from the intestinal epithelium was observed in the presence of SLG-30, suggestive of the safety of this compound. Additionally, SLG-30 increased the membrane fluidity at the protein portion of the cells membranes. On the top of that, SLG-30

decreased the transepithelial electrical resistance (TEER) values of Caco-2 cells significantly, suggestive of loosening of the TJs. In this context, the expression levels of claudin-1 and claudin-4 significantly deceased with 57% and 64%, respectively, compared with the control group, in the presence of SLG-30 at a concentration of 0.5% (v/v), then started to recover after washing out SLG-30 from the intestines. These findings indicate that SLG-30 is an effective absorption enhancer for improving the intestinal absorption of poorly absorbed drugs, without causing serious damage to the intestinal epithelium. The mechanisms might be changing the membrane fluidity, and loosening of TJs by decreasing the expression of claudin-1 and claudin-4, enhancing by that the absorption through the transcellular and paracellular pathway, respectively.

Chapter 2. Enhanced oral delivery of alendronate by sucrose fatty acids esters in rats and their absorption-enhancing mechanisms

Bisphosphonates, carbon-substituted pyrophosphate analogs, are useful in reducing the hazard of future fractures in osteoporosis patients who have already sustained a fracture due to the disease. However, the intestinal absorption of alendronate (ALN), a bisphosphonate drug, after oral administration is very poor. In this study, sucrose fatty acid esters as promising absorption enhancers were used to enhance the intestinal absorption of ALN using an *in situ* closed-loop method in rats. The intestinal absorption of ALN was significantly enhanced in the presence of sucrose fatty acid esters. The best absorption enhancing effect was observed in the presence of L-1695 in a dose-dependent manner, with 11 times higher than the control group at a concentration of 5.0% (w/v). Moreover, there is almost no regional differences in the intestinal absorption of ALN in the small and the large intestines. In addition, no considerable increase was observed in the activity of LDH or in protein release from the intestinal epithelium in the presence of sucrose fatty acid esters at concentrations equivalent to or lower than 1.0% (w/v), suggesting that these compounds are safe. Furthermore, mechanistic studies revealed increased membrane fluidity in the presence of sucrose fatty acid esters at the inner portion between the phospholipids bilayers and at extracellular faces of the phospholipids bilayers of the membrane. Additionally, sucrose fatty acid esters at all studied concentrations significantly decreased the TEER values of Caco-2 cells. TEER values recovered to the baseline after removing sucrose fatty acid esters and ALN. Therefore, the loosening of the TJs might be another underlying mechanism by which sucrose fatty acid esters improve the intestinal absorption of ALN. L-1695 at a concentration of 2.0% (w/v) decreased the levels of claudin-1 and claudin-4, with 24% and 49%, respectively, compared with the control group. Such effect being reversible. These findings suggest that sucrose fatty acid esters are effective absorption enhancers for improving the intestinal absorption of ALN, without causing serious damage to the intestinal epithelium, through the transcellular and paracellular routes, respectively.

These findings give us basic information about enhancing the intestinal absorption of poorly absorbed drugs including peptide and protein drugs.

Introduction

Development of new drug molecule is expensive and time consuming. Improving safety-efficacy ratio of "old" drugs has been attempted using different methods such as individualizing drug therapy, dose titration, and therapeutic drug monitoring. Other than a lack of *in vivo* efficacy or unintended toxicological issues, failures are often associated with inappropriate physico-chemical characteristics contributing to poor absorption and poor pharmacokinetics. Therefore, it is of great relevance to consider the biopharmaceutical and pharmacokinetics properties of the drugs during the assessment of new drugs for industry.

The term "ADME" denotes the absorption, distribution, metabolism, and excretion of drugs and drug candidates upon administration to animals or humans. The ADME processes occurring after oral administration of drugs are depicted (Fig. 1). The overall goal of ADME in drug discovery and development is to predict the behavior of a drug candidate in humans. Pharmacokinetics (PK) parameters in animal species that will be used in pharmacological and safety assessment models provide very important insights (systemic and tissue exposures) for those studies. The results of PK studies in several animal species generate the data for physiologically based models or allometric scaling [1] to predict the basic pharmacokinetic behavior of a compound in humans.

The oral route is by far the most preferred route of administration, although significant advances have been made with other routes as well. The lack of pain and discomfort associated with injections, and the elimination of possible infections caused by inappropriate use or reuse of needles are some of the advantages offered by the oral route. Moreover, most patients prefer swallowing medicines to other routes of administration, and oral formulations are also less expensive to other dosage forms.

In this contest, the key "A" of ADME, absorption, in the oral route is considered as the most important factor for any drug to start functioning. Without absorption, there is no pharmacokinetics and most importantly, there is no efficiency. However, many obstacles have to be overcome in the gastrointestinal (GI) tract for better absorption of the target drugs.

The key "A" of ADME, absorption

Absorption of a drug is the first process a molecule must navigate in order to reach the systemic circulation. The properties of both the drug and the route of administration will influence the overall bioavailability of the compound.

1- Barriers to oral drug delivery

The body contains many biological barriers that serve to protect its interior from a variety of external invaders and toxins. These obstructions to drug delivery can be categorized as physiological, biochemical, and chemical barriers.

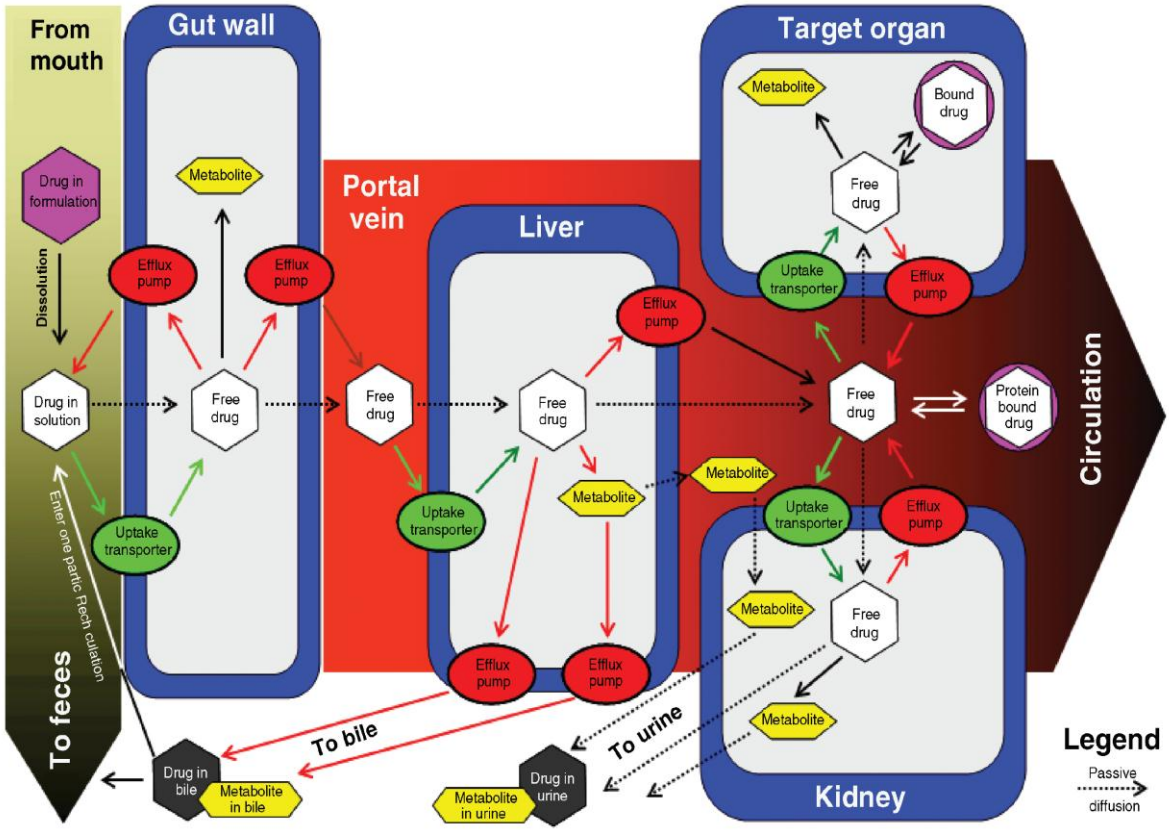


Fig. 1. The processes during the absorption, distribution, metabolism, and excretion of drugs.

1-1- Transcellular pathway

Drugs with the appropriate physiochemical characteristics can pass through the cells by a passive diffusion, as shown in Fig. 2. In the case of peptides or peptidomimetic drugs, their physicochemical properties may not be suitable for permeation through the cells membrane via the transcellular pathway. The drug molecules must travel through the lipid bilayers, which consists of four regions. These regions form the rate-limiting barriers to the passive flow of molecules. It is widely accepted that lipophilicity plays an important role in determining the transport mechanism. However, early *in vivo* studies concluded that the intestinal absorption diminishes when lipophilicity is very high (usually log P > 5) [2]. The drug molecules must also traverse the cytosol before exiting through the basolateral membranes. Within the cytosol, various drug-metabolizing enzymes exist, which can metabolize the drug molecules and can lower the drug transport via this pathway.

1-2- Paracellular pathway

This physiological barrier exists to provide protection from the entry of toxins, bacteria, and viruses from the apical side to the basolateral side, and it allows the passage of selective molecules and cells. Paracellular transport involves the transport of molecules via water-filled pores/channels between cells.

Approximately 0.01–0.1% of the total intestinal surface area consists of water-filled pores. Taking into consideration that the intestinal epithelium has a surface area of ~2×10^6 cm^2 [3], paracellular route corresponds to ~200–2000 cm^2.

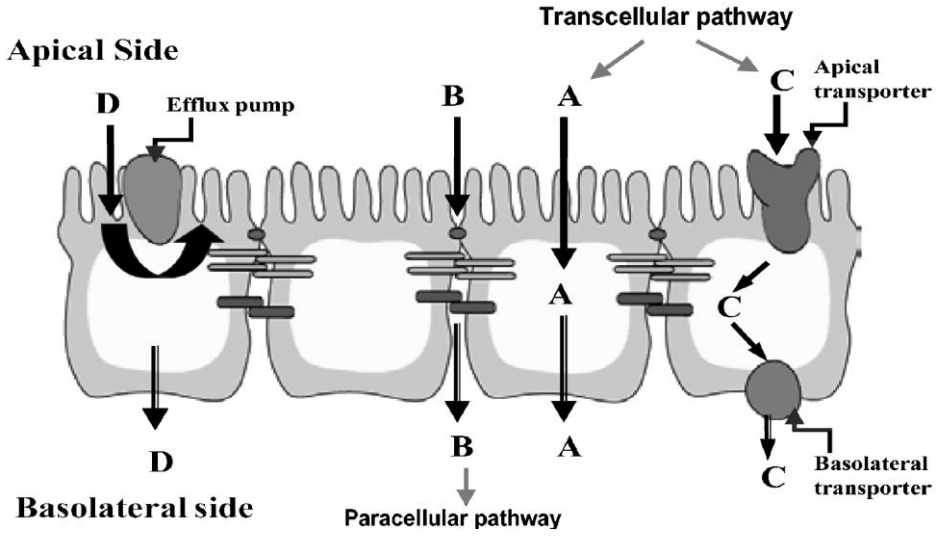


Fig. 2. Absorption pathways in the intestinal tract. Pathway A is the transcellular route in which drug can passively permeates the cell membranes. Pathway B is the paracellular route in which the drug passively diffuses via the intercellular junctions. Pathway C is the route of active transport of the drug by transporters. Pathway D is the route of drug permeation, which is controlled by efflux pumps.

This route is preferred by low molecular weight hydrophilic compounds such as small peptide fragments generated from the breakdown of proteins. Additionally, the presence of tight junctions or zonula occludens between the epithelial cell layers of GI tract severely limit penetration ability of polar macromolecules. The diffusion of polypeptides via a paracellular route depends on their physicochemical properties, molecular dimension and overall ionic charge [4]. The bioavailability of drugs decreases rapidly with increase in molecular weight beyond 700 Da [5]. The tight epithelial junctions of colon are impermeable to molecules with radii larger than 8–9 A˚. Since this pathway is important for absorption and can be tremendously exploited for drug delivery system, a little explanation about the structure of paracellular pathway is necessary. The intercellular junctions can be divided into three different regions: (1) tight junctions (zonula occludens), (2) adherens junctions (zonula adherens), and (3) desmosomes. At the most apical portion of the cells, the tight junctions or zonula occludens function to bring adjacent cells into close apposition. This is defined as the gate function of the tight junction [6], as shown in Fig. 3. These areas of apposition have been referred to as "kisses", as seen by freeze-fracture electron microscopy [7]. Tight junctions cause cell surface polarity that produces the fence function and restricts free diffusion of lipids and proteins from the apical plasma membrane to the basolateral surface [8]. Opening tight junctions may allow the movement of many types of drugs, without the need for chemical modification of the compounds. Therefore,

absorption enhancers, which can open TJs, may be useful in this context. Paracellular permeation of drug through the intercellular junctions depends on the pore size of the tight junctions.

In 1998, Furuse et al. identified two novel components of TJs strands, claudin-1 and claudin-2 [9]. These proteins, each with a molecular mass of ~ 23 kDa and is consisting of four transmembrane domains, are localized in TJs strands [10]. The exogenous expression of claudins led to formation of TJs strands in claudin-absent fibroblast cells [11]. Accordingly, claudins are critical for the formation of TJs strands. The family of claudins consists of more than 24 members; interestingly, the expression profiles and barrier function of each member varies with tissue type [12].

Additionally, there are occludin and three scaffolding proteins associated with the tight junctions, which are ZO-1, ZO-2, and ZO-3, as illustrated in Fig. 4. ZO-1 stabilizes the tight junction by interacting with occludin and claudin through cross-linking them to the actin cytoskeleton.

1-3- Chemical barriers to oral drug delivery

The chemical structure of a drug determines its solubility and permeability profiles. In turn, the concentration at the intestinal lumen and the permeation of the drug across the intestinal mucosa are responsible for the rate and extent of absorption [13]. Unfavorable physicochemical properties have been a limiting factor in the oral absorption of peptides and peptidomimetics [14]. According to the Biopharmaceutics Classification System (BCS), as shown in Fig. 5, which classifies the therapeutic agents using the parameters solubility and intestinal permeability, drugs can be classified as the following:

Class I - high permeability, high solubility, (e.g. metoprolol). Those compounds are well absorbed and their absorption rate is usually higher than excretion.

Class II - high permeability, low solubility, (e.g. glibenclamide, bicalutamide, ezetimibe). The bioavailability of those products is limited by their solubility rate.

Class III - low permeability, high solubility, (e.g. cimetidine, peptide drugs and alendronate). The absorption is limited by the permeation rate but the drug is solubilized very fast.

Class IV - low permeability, low solubility, (e.g. hydrochlorothiazide, amphotericin B and crucumin). Those compounds have a poor bioavailability. Usually they are not well absorbed over the intestinal mucosa and a high variability is expected.

Therefore, beside the permeability barrier, there is the solubility problem, which limits drug absorption. There is a trend in new drug molecules toward larger molecular weights, which often leads to lower solubility. The ability to formulate a soluble form of a drug is becoming both more important and more challenging. This has resulted in extensive researches on methods to increase drug solubility.

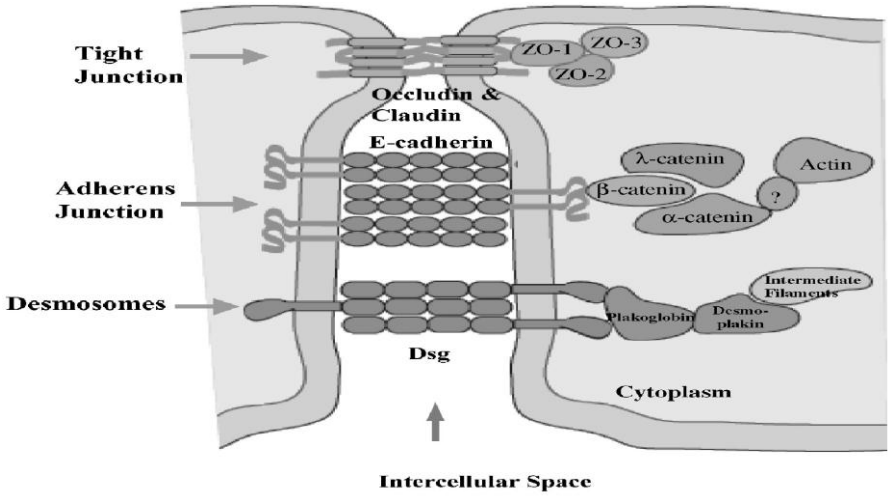


Fig. 3. The intercellular junction is mediated by different proteins at different levels: (1) tight junction (zonula occludens), (2) adherens junction (zonula adherens), and (3) desmosomes.

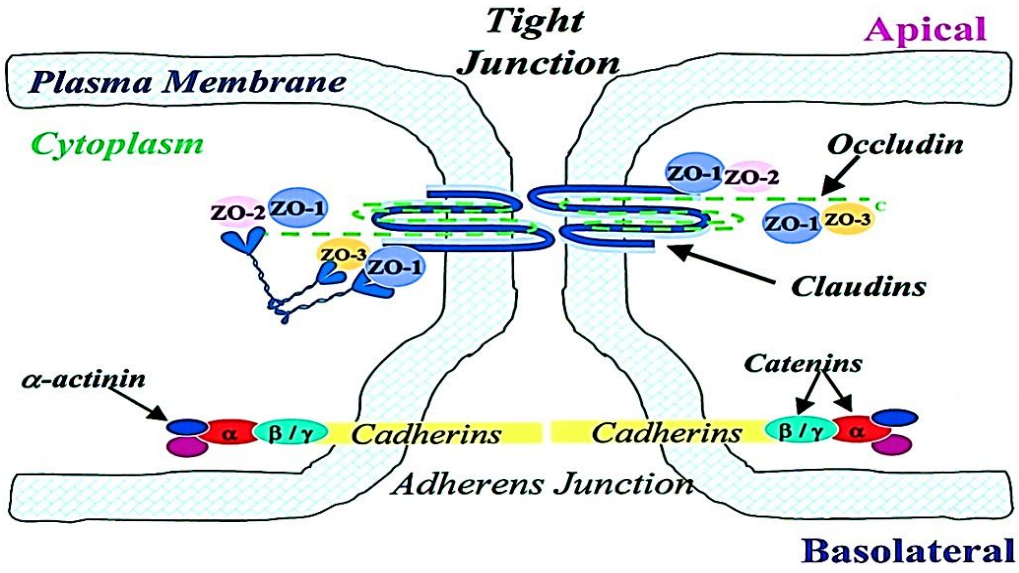


Fig. 4. The composition of tight junction and adherens junction.

Additionally, there are other important barriers to oral delivery such as chemical barriers represented by degradation enzymes [15]. Efflux pumps mainly represented by P-glycoprotein (P-gp) are also another kind of barriers act against the absorption of durgs [16].

2- Approaches to overcome barriers to oral delivery

Various strategies for improving oral bioavailability of poorly absorbed drugs have been investigated. One aspect of oral absorption is the solubility of a drug in the intestinal fluid.

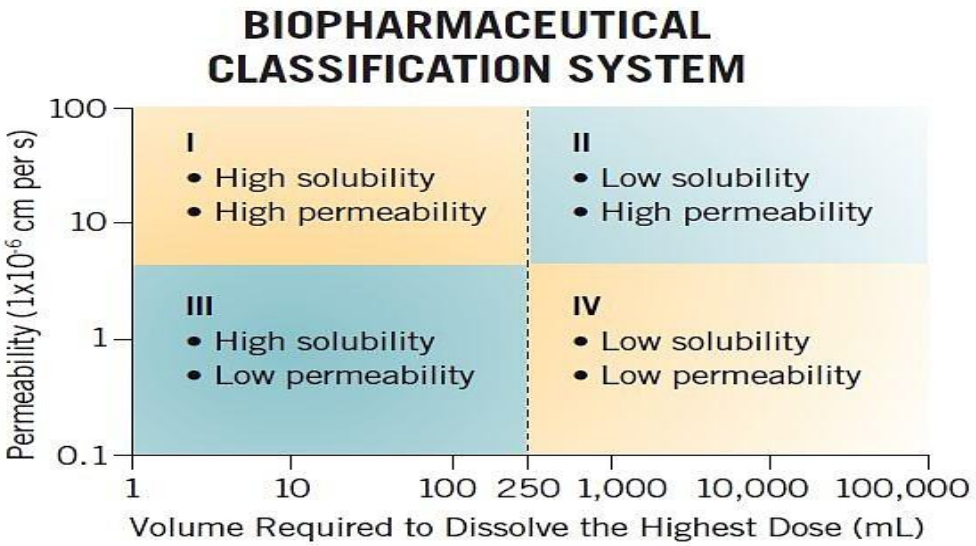


Fig. 5. The Biopharmaceutics Classification System (BCS) as defined by the FDA.

The addition of polar groups and conjugation of polymers, such as low molecular weight chitosan (LMWC) or PEG, are some common strategies to improve drug solubility [17]. Moreover, co-administration of protease inhibitors can lower the enzymatic barrier and prevent degradation of proteins and peptides in the GI tract thereby facilitating intestinal absorption [18]. Additionally, many formulations, have been studied in order to overcome multiple barriers to the intestinal absorption of poorly absorbed dugs especially peptide and protein drugs. Examples can include mucoadhesive systems [19], water-in-oil microemulsions [20], dendrimers [21] and liposomes [22] have been successfully applied in the delivery of wide range of therapeutics including lipids, nucleotides, proteins and plasmids. Interestingly, colon targeted drug delivery represents a novel concept for improving the systemic bioavailability of orally administered drugs including protein and peptide therapeutics [23].

2-1- Absorption enhancers

Absorption enhancers are compounds which can enhance the intestinal absorption of drugs for enhancing their oral bioavailability [24]. These compounds allow therapeutic agents to permeate across biological membranes into systemic circulation and reach the site of action to exert pharmacological effect [25]. However, the selection of absorption enhancer and its efficacy depends on the physicochemical properties of the drugs, especially protein and peptide drugs, regional differences in intestinal membrane, nature of the vehicle and other excipients. Absorption enhancers have to be safe, pharmacologically and chemically inert, non-irritant, and non-allergenic. Various absorption enhancers have been investigated for the enhancement of drugs, especially protein and peptide absorption through the intestinal membrane. These

enhancers can be classified into surfactants, chelating agents, bile salts, cationic and anionic polymers, acylcarnitines, fatty acids and their derivatives (Table 1).

Some surfactants are believed to disturb the integrity of the plasma membrane [26]. Surfactant-enhanced membrane permeability is generally assumed non-specific and cytotoxic [26].

EDTA, a calcium chelator, enhances mucosal absorption of drugs. EDTA modulates TJ barrier integrity by opening intracellular TJ seals [27]. Various fatty acids, including caprate, caprylate, and laurate can enhance the membrane permeability. A series of analyses aimed at determining the mode of action of sodium caprate indicated that the compound activates phospholipase C, elevates intercellular calcium levels, and subsequently stimulates contraction of calmodulin-dependent actin–myosin filaments, thereby opening TJs seals [28].

Cationic chitosans increase epithelial paracellular permeability [29]. Chitosans bind to the epithelial cell membrane through a charge-dependent interaction, resulting in F-actin depolymerization and separation of TJs components. This event triggers enhanced epithelial permeability. Poly-lysines also enhance epithelial paracellular permeability by opening TJs seals [30].

Cyclodextrins have been evaluated as absorption enhancers for enhancing the absorption of calcitonin in *in situ* absorption studies in rats [31].

Nitric oxide (NO) donors such as SNAP [S-nitroso-N-acetyl-penicillamin], NOC5 [3-(2-hydroxy-1-(methylethyl)-2-nitrosohydrazino)-1-propanamine], and NOC12 [N-ethyl-2-(1-ethyl-hydroxy-2-nitrosohydrazino)-ethanamine] are effective absorption enhancers in the small and large intestines [32] SNAP enhanced the absorption of CF with 5 times comparing to the control. NO donors improved the intestinal absorption of insulin and [$Asu^{1,7}$]-eel calcitonin from all the intestinal regions [33]. The mechanism might be ascribed to the dilation of tight junction in the epithelium via paracellular route. The effects of NO donors significantly decreased in the presence of carboxy-PTIO [2-(4-carboxyphenyl) 4,4,5,5-tetramethylimidazole-1-oxyl 3-oxide sodium salt], a typical NO scavenger [32].

Lin et al., [34] studied the effects of PAMAM dendrimers (G0–G3) on the absorption of CF, FD4, FD10, calcitonin and insulin. G2 PAMAM dendrimer was the most effective to enhance the small intestinal absorption of CF, FD4 and calcitonin with 11, 3 and 4 times comparing to the control groups, respectively. However, they had no effect on the absorption of FD10 and insulin. Thus, better enhancers are needed to improve the intestinal absorption of drugs safely.

Table 1. Commonly used absorption enhancers and their mechanisms of action.

Category	Examples	Mechanisms of action
Bile salts	Sodium deoxycholate, Sodium taurocholate, Sodium glycodeoxycholate, Sodium taurodihydrofusidate, Sodium glycodihydrofudisate	Form reverse micelles and disrupt membrane, open up tight junctions, enzyme inhibition and mucolytic activity.
Chelators	EDTA, Citric acid, Salicylates	Interferes with calcium ions, chelation disrupts intracellular junctions and decreases transepithelial electrical resistance.
Surfactants	Sodium lauryl sulfate, Laureth-9, Poly oxyethylene ethers	Perturbation of intercellular lipids, lipid order, orientation and fluidity. Inhibition of efflux mechanisms.
Fatty acids and derivatives	Oleic acid, Linoleic acid, Caprylic acid, Capric acid, Acylcarnitines, Mono and Di-Glycerides	Increase fluidity of phospholipid membranes, contraction of actin-myosin filaments, opening of tight junctions.
Cationic polymers	Chitosan and its derivatives	Combined effect of mucoadhesion and opening of tight junctions via ionic interactions with the cell membrane.
Anionic Polymers	Carbopol and polyacrylic acid derivatives	Combined effect of enzyme inhibition and opening of tight junctions through removal of extracellular calcium ions.
	N-Acetyl cysteine	Reduce the viscosity of mucus layer by breaking down disulfide bonds.
Acylcarnitines	Palmitoylcarnitine chloride, Lauroyl-l-carnitine chloride.	Membrane disruption, opening of tight junctions with a calcium independent mechanism

Chapter I:

Absorption-enhancing effects of gemini surfactant on the intestinal absorption of poorly absorbed hydrophilic drugs including peptide and protein drugs in rats

The first-generation absorption enhancers are surfactants of anionic, cationic, and nonionic nature, bile salts, chelating agents, and fatty acids, and are considered to be promising pharmaceutical agents in improving the intestinal absorption of drugs [35] by disturbing the integrity of the plasma membrane [25]. It has been previously reported that various surfactants could enhance the intestinal absorption of drugs, but their actions are assumed to be non-specific and cytotoxic. Additionally, their effect on TJs is not well addressed.

However, it was recently reported that gemini surfactants, consisting of two monomeric surfactants and linked through a spacer, are an important class of functionalized amphiphilic molecules. These surfactants are superior to the single-chain conventional surfactants, owing to better wetting properties due to their greater surface activities i.e., more efficiency to reduce the surface and interfacial tension. Gemini surfactant showed good efficiency in terms of increasing the permeation of hydrophilic ionized compounds namely lidocaine HCl, caffeine, and ketoprofen, which do not easily cross the stratum corneum without relevant changes in the skin structure [36]. Permeation of tetracaine and ropivacaine across the skin was also improved using serine-based gemini surfactants, namely the cationic ones with long alkyl chains and shorter spacer [37].

However, no studies have been performed to determine the effects of gemini surfactants on the intestinal absorption of drugs, including peptides and protein drugs. Therefore, the gemini surfactant, SLG-30, which basically consists of amino acids and two lauryl chains, was selected as a representative of gemini surfactants to study their efficiency on improving the intestinal absorption of poorly absorbed drugs. The effects of dilauramidoglutamide lysine 29% (SLG-30), on the intestinal absorption of 5(6)-carboxyfluorescein (CF), fluorescein isothiocyanate-dextrans (FDs) with various molecular weights, calcitonin and insulin, as models of poorly absorbed drugs were studied. As shown in Fig. 6, the hydrophilic moiety of this surfactant has two derivatives of amino acid i.e., two glutamic acid moieties, linked through a lysine spacer [38]. Furthermore, the effects of these surfactants on the intestinal membrane damage were evaluated by measuring the lactate dehydrogenase (LDH) activity and the protein release from the intestinal membranes in rats. This is the first study that discusses the effects of gemini surfactants on the improvement of the intestinal absorption of poorly absorbed drugs.

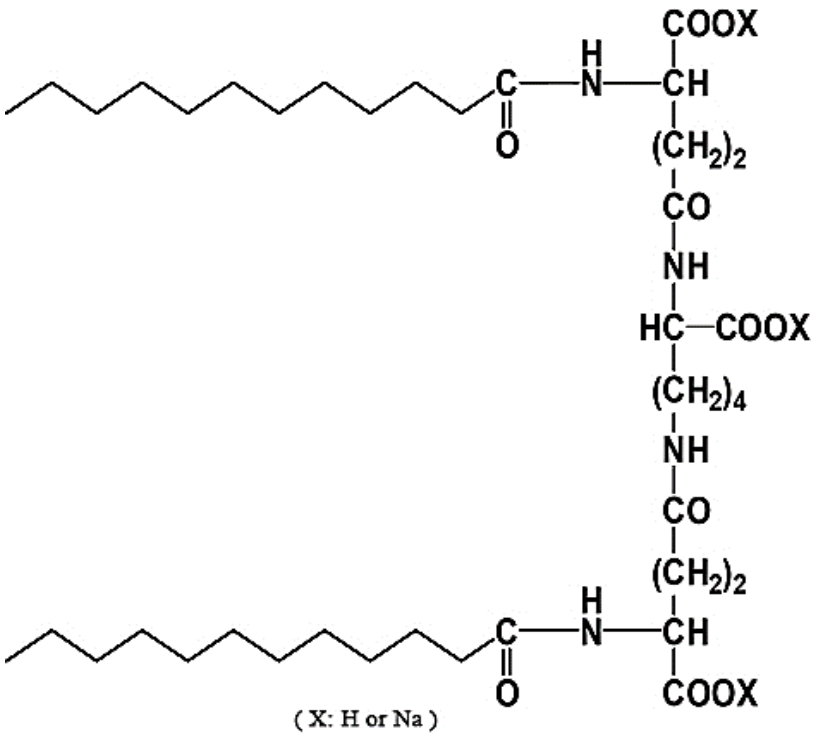


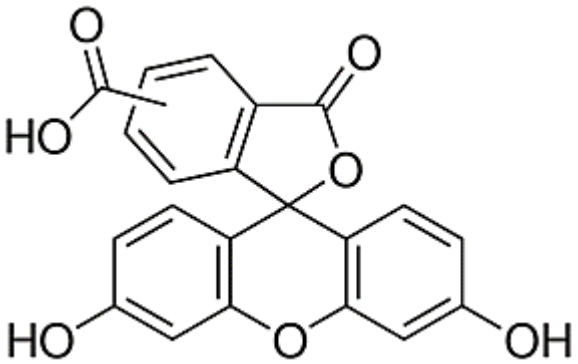
Fig. 6. The chemical structure of a gemini surfactant, SLG-30. The hydrophilic moiety of this surfactant has two derivatives of amino acid i.e., two glutamic acid moieties, linked through a lysine spacer, while the hydrophobic moieties are lauric acid. (*Int. J. Pharm,* **499**, 58–66 (2016), Fig. 1).

1- Materials and methods

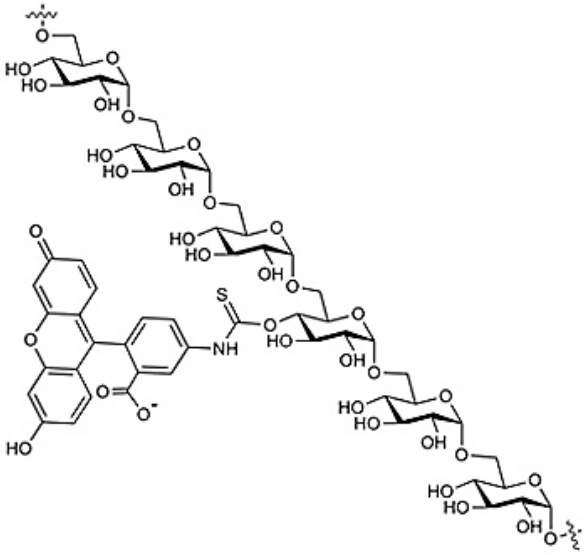
1-1- Materials

Male Wistar rats, weighing 250-300 g, were purchased from SLC, INC. (Hamamatsu, Shizuoka, Japan). CF was obtained from Eastman Kodak Co. (Rochester, NY, USA), FDs with average molecular weights of 4 KD (FD4), 10 KD (FD10) and 70 KD (FD70) (Fig. 7), DPH (1,6-diphenyl-1,3,5-hexatriene) and Hank's balanced salt solution (HBSS) were supplied by Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), Dansyl chloride (DNS-Cl) was purchased from Santa Cruz Biotechnology, Inc. (Texas, USA). SLG-30 was kindly supplied by Asahi Kasei Chemical (Tokyo, Japan). Insulin was purchased from Nacalai Tesque (Kyoto, Japan). Salmon calcitonin acetate, the Calcium E Test kit, the Glucose B Test kit, sodium glycocholate (NaGC), sodium laurate, tma-DPH (1-(4-(trimethylamino)phenyl)-6-phenylhexa-1,3,5-hexatriene-p-toluenesulfonate) and the LDH-Cytotoxicity Test Wako were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Bicinchoninic acid (BCA) protein assay kit was obtained from Pierce Biotechnology Inc. (Rockford, USA). Anti-claudin-1, anti-β-actin (rabbit monoclonal antibodies) and goat anti-rabbit IgG HRP-linked antibodies were purchased from Cell Signaling Technology® (Danvers, Ma, USA). Anti-claudin-4 (mouse monoclonal antibodies) and rabbit anti-mouse IgG HRP-linked antibodies were purchased from Invitrogen™ (Carlsbad, CA, USA). Chemi-Lumi One Ultra kit,

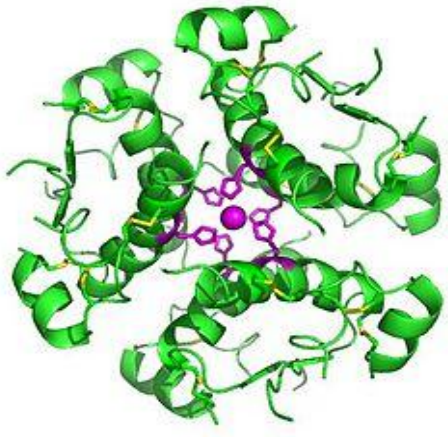
Dulbecco's modified Eagle medium (DMEM) with 4500 mg/L glucose, nonessential amino acids (MEM–NEAA), antibiotic-antimycotic mixture stock (10,000 U/mL penicillin, 10,000 µg/mL streptomycin, and 25 µg/ml amphotericin B in 0.85% sodium chloride), and 0.25% trypsin-1mM EDTA solution were purchased from Nacalai Tesuque (Kyoto, Japan). Human colon adenocarcinoma-derived Caco-2 cell line was purchased from Dainippon Sumitomo Pharma Co., Ltd. (Osaka, Japan). Fetal bovine serum (FBS) was purchased from Gibco® life technologies (Grand Island, USA). All other reagents used in the experiments were analytical grade.



5(6)-carboxyfluorescein
MW = 376 Da



Fluorescein isothiocyanate–dextran
FD4; MW = 4 KDa
FD10; MW = 4 KDa
FD70; MW = 4 KDa



Insulin
MW = 4 KDa

Calcitonin
MW = 4 KDa

Fig. 7. Illustration of model drugs used in chapter I.

1-2- Preparation of the dosing solution

For the *in situ* closed-loop absorption studies, dosing solutions of CF, FDs (FD4, FD10 and FD70), calcitonin and insulin were prepared in a phosphate-buffered saline (PBS, pH 7.4) at concentration of 0.04 mg/ml, 0.66 mg/ml, 6.7 IU/ml and 6.7 IU/ml, respectively. The volume of administration was 3 ml/rat in order to achieve the doses of 0.5 mg/kg, 8 mg/kg, 80 μg/kg and 80 IU/kg, respectively. A solution containing SLG-30 0.5% (v/v) was prepared for the pre-treatment study, in order to determine the reversibility of the absorption enhancing effect of SLG-30.

1-3- The intestinal absorption of drugs by an *in situ* closed-loop method

The intestinal absorption of drugs was examined by an *in situ* closed-loop method [18]. The experiments were carried out in accordance with the guidelines of the Animal Ethics Committee at Kyoto Pharmaceutical University. The rats were fasted overnight for ~ 16 h pre-dosing, but water was freely available. After inducing anesthesia with sodium pentobarbital at a dose of 32 mg/kg body weight intraperitoneally, the rats were placed under a heating lamp to maintain the body temperature at around 37°C and the intestines were exposed by a midline-abdominal incision. After ligating the bile duct, the intestines were washed with phosphate-buffered saline (PBS, pH 7.4) and the remaining buffer solution was expelled with air. Intestinal cannulation was performed at both ends using a polyethylene tubing and the distal parts of the small or the large intestines were clipped by forceps. The dosing solutions, with or without absorption enhancers, kept at 37 °C, were directly introduced into the lumen of the intestinal loop through a cannulated opening in the proximal part of the small or large intestinal loop, which was then closed by clipping with another forceps. The jugular vein was exposed and ~0.3 ml of the blood samples were collected via a direct puncture into heparinized syringes, at predetermined time intervals up to 240 min. Rats were kept under anesthesia by injection of sodium pentobarbital at a dose of 5 mg/kg intraperitoneally every 2 h. The samples were immediately centrifuged at 12,000 rpm (15,000 x g) for a period of 5 min to obtain the plasma fraction, which was stored in ice for further analysis. The drug concentrations in these plasma samples were determined by the methods as described below.

For the pretreatment studies, SLG-30 0.5% (v/v) solution was administered into the loop of the small intestine. SLG-30 solution was removed by washing the small intestine with the PBS buffer after pre-treatment for 10, 60 and 120 min, respectively. After washing the small intestine with the PBS buffer, the CF solution was administered into the small intestinal loop as indicated above and the blood samples were collected for determination of CF concentrations.

The peak drug concentrations (C_{max}) and the time to reach the peak drug concentrations (T_{max}) in plasma were directly determined from the plasma concentration–time profiles. The area under the curve (AUC) was calculated by the trapezoidal method, from pre-dose (time zero) to the final sample. The absorption

enhancement ratios of the drugs, with or without SLG-30, were calculated as follows:

Absorption enhancement ratio = $AUC_{\text{with enhancer}} / AUC_{\text{control without enhancer}}$

1-4- Analytical methods

The fluorescence intensities of CF, FD4, FD10 and FD70 were determined in plasma treated with the same volume of acetonitrile with a fluorescence spectrophotometer, Powerscan® HT supplied by BioTek Instruments (Winooski, Vermont, USA) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. There was no significant background fluorescence in the plasma, when the fluorescence intensities of CF and FDs were determined in this study. The plasma concentrations of calcium were measured by the Calcium E Test kit, whereas the plasma levels of glucose were measured by the glucose oxidase method with the Glucose B Test Kit. The decrease in the plasma levels of calcium and glucose (D%), were calculated by a modification of the method of Hirai et al. [39], based on the following equation:

$$D\% = \left(1 - \frac{AUC_{0\rightarrow240 \text{ or } AUC_{0\rightarrow360}}}{100\% \times 240\ \text{min or } 360\ \text{min}}\right) \times 100$$

Here, the time was 240 min for calcitonin in the small intestine and 360 min for both, calcitonin and insulin in the large intestine. The area above the curve (AAC) was calculated by the summation of the areas above the plasma glucose levels, in the percentage change vs. time curve plots, using the trapezoidal method. The area above 100% line was ruled out for calculation of the $AUC_{0\rightarrow240}$ and the $AUC_{0\rightarrow360}$.

The pharmacological availability denoted as PA%, was calculated using the following equation:

$$PA\% = \left(\frac{D\%_{\text{P.O}}}{D\%_{\text{i.v}}}\right) \times \left(\frac{\text{Dose}_{\text{i.v}}}{\text{Dose}_{\text{P.O}}}\right) \times 100$$

The LDH activity was determined using the LDH CII assay kit and the protein release was measured using a BCA protein assay kit, with bovine serum albumin as the standard.

1-5- Estimation of intestinal membrane damage

To evaluate small intestinal membrane damage in the presence of absorption enhancers, the LDH activity and the protein release from the small intestinal membrane were measured by an *in situ* closed-loop method, as reported previously [40,41]. Various concentrations of SLG-30 were administered into the small intestinal loop, as described previously, and compared to the results obtained with 3% Triton X-100, used as a positive control. Four hours post-administration, the perfused rat small intestine was washed thrice with 10 ml of PBS. The PBS solution was thereafter, centrifuged for 7 min at 200 × g at a temperature of 4 °C. One ml of the supernatant was used for the determination of the LDH activity and the protein release from the small intestinal membrane.

1-6- Morphological observations

SLG-30 at various concentrations was administered into the small intestinal loop, as described earlier. I used 3% Triton X-100 as a positive control. After completion of the experiment, the small intestine was removed and cut into three equal segments consisting of the proximal, the median, and the distal parts. A 5-cm piece from the median part (which is expected to be the jejunum) was dissected, rinsed in ice cold PBS (pH 7.4), and fixed with 4% buffered-formaldehyde. The intestinal segments were paraffin-embedded, conventionally sectioned, stained with haematoxylin-eosin (H&E) and examined by light microscopy under a BZ-8000 Fluorescence Microscope, KEYENCE Corporation (Osaka, Japan).

1-7- Preparation of brush border membrane vesicles (BBMVs)

BBMVs were prepared by the methods reported previously [42–44] with a slight modification. Briefly, an *in situ* small intestinal loop was prepared in each rat, as mentioned above, then PBS washing solution (pH 7.4) was administered into the loop, the fat was trimmed off the small intestine and mesentery. Then, the whole small intestine was soaked with ice-cold PBS (pH 7.4). The small intestine was divided into 10-cm segments. Mucosa was scraped out with a slide glass from each of those segments and used for the subsequent studies. BBMVs were prepared by the divalent cation precipitation method using $MgCl_2$ in the presence of ethylenebis (oxyethylenenitrilo) tetraacetic acid (EGTA) [42–44]. Briefly, the collected mucosa was homogenized within a buffer containing (mannitol 300 mM, EGTA 5 mM, Tris (pH 7.4) 12 mM) by using a tissue homogenizer. An aqueous solution of 10 mM magnesium chloride was added to the homogenate. The homogenate was centrifuged at 3000 × *g* for 15 min. The supernatant was then centrifuged at 32,000 × *g* for 30 min. The pellet was re-suspended in a buffer of (mannitol 300 mM, EGTA 5 mM, Tris (pH 7.4) 12 mM) by using a 26-G needle. The protein concentration was determined by a BCA method using bovine serum albumin as a standard, and the final concentration was adjusted to 1 mg/ml in each eppendorf tube. The samples were frozen by liquid nitrogen and kept at -80 °C for further studies.

1-8- Measurement of membrane fluidity by fluorescence polarization

BBM vesicles (100 µg protein) were incubated with 1 µM DPH, or with 0.5 µM tma-DPH, or with 5 µM DNS-Cl in HEPES-Tris buffer (HEPES 25 mM, KCL 5.4 mM, $CaCl_2$ 1.8 mM, $MgSO_4$ 0.8 mM, NaCl 140 mM, glucose 5 mM, pH 7.4 modified by 1 M Tris) in the dark at 37°C for 30 min [43,45]. Then, various concentrations (0.1% v/v, 0.25% v/v, 0.5% v/v and 1.0% v/v) of SLG-30 were added. Then, the samples were incubated in the dark for 1 min at 37ºC. For the control group, same procedures were carried out by adding HEPES-Tris buffer only. The fluorescence intensities and the steady state polarization of fluorescence expressed as the fluorescence anisotropy, r, of the labeled membrane vesicles were measured at 37ºC. The excitation and emission wavelengths used during the measurements were λ_{ex} = 360 nm, λ_{em} =

430 nm for DPH and tma-DPH, λ_{ex} = 380 nm, λ_{em} = 480 nm for DNS-Cl. The measurements were carried out by using a Hitachi Spectrofluorometer (F-2000 Spectrofluorometer, Hitachi Seisakusho Corp, Yokohama, Japan) equipped with a polarizer set.

The fluorescence anisotropy (r) was calculated using the following equation:

$$r = \frac{I_V - I_H}{I_V + 2I_H}$$

where I_V and I_H represent the fluorescence intensities perpendicular and parallel, respectively, to the polarized excitation plane [46].

1-9- Measurement of TEER and the transport of CF using Caco-2 cell monolayers

Caco-2 cells (passage 45) were cultured in 175-cm^2 culture flask (Thermo Fisher Scientific™, Massachusetts, USA). The culture medium consisted of DMEM containing 10% FBS, 0.1 mM MEM–NEAA, 2 mM glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin [47]. Cells were cultured in a humidified atmosphere of 5% CO_2 at 37 °C. The culture medium was changed every two days. When the cultured Caco-2 cells became sub-confluent, Caco-2 cells were seeded onto 12 mm Transwell® with 0.4 µm Pore Polycarbonate Membrane Insert (Corning Inc., New York, USA) at a density of 1×10^5 cells/insert. The transepithelial transport studies were performed when the transepithelial electrical resistance (TEER) values were more than 500 Ω/cm^2 (i.e., after 21 days) [48]. Briefly, after removing the incubation medium by aspiration, the apical and basal sides were washed thrice by Hank's Balanced Salt (HBSS) solution (pH 7.4). The cells were incubated with HBSS (pH 6.0) for the apical side and HBSS (pH 7.4) for the basal side for 20 min at 37 ˚C. Hank's balanced salts solution (HBSS) supplemented with 1 mg/ml glucose was used in all cell experiments. After removing the washing solution by aspiration, 500 µl of 10 µM CF in HBSS (pH 6.0) solution with or without various concentrations of SLG-30 (0.001% v/v, 0.01% v/v, 0.025% v/v, 0.05% v/v and 0.1% v/v), NaGC at concentrations of (2 mM and 20 mM) or sodium laurate at a concentration of (0.05% w/v) were added to the apical side at zero time, whereas precisely 1500 µl of HBSS (pH 7.4) were added to the basal side at 37 ˚C. The cells were kept at 37°C and 5% CO_2 and were continuously agitated on a shaker during the transport experiments.

TEER values were measured by Millicell® (ERS-2 Volt-Ohm Meter, Massachusetts, USA) at predetermined times up to 24 h and the initial values were considered as 100% . Samples from the basal side were withdrawn at predetermined times up to 24 h. The samples were replaced with an equal volume of the concentrations of HBSS (pH 7.4). CF was determined as previously mentioned. The apparent permeability coefficients P_{app} (cm/s) for the transported CF was determined by the following equation: $P_{app} = \frac{dX_R}{dt} \cdot \frac{1}{A} \cdot \frac{1}{C_0}$, where P_{app} is the apparent permeability coefficient in centimeters per second, X_R is the

amount of the drugs in moles in the receptor side, $\frac{dX_R}{dt}$ is the flux across the monolayer, A is the diffusion area (i.e., in square centimeters), C_0 is the initial concentration of CF in the donor side in moles per milliliter.

1-10- Assessment of Caco-2 cells membrane damage

After the transport studies were over, the solution of apical side of each well was collected. The influences of SLG-30 on the membrane damage were determined by tracking the LDH release [49,50] of The cell precipitate and the monolayers left on the filters were solubilized with Triton X-100 1% (v/v). The apical solutions and the lysate were centrifuged for 7 min at 200 × *g* at a temperature of 4 °C. The activities of LDH in the supernatant were measured. ($LDH_{release}$) stands for the activity of LDH released into the apical side in the presence of SLG-30. (LDH_{cell}) stands for the activity of LDH released from the cell lysate. The percentage of LDH released was calculated by the following equation: $LDH_{release}\% = LDH_{release} x100 / (LDH_{release} + LDH_{cells})$

1-11- Western Blotting

Western blotting was evaluated by the methods described previously with slight modification [51,52]. Three male Wistar rats weight 250 g were treated to perform an *in situ* closed-loop method, as mentioned above, on the small intestine. A control study, a treatment study and a pretreatment study were conducted on the first, second and third rat, respectively. For the control study, only PBS solution (pH 7.4) was administered into the small intestine then the rat was sacrificed, while for the treatment study, SLG-30 was administered, without washing it out, for 1 h, then the rat was sacrificed. The pretreatment study was performed by administrating SLG-30 for 1 h then washing it out with PBS solution (pH 7.4), then the rat was sacrificed after 4 h. The small intestine (60 cm) of three rats was taken and treated, as mentioned above, for extracting the brush border membrane vesicles. The total protein amount of each sample was adjusted to 30 μg. The protein expression levels of the claudin family in homogenate of the small intestine membrane were evaluated by Western blotting. Briefly, equal amounts of protein samples (30 μg protein) were mixed with SDS buffer solution and separated onto SDS-polyacrylamide 15% gels electrophoretically. The separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking in 5% of skim milk in Tris-buffered saline (pH 7.4) for 1 h at room temperature, the PVDF membrane was incubated overnight in blocking buffer with diluted (1:1000) monoclonal antibodies for claudin-1, claudin-4 and β-actin at 4 °C. Subsequently, the PVDF membrane was washed three times using Tris-buffered saline containing 0.05% Tween 20 (TTBS), followed by incubation with peroxidase-conjugated anti-rabbit IgG antibody for claudin-1 and β-actin, and with peroxidase-conjugated anti-rabbit IgG antibody for claudin-4 for 1h at room temperature. The signals were visualized by luminescence imaging (Fujifilm Luminescent

Image Analyzer LAS4000 System, Tokyo, Japan). The intensity of each signal was corrected using the values obtained from the β-actin bands, and the relative protein intensity was expressed as folds of the content in the normal group.

1-12- Statistical analyses

The results are expressed as the mean ± S.E. of at least three experiments. The statistical significances between the groups were analyzed using the Dunnet's test; $p < 0.05$ was considered significant. The significance levels are denoted as (**) when the value of $p < 0.01$, (*) when the value of $p < 0.05$, and (n.s.) when the p values were not statistically significant. The number of experiments is indicated by (n).

2- Results

2-1- The effects of SLG-30 on the intestinal absorption of poorly-absorbed drugs

The effects of multiple concentrations of SLG-30 (i.e., 0.1% v/v, 0.25% v/v, 0.5% v/v and 1% v/v) on the intestinal absorption of CF were estimated by an *in situ* closed-loop method. As shown in Fig. 8A, SLG-30 significantly enhanced the intestinal absorption of CF at all the concentrations studied, in a concentration-dependent manner, and the maximum absorption-enhancing effect was seen in the presence of SLG-30 0.5% (v/v), with $AUC_{0\rightarrow240}$ 16 times higher than with the control, as shown in Table 2. In the study of FDs at various molecular weights, the intestinal absorption of FD4 was significantly increased in the presence of SLG-30 as seen with CF, with the absorption enhancement ratio being 13 times higher for the concentration 0.5% (v/v) than with the control, as shown in Fig. 8B and Table 2. In the case of higher-molecular weight hydrophilic molecules, FD10 and FD70, the intestinal absorption of these also increased in the presence of SLG-30 especially at concentration of 0.5% (v/v); however, with the absorption enhancement ratios being only 6 and 3 times higher, respectively, than with the control, as shown in Fig. 8C and D and Table 2. Fig. 9 shows the relationship between the absorption enhancement ratios of the drugs and their molecular weights after co-administration with SLG-30 0.5% (v/v). The absorption enhancement ratios of the drugs after co-administration with SLG-30 0.5% (v/v) decreased, as their molecular weights increased.

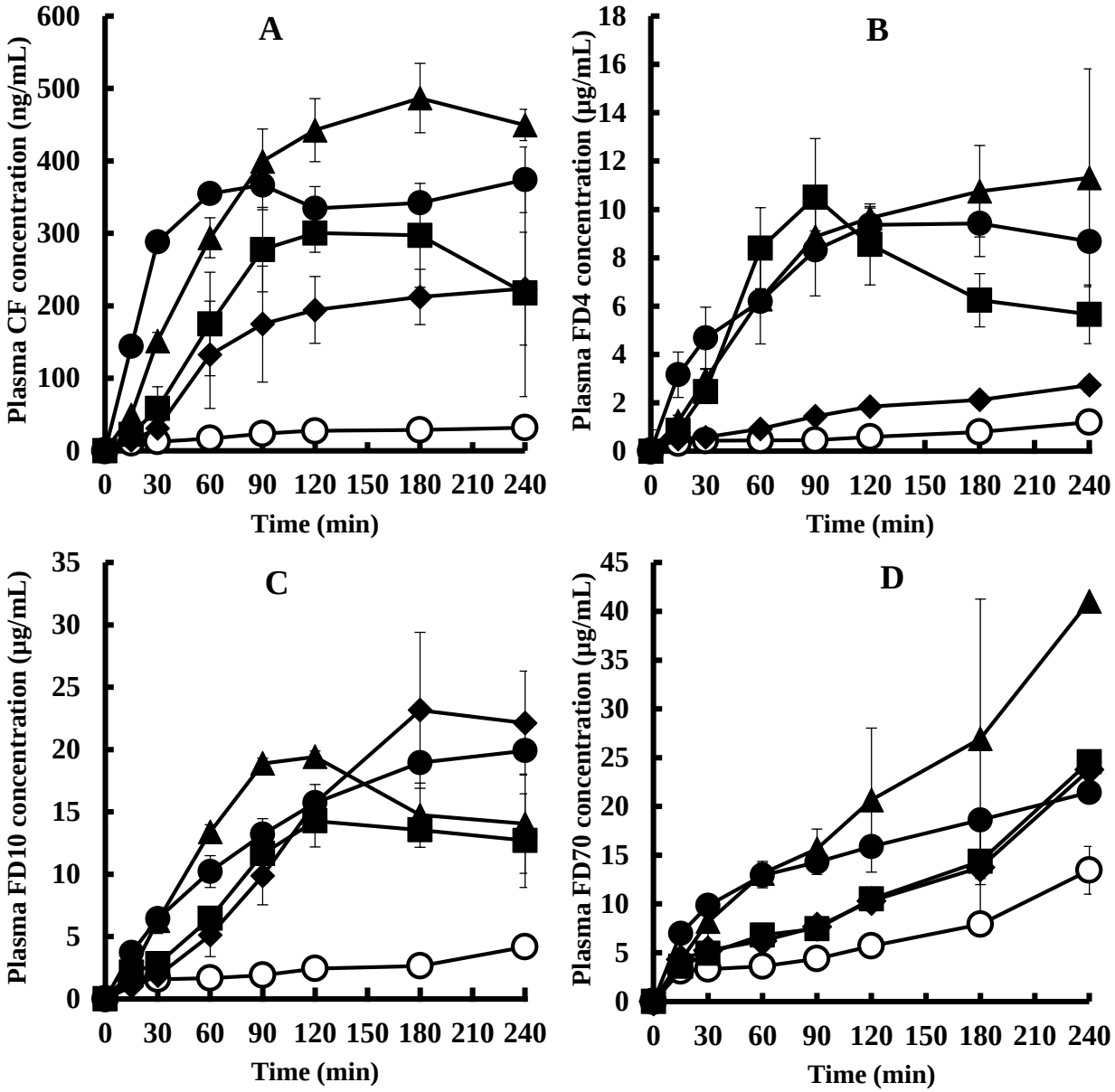


Fig. 8. Effects of SLG-30 on the absorption of (A) 5(6)-carboxyfluorescein (0.5 mg/kg), (B) fluorescein isothiocyanate-labelled dextran (8 mg/kg) FD4, (C) FD10, and (D) FD70 from the rat small intestine by an *in situ* closed-loop method. The results are expressed as the mean ± S.E. (n=3).Keys: (○) Control, (◆) SLG-30 0.1% (v/v), (■) SLG-30 0.25% (v/v), (▲) SLG-30 0.5% (v/v), (●) SLG-30 1.0% (v/v). (*Int. J. Pharm*, **499**, 58–66 (2016), Fig. 2).

2-2- The effects of SLG-30 on the absorption of peptides and protein drugs in the small intestine

To establish whether SLG-30 was effective in enhancing the absorption of peptides and protein drugs in the rat small intestine, calcitonin and insulin were selected as models of peptide and protein drugs and were

examined the effects of SLG-30 on the intestinal absorption of these drugs by an *in situ* closed-loop method.

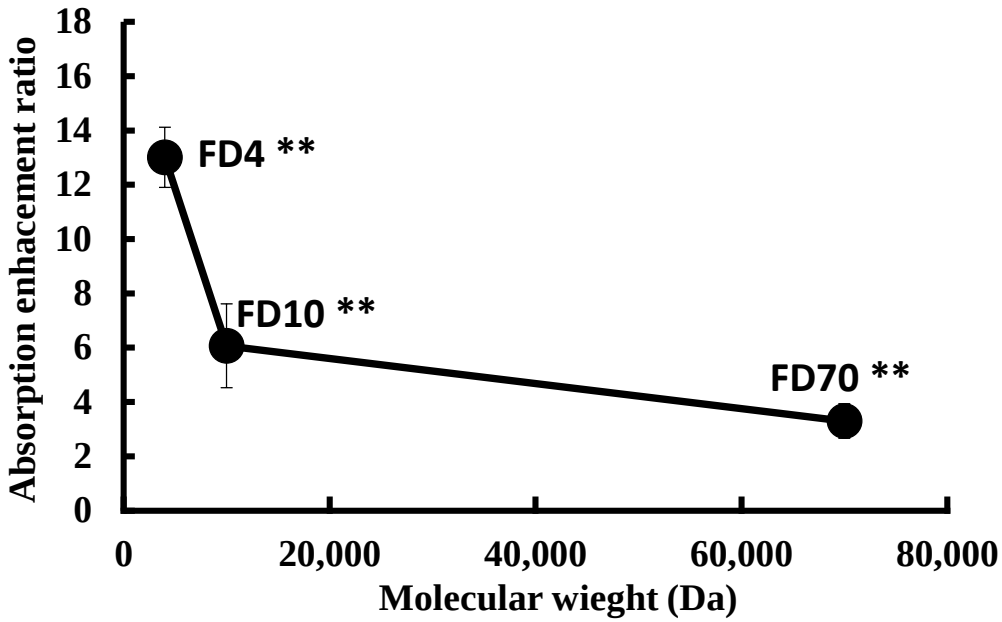


Fig. 9. The relationship between the absorption enhancement ratio and the molecular weight of drugs after co-administration of these model drugs with SLG-30 0.5% (v/v) into the rat small intestine by an *in situ* closed-loop method. The results are expressed as the mean ± S.E. (n=3). ** $p<0.01$, when compared with the control. (*Int. J. Pharm*, **499**, 58–66 (2016), Fig. 3).

Hypocalcaemic effects were observed after the intestinal administration of calcitonin with SLG-30 especially at concentration of 0.5% (v/v), showing 29% decrease in the plasma levels of calcium, as shown in Fig. 10A, with AAC being 5.5 times higher than in the control as shown in Table 3. However, no hypoglycemic effect (n.s.) was found after the small-intestinal administration of insulin with various concentrations of SLG-30.

2-3- The effects of SLG-30 on the absorption of peptides and protein drugs in the large intestine

Anatomically, the absorption area of the large intestine is smaller than that of the small intestine; however, the high activity of proteolytic enzymes in the small intestine hinders the absorption of intact peptides and proteins, thereby decreasing the bioavailability of such drugs. Therefore, the absorption enhancing effect of SLG-30 on calcitonin and insulin in the large intestines was studied and compared the results with those in the small-intestine group.

As shown in Fig. 10B, hypocalcaemic effects (* $p<0.05$) were observed after the co-administration of calcitonin with various concentrations of SLG-30 in the large intestine, as compared to the small intestine. The decrease in the plasma levels of calcium were similar among the groups at all the concentrations of

SLG-30, as shown in Table 3. However, significant hypoglycemic effects (** $p<0.01$) were observed after the co-administration of SLG-30, in the large intestine, as shown in Fig. 10C. Table 3 shows that the plasma levels of glucose decreased to 63% when insulin was co-administrated with SLG-30 1% (v/v), showing a PA value of 7.18%. In case of pretreatment of the large intestine with SLG-30 0.5% (v/v) for 2 h, followed by administration of insulin, the plasma levels of glucose did not decrease as much as that during the co-administration of insulin with SLG-30. The decrease in the plasma levels of glucose was about 10.7%, with a PA value of 1.2%.

Table 2 Summary of the pharmacokinetic parameters of CF, FD4, FD10 and FD70 after their co-administration with SLG-30 into rat small intestines by an *in situ* closed-loop method.

	Conc.	C_{max} (μg/mL)	T_{max} (min)	$AUC_{0\rightarrow240}$ (μg・min/mL)	Enhancement ratio
CF					
Control		—	—	5.5 ± 0.7	—
SLG-30	0.10%	0.26 ± 0.05	150 ± 30	38 ± 6.0 **	7.0
	0.25%	0.38 ± 0.02	150 ± 30	53 ± 2.0 **	9.7
	0.50%	0.49 ± 0.05	180 ± 00	87.2 ± 6.0 **	16
	1.0%	0.38 ± 0.02	80 ± 10	76 ± 3.0 **	13.9
FD4					
Control		—	—	150 ± 14.0	—
SLG-30	0.10%	2.0 ± 0.20	180 ± 00	380 ± 38.0	2.5
	0.25%	11 ± 2.00	80 ± 10	1560 ± 290.0 **	10.4
	0.50%	11.5 ± 1.50	160 ± 20	1950 ± 232.0 **	13.0
	1.0%	10 ± 1.00	160 ± 20	1811 ± 269.0 **	12.1
FD10					
Control		—	—	544 ± 36.0	—
SLG-30	0.10%	24 ± 6.00	160 ± 20	3259 ± 378.0 **	6.0
	0.25%	16 ± 0.70	140 ± 20	2459 ± 48.0 **	4.5
	0.50%	19.5 ± 0.50	120 ± 00	3305 ± 344.0 **	6.1
	1.0%	19 ± 0.70	160 ± 20	3316 ± 57.0 **	6.1
FD70					
Control		—	—	1472 ± 133.5	—
SLG-30	0.10%	23.75 ± 1.80	240 ± 00	2567 ± 40.0	1.8
	0.25%	24.6 ± 5.00	168 ± 72	2637.5 ± 132.0	1.8
	0.50%	41.5 ± 14.0	200 ± 40	4855 ± 853.0 **	3.3
	1.0%	21.5 ± 0.90	220 ± 20	3564 ± 209.0 *	2.4

The results are expressed as the mean ± S.E. (n=3). ** $p<0.01$, * $p<0.05$, when compared with the control. (*Int. J. Pharm*, **499**, 58–66 (2016), Table 1).

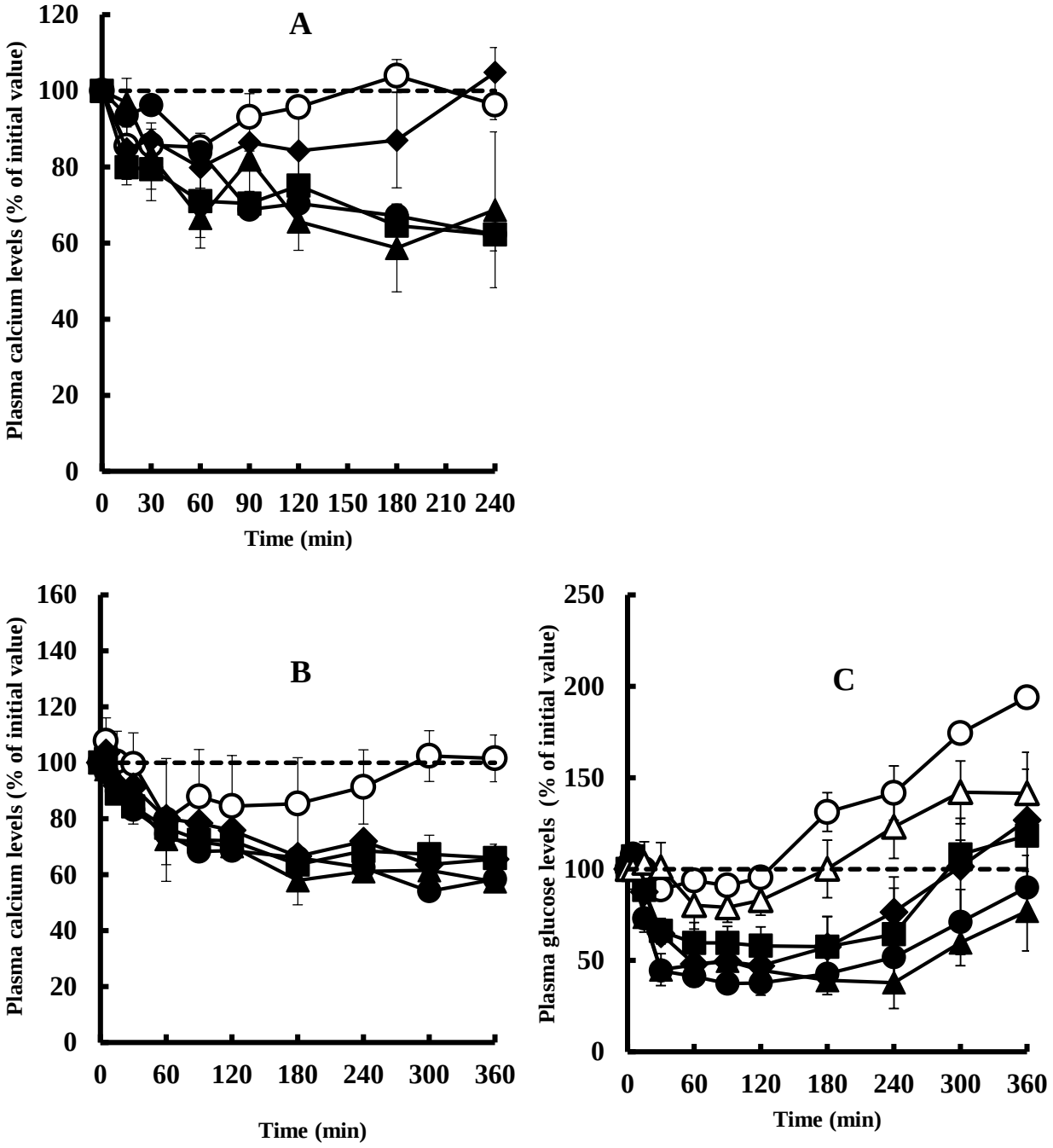


Fig. 10. Effects of SLG-30 on the absorption of (A) calcitonin from the rat small intestines, (B) calcitonin from the rat large intestines and (C) insulin from the rat large intestines, by an *in situ* closed-loop method. The absorption of calcitonin when administered at a dose of 80 μg/kg, was estimated by measuring the calcium levels in the plasma, whereas the absorption of insulin when administered at a dose of 80 IU/kg, was estimated by measuring the glucose levels in the plasma. The results are expressed as the mean ± S.E. (n=3). Keys: (○) Control, (◆) SLG-30 0.1% (v/v), (■) SLG-30 0.25% (v/v), (▲) SLG-30 0.5% (v/v), (●) SLG-30 1.0% (v/v), (△) Pretreatment with SLG-30 0.5% (v/v) for 2 h. (*Int. J. Pharm*, **499**, 58–66 (2016), Fig. 4).

2-4- *In vivo* comparison study between SLG-30 and conventional surfactants including sodium glycocholate and sodium laurate

In order to compare the effectiveness of SLG-30 with that of conventional absorption enhancers, absorption studies for SLG-30 0.5% (v/v) and 20 mM sodium glycocholate (NaGC) by an *in situ* closed-loop method by using CF were carried out. As shown in Fig. 11, the absorption of CF was significantly enhanced in the case of SLG-30 0.5% (v/v), and the AUC_{0-240} of CF was 16 times (** $p < 0.01$) higher than the control group, while it was 4.5 times in the case of 20 mM NaGC. The absorption enhancement ratio of CF after using SLG-30 was about 3.5 times highr than that of 20 mM NaGC (Table 4). Moreover, SLG-30 contains two moieties of the fatty acid (lauric acid) (Fig. 6). In order to clarify whether SLG-30 is effective as a whole compound or the effectiveness is related to the fatty acid only [35], the absorption enhancement effect of sodium laurate 0.5% (w/v) in the small intestines was studied by an *in situ* closed-loop method. It was found that T_{max} of CF in the case of sodium laurate is about 15 min then the concentrations of CF deceased quickly and continuously until the end of experiment, while SLG-30 greatly increased the concentrations of CF until T_{max} (180 min) then started to decrease, as shown in Fig. 11, and Table 4. The absorption enhancement ratio of CF for SLG-30 was also higher than that of sodium laurate with about 6 times.

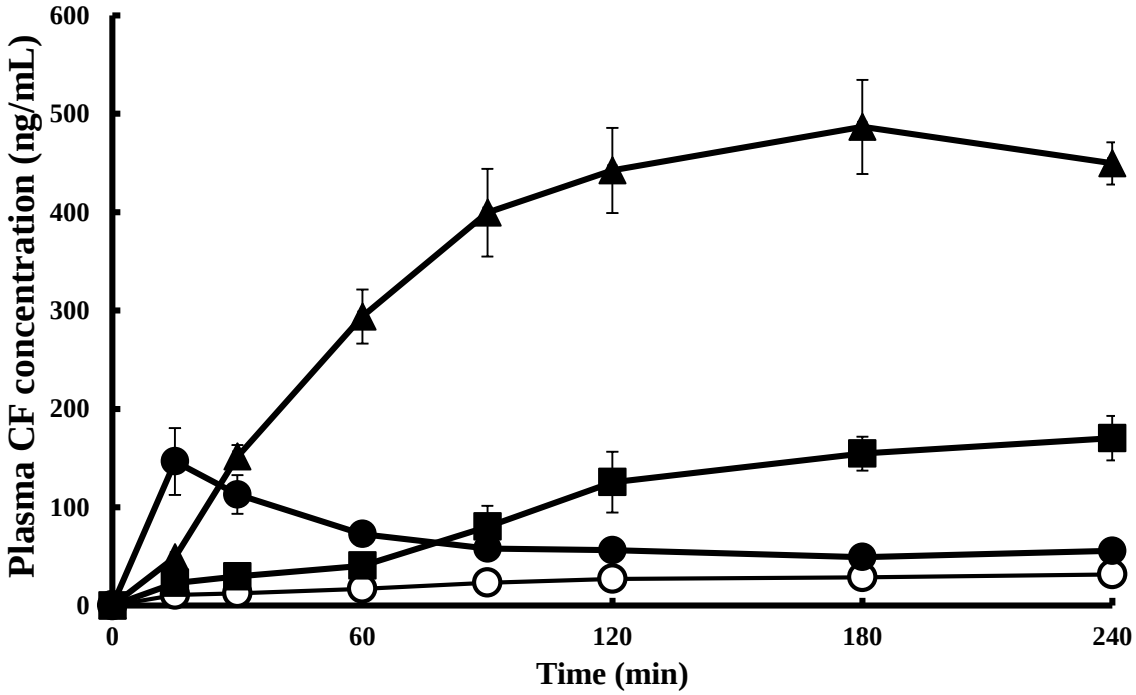


Fig. 11. *In vivo* comparison of absorption enhancing effects between gemini surfactant, SLG-30, and conventional absorption enhancers including sodium glycocholate and sodium laurate. Results are expressed as the mean ± S.E. (n = 3). Keys: (◯) Control, (▲) SLG-30 at a concentration of 0.5% (v/v), (●) sodium laurate at a concentration of 0.5% (w/v), (■) sodium glycocholate at a concentration of 1.0% (v/v).

Table 3 Pharmacodynamic parameters of calcitonin and insulin after their co-administration with SLG-30 into rat small and large intestines by an *in situ* closed-loop method.

	Conc.	AAC (% min)	D%	PA%	Enhancement ratio
Small intestines					
Calcitonin					
Control	**—**	**1250 ± 250**	**5.2 ± 1.1**	**0.08 ± 0.01**	**—**
SLG-30	**0.10%**	**2900 ± 2300**	**12.0 ± 9.9**	**0.18 ± 0.10**	**2.3**
	0.25%	**6900 ± 1200**	**28.8 ± 5.5**	**0.44 ± 0.08**	**5.5**
	0.50%	**6950 ± 1800**	**28.9 ± 7.5**	**0.44 ± 0.10**	**5.5**
	1.0%	**6000 ± 1700**	**25.2 ± 7.1**	**0.38 ± 0.10**	**4.8**
Large intestines					
Calcitonin					
Control	**—**	**2800 ± 480**	**7.8 ± 13.3**	**0.12 ± 0.20**	**—**
SLG-30	**0.10%**	**14850 ± 2550**	**41.3 ± 7.2 ***	**0.63 ± 0.10 ***	**5.3**
	0.25%	**15950 ± 2100**	**44.3 ± 5.9 ***	**0.67 ± 0.08 ***	**5.7**
	0.50%	**16800 ± 1850**	**46.6 ± 5.1 ***	**0.71 ± 0.07 ***	**6.0**
	1.0%	**16500 ± 2050**	**45.9 ± 9.8 ***	**0.70 ± 0.08 ***	**5.9**
Insulin					
Control	**—**	**750 ± 133**	**5.3 ± 0.2**	**0.60 ± 0.2**	**—**
SLG-30	**0.1%**	**19400 ± 1600**	**53.8 ± 4.5 ****	**6.12 ± 0.5 ****	**25.8**
	0.3%	**17200 ± 1200**	**47.7 ± 3.4 ****	**5.41 ± 0.3 ****	**23.0**
	0.5%	**21300 ± 4850**	**59.1 ± 13.4 ****	**6.71 ± 1.5 ****	**28.5**
	1.0%	**22800 ± 2050**	**63.2 ± 5.7 ****	**7.18 ± 0.6 ****	**30.5**
Pretreatment with	**0.5%**	**1927 ± 568**	**10.7 ± 3.2**	**1.2 ± 0.3**	**2.5**

The absorption of calcitonin (80 μg/kg) and insulin (80 IU/kg) was estimated by measuring the calcium and glucose levels in the plasma, respectively. The results are expressed as the mean ± S.E. (n=3). ** $p<0.01$, * $p<0.05$, when compared with the control. (*Int. J. Pharm*, **499**, 58–66 (2016), Table 2).

Table 4 Summary of the pharmacokinetic parameters of CF (0.5 mg/kg) after its administration with SLG-30 0.5% (v/v), sodium laurate 0.5% (w/v), and sodium glycocholate 20 mM into rat small intestines by an *in situ* closed-loop method.

	Conc.	C_{max} (μg/mL)	T_{max} (min)	$AUC_{0\rightarrow240}$ (μg・min/mL)	Enhancement ratio
CF					
Control		**—**	**—**	**5450 ± 700**	**—**
SLG-30	**0.5% (v/v)**	**487 ± 4**	**180 ± 0**	**87200 ± 6000 ****	**16**
Sodium laurate	**0.5% (w/v)**	**375 ± 2**	**15 ± 0**	**14700 ± 1950**	**2.7**
Sodium glycocholate	**20 mM**	**172 ± 2**	**180 ± 0**	**24500 ± 3700 ***	**4.5**

The results are expressed as the mean ± S.E. (n=3). ** $p<0.01$, * $p<0.05$, when compared with the control.

2-5- The reversible effect of SLG-30 on the intestinal absorption of CF

When absorption enhancers are used in clinical practice, it is essential that the absorption enhancement effect be reversible, otherwise foreign particles, like viruses, could enter the systemic circulation. To establish the reversibility of the absorption enhancing effect of SLG-30, the effects of pretreatment with SLG-30 on the absorption of CF from the rat small intestine were examined. The SLG-30 was washed out with PBS prior to the administration of CF. Fig. 12 shows the effects of pretreatment with SLG-30 0.5% (v/v) on the absorption of CF administered at a dose of 0.5 mg/kg, from the small intestine, by an *in situ* loop-method. As indicated in Fig. 12, the plasma concentrations of CF significantly increased by the co-administration with SLG-30 0.5% (v/v) with the AUC $_{0\rightarrow240}$ values being 16 times higher than the control; however, the intestinal absorption of CF was only partially enhanced after pretreatment with SLG-30 0.5% (v/v) with the AUC $_{0\rightarrow240}$ values being only 4 times higher than the control at 10, 60 and 120 min. These findings suggest that the absorption enhancing effect of SLG-30 was partially reversible and was reduced after its removal from the rat small intestine, during the duration of the experiment.

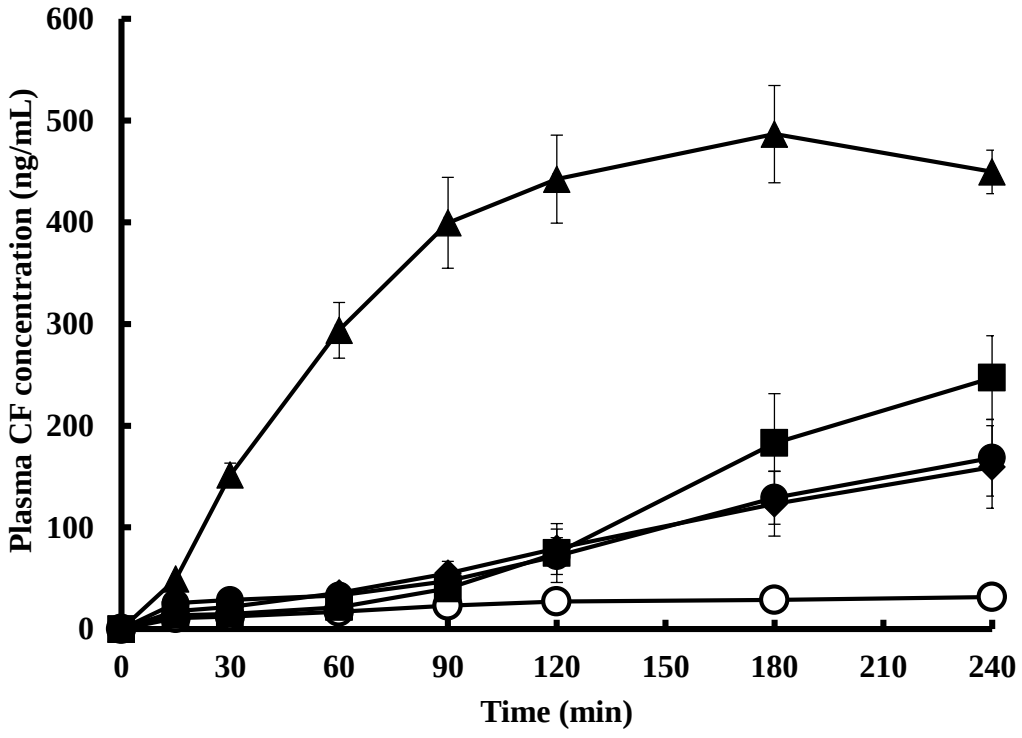


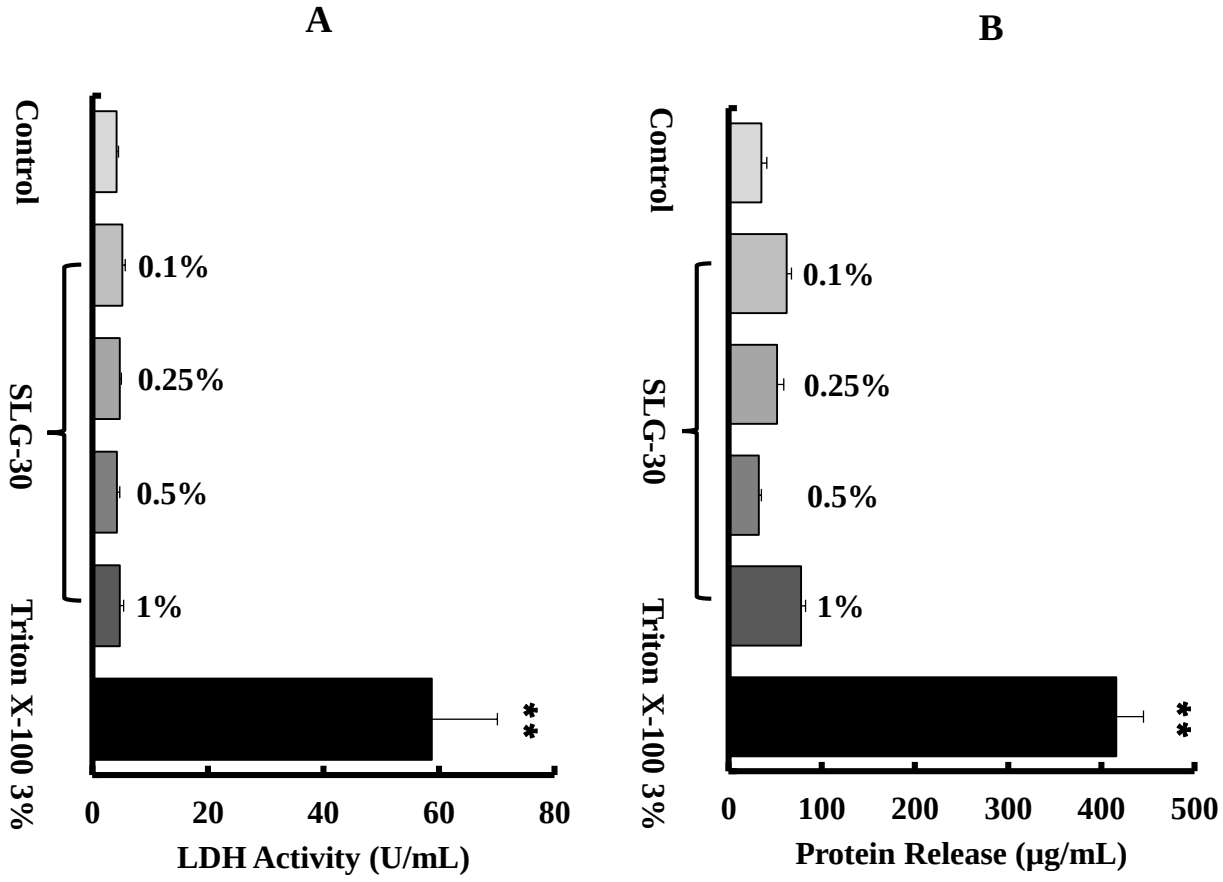
Fig. 12. Effects of pretreatment with SLG-30 (0.5% v/v) on the absorption of 5(6)-carboxyfluorescein (0.5 mg/kg) from the small intestines by an *in situ* closed-loop method. The results are expressed as the mean ± S.E. (n=3). Keys: (◯) Control, (▲) Co-administration with SLG-30 0.5% (v/v), (■) Pretreatment with SLG-30 0.5% (v/v) for 10 min, (◆) Pretreatment with SLG-30 0.5% (v/v) for 60 min, (●) Pretreatment with SLG-30 0.5% (v/v) for 120 min. (*Int. J. Pharm*, **499**, 58–66 (2016), Fig. 5).

2-6- Assessment of membrane damage

To evaluate the extent of small-intestinal membrane damage in the presence of SLG-30, the LDH activity and the protein release from the small-intestinal membrane were measured by an *in situ* closed-loop method. As a result, almost no significant increase, either in the LDH activity or the protein release were observed, when compared to the positive control, Triton X-100 3%, as shown in Fig. 13A and 13B, suggesting that SLG-30 might not cause serious membrane damage to the small-intestinal membrane.

2-7- Morphological observations

As shown in Fig. 13C, the positive control, Triton X-100 3%, caused serious membrane damage to the rat small intestine; however, no morphological changes were observed in the presence of SLG-30 at various concentrations of 0.25% (v/v), 0.5% (v/v) and 1% (v/v), when compared with control in the non-treated jejunum. These morphological studies confirm the safety of SLG-30 on the intestinal membrane at the highest concentration of 1% (v/v).



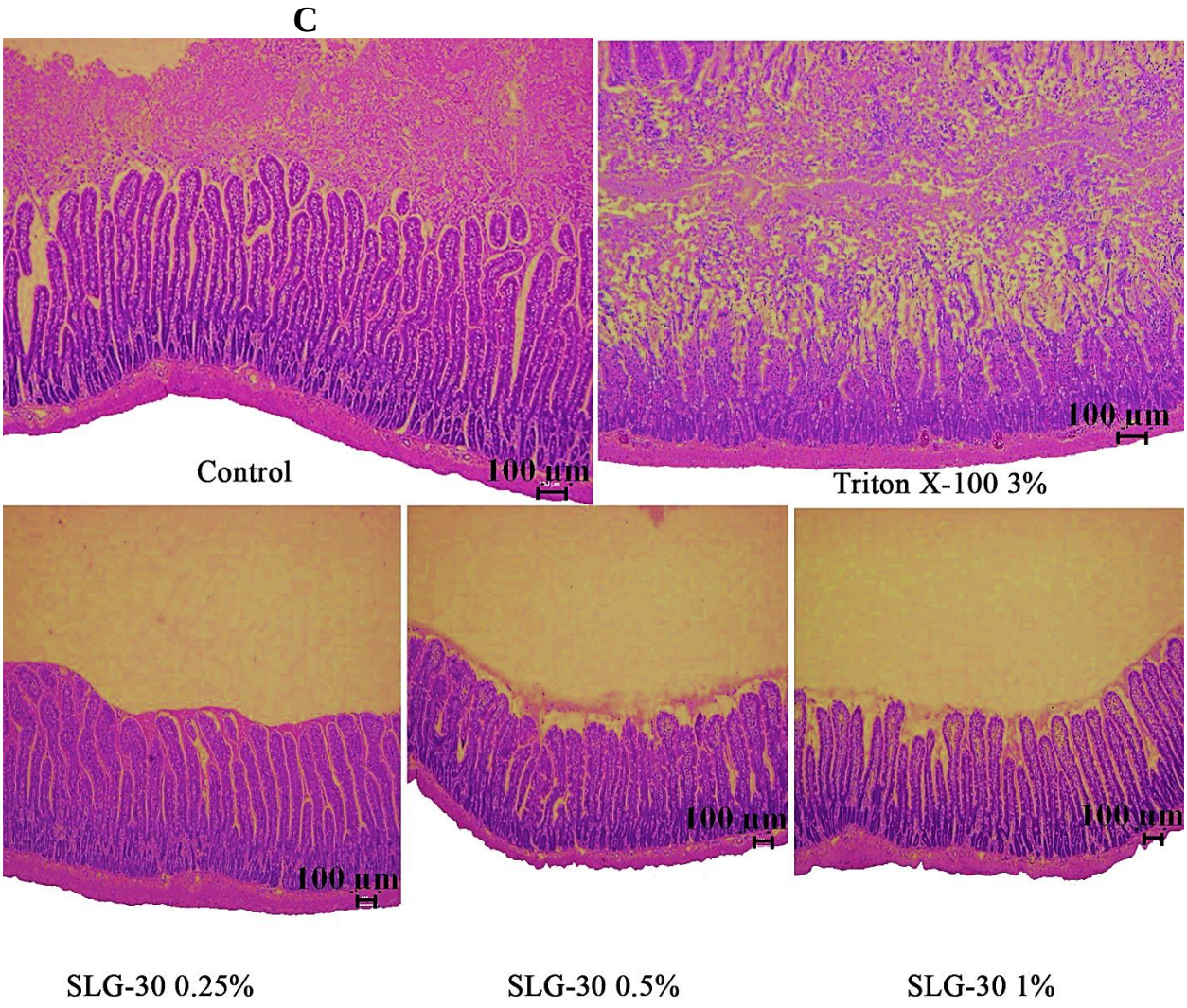


Fig. 13. Evaluation of rat small intestinal-membrane damage in the presence of SLG-30 at multiple concentrations of 0.1% (v/v), 0.25% (v/v), 0.5% (v/v) and 1% (v/v). The membrane damage was determined by measuring (A) the activities of LDH, (B) the amount of protein released from the intestinal membranes and (C) the morphological observations of the rat jejunum. The results are expressed as the mean ± S.E. (n=6). ** $p<0.01$, n.s. not significantly different, when compared with the control. (*Int. J. Pharm*, **499**, 58–66 (2016), Fig. 6).

2-8- Effects of SLG-30 on the membrane fluidity of rat small intestines

In order to study the changes in membrane fluidity, DPH, tma-DPH, and DNS-Cl, which can attach to specific parts of membrane bilayers, were used. The change of anisotropy of the fluorescence of these markers means changes in membrane fluidity. As shown in Fig. 14A, B and C, cholesterol, used as a positive control, increased the fluorescein anisotropy, which indicates a decrease in the membrane fluidity by using cholesterol. Fig. 14A shows that SLG-30 decreased (* $p < 0.05$) the fluorescence anisotropy of DPH compared to the control group. SLG-30 did not decrease the fluorescence anisotropy of tma-DPH, as

shown in Fig. 14B. Furthermore, Fig. 14C shows that SLG-30 caused dose dependent reductions in the fluorescence anisotropy of DNS-Cl (** $p < 0.01$) compared to the control group. These findings suggest that the cell membrane fluidity of the small intestines in the protein portion could be significantly increased by SLG-30 with a small effect on the inner portion of the membrane's bilayers.

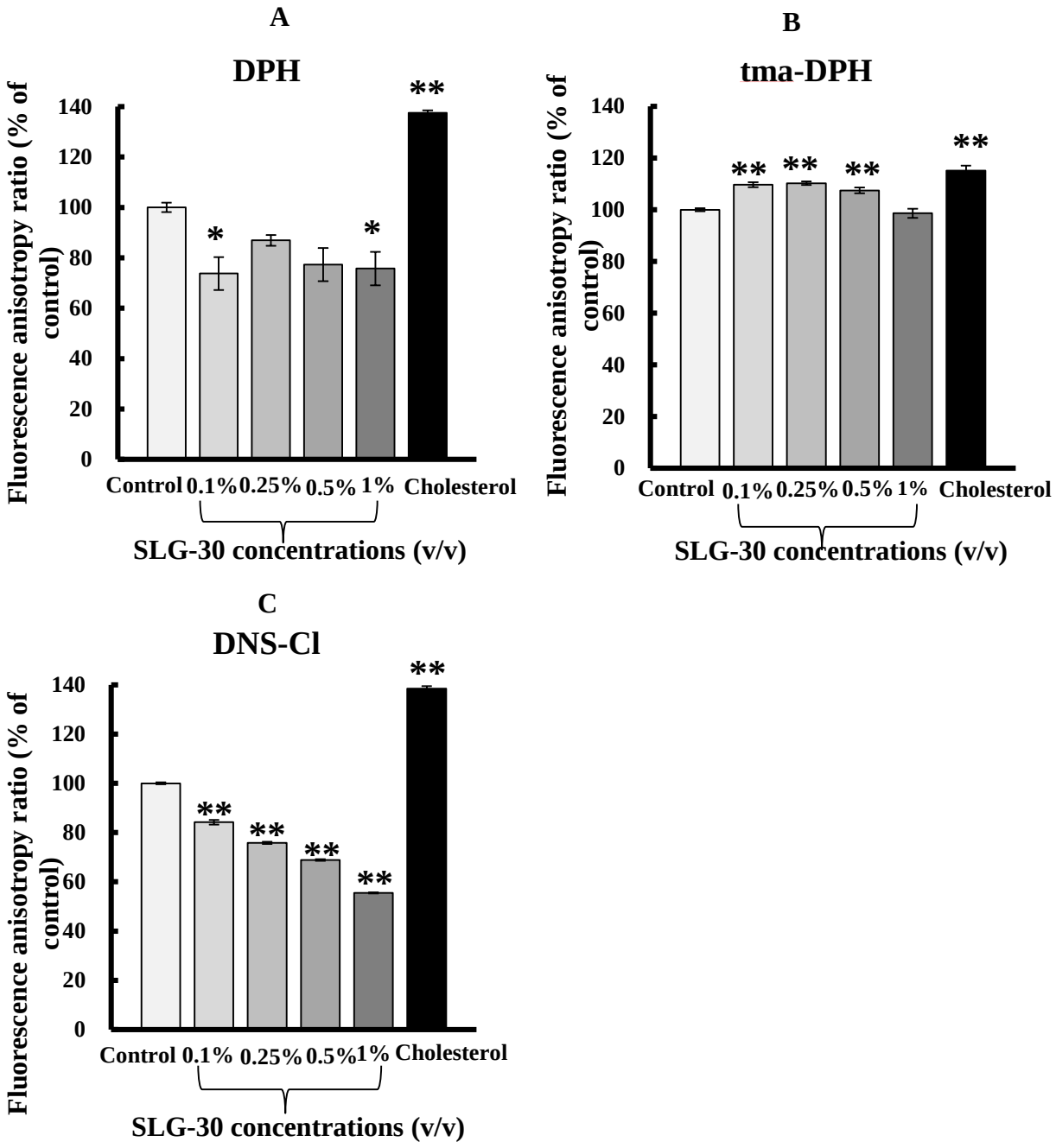


Fig. 14. Effects of SLG-30 on the fluorescence intensity of DPH, tma-DPH, and DNS-Cl. All samples contained DPH, tma-DPH, and DNS-Cl at a final concentration of 1 μM, 0.5 μM and 0.5 μM, respectively. The results are expressed as the mean ± S.E. (n = 4). ** $p < 0.01$, n.s. not significantly different when compared with the control.

2-9- Effect of SLG-30 on TEER and the transport of CF in Caco-2 cell monolayers

CF solutions in HBSS with or without various concentrations of SLG-30 (i.e., 0.001% v/v, 0.01% v/v, 0.25% v/v, 0.05% v/v and 0.1% v/v) were added to the apical side at 37 ˚C. As shown in Fig. 15A, a significant decrease (** $p < 0.01$) in the TEER values (as a percentage of the initial value at time zero before adding the solution of SLG-30 and/or CF, i.e., TEER values were higher than 500 Ω/cm^2) was obtained in the presence of SLG-30 at all concentrations studied even at a very small concentration 0.001% (v/v) for the first hour. TEER values did not recover to the baseline (without removing SLG-30) for the higher concentrations but it started to recover for the small concentrations. In corresponds with the TEER results, as shown in Fig. 16, the permeability of CF after 24 h significantly increased (** $p < 0.01$) when CF was co-administrated with SLG-30 at all concentrations. The enhancement ratios of permeability were (6, 7, 9, 11 and 12) times higher than the control group for SLG-30 concentrations (0.001% v/v, 0.01% v/v, 0.25% v/v, 0.05% v/v and 0.1% v/v), respectively.

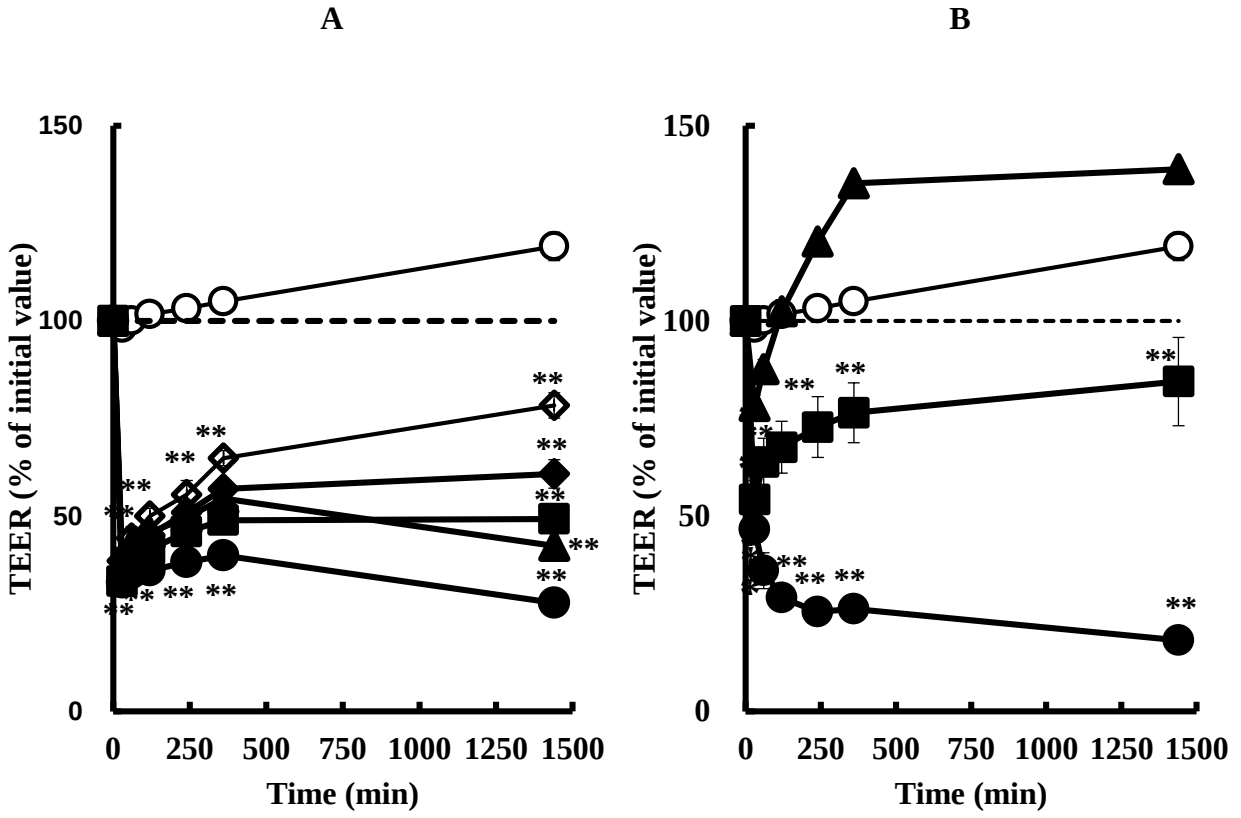


Fig. 15. Transepithelial electrical resistance (TEER) values in the presence of (A) SLG-30 and (B) sodium glycocholate and sodium laurate in Caco-2 cell monolayers. The results are expressed as the mean ± S.E. (n = 3). ** $p < 0.01$, n.s. not significantly different when compared with the control. Keys for (A): (◯) Control, (◇) SLG-30 0.001% (v/v), (◆) SLG-30 0.01% (v/v), (■) SLG-30 0.025% (v/v), (▲) SLG-30 0.05% (v/v), and (●) SLG-30 0.1% (v/v). Keys for (B): (◯) Control, (▲) 2 mM sodium glycocholate, (■) 20 mM sodium glycocholate, (●) sodium laurate 0.05% (w/v).

Parallel studies were performed with (2 mM and 20 mM) NaGC and sodium laurate 0.05% (w/v). As shown in Fig. 15B, TEER values significantly decreased (** $p < 0.01$) in the presence of sodium laurate 0.05% (w/v) without recovering to the baseline during 24 h of experiments. In the case of 20 mM NaGC, TEER value significantly decreased (** $p < 0.01$) then recovered to the baseline at the end of the experiment. NaGC 2 mM had almost no effect on TEER values. As shown in Fig. 16, sodium laurate significantly increased (** $p < 0.01$) the permeability of CF across Caco-2 cells with 94 times higher than the control group. 20 mM NaGC also significantly increased (** $p < 0.01$) the permeability of CF with a lesser extent comparing with SLG-30 concentrations (i.e., 5 times higher than the control group). 2 mM NaGC had almost no effect on the permeability of CF across Caco-2 cells (Fig. 16).

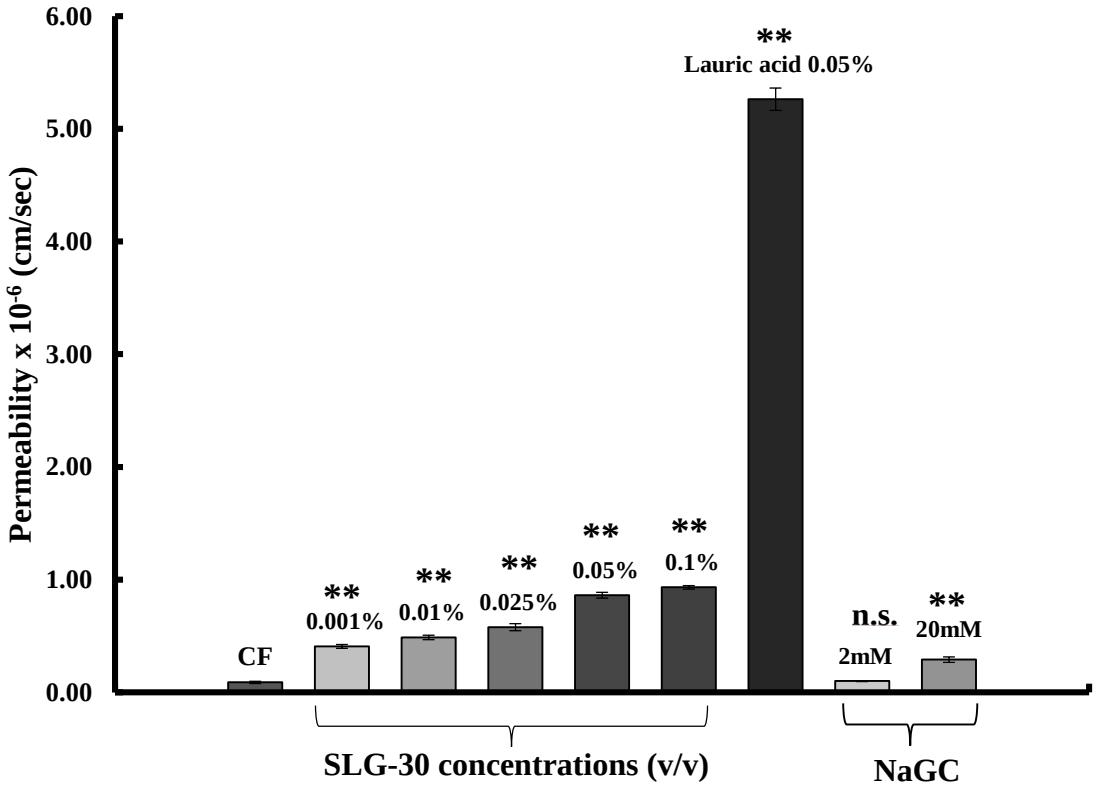


Fig. 16. Permeability of CF in the presence of SLG-30, sodium glycocholate and sodium laurate across Caco-2 cell monolayers. The results are expressed as the mean ± S.E. (n = 3). ** $p < 0.01$, n.s. not significantly different when compared with the control.

2-10- Assessment of membrane damage in Caco-2 cells

To assess the damage extent of Caco-2 cells caused by SLG-30, sodium laurate or NaGC, LDH activity was measured at the end of the transport studies. The solution was collected from the apical side of each well then centrifuged at 200 × *g* for 7 min at 4 °C. The supernatant was measured for released LDH activity

(LDH $_{release}$). As shown in Fig. 17A, SLG-30 did not increase the activity of LDH released from Caco-2 cells comparing with the control group. Moreover, the activity of LDH released from Caco-2 cells significantly increased after using sodium laurate 0.05% (w/v). Neither 2 mM nor 20 mM of NaGC increased the activity of LDH. These findings suggested that SLG-30 did not cause serious damages to the cell membranes. Fig. 17B shows the relationship between the effects of SLG-30 and conventional absorption enhancers on the permeability of CF across Caco-2 cells on one side and their toxic effects on the other side. The absorption enhancing effect of SLG-30 was concentration-dependent, while no serious intestinal membrane damage was seen irrespective of SLG-30 concentrations.

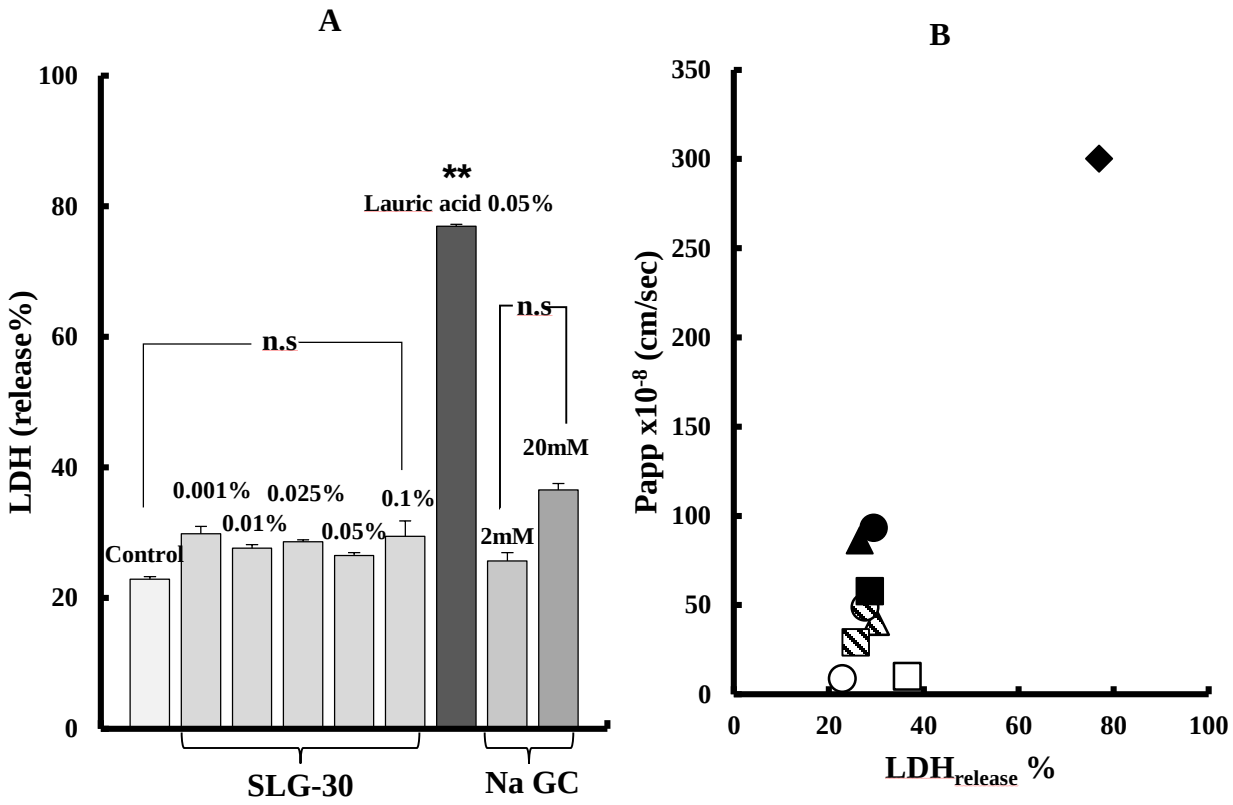


Fig. 17. (A) Evaluation of membrane rupture of Caco-2 cells monolayers in the presence of SLG-30, sodium glycocholate and sodium laurate. (B) Relationship between the effects of SLG-30 and conventional absorption enhancers on the permeability of CF across Caco-2 cells and their toxic effects. The results are expressed as the mean ± S.E. (n = 6). ** $p < 0.01$, n.s. not significantly different when compared with the control. Keys for (B): (◯) Control, (◬) SLG-30 0.001% (v/v), (▨) SLG-30 0.01% (v/v), (■) SLG-30 0.025% (v/v), (▲) SLG-30 0.05% (v/v), and (●) SLG-30 0.1% (v/v). (▧) 2 mM sodium glycocholate, (□) 20 mM sodium glycocholate, (◆) sodium laurate 0.05% (w/v).

2-11- Estimation of claudin proteins by western blotting

The suggested mechanism for the effect of SLG-30 on intestinal absorption is the enhancement of membrane permeability through the paracellular pathway by unfastening the nodes of tight junctions. Fig.

18A shows the bands of western blotting for claudin-1 and claudin-4 after treatment with SLG-30 0.5% (v/v) and with pre-treatment with SLG-30 0.5% (v/v). The intensity of claudin-1 and claudin-4 decreased after treatment with SLG-30 0.5% (v/v), as indicated in Fig. 18B and C.

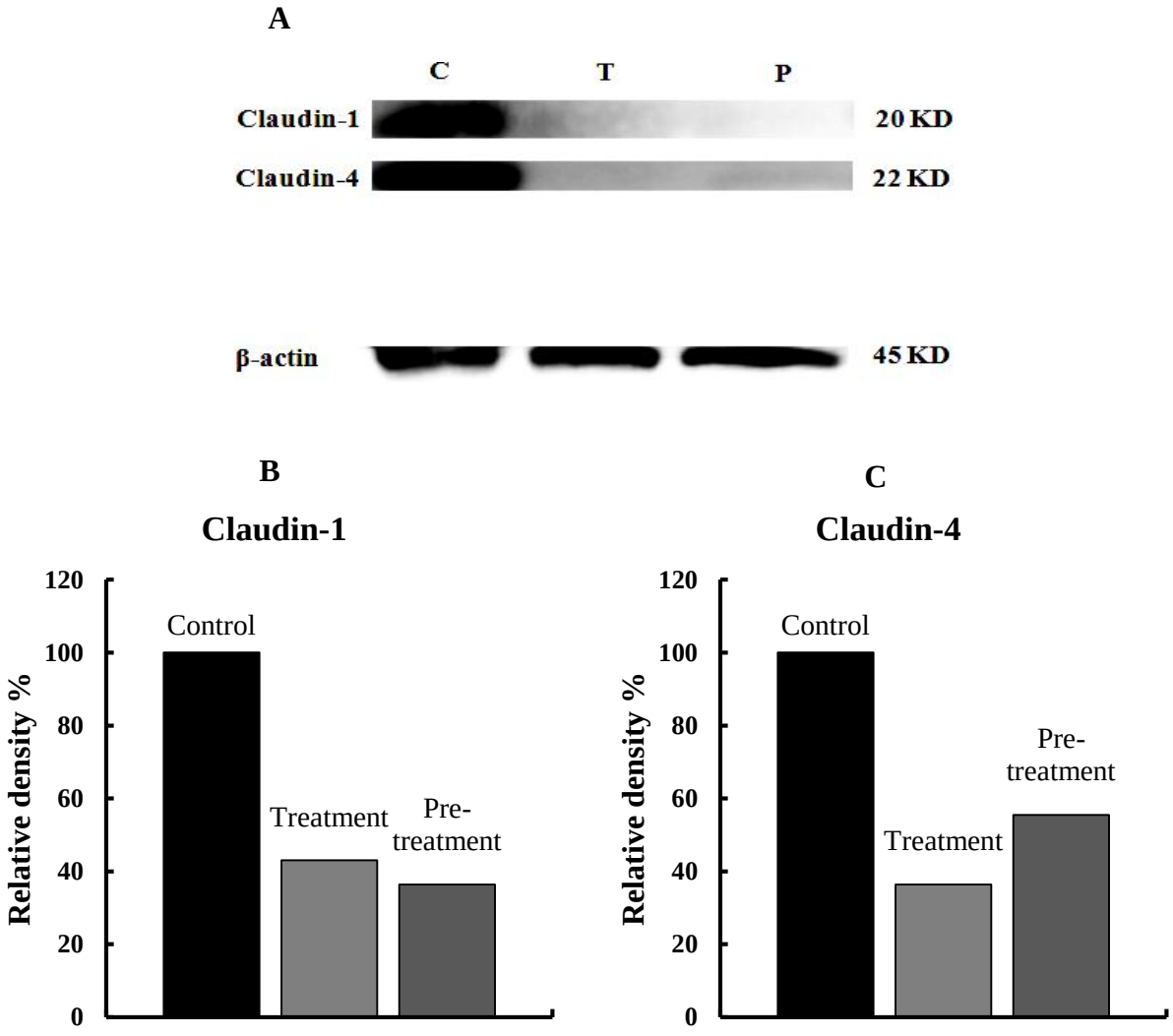


Fig. 18. Expression levels of claudin-1 and claudin-4 in the rat small intestine after treatment with SLG-30 0.5% (v/v) for 1 h. Control; without treatment, treatment; treatment with SLG-30 0.5% (v/v) for 1 h, pre-treatment; pre-treatment for 1 h. Proteins were extracted after 4 h.

3- Discussion

In the present study, the effects of the gemini surfactant, SLG-30, on the intestinal absorption of CF, FD4, FD10 and FD70 were examined. CF and FDs were used as models of low- and high-molecular-weight hydrophilic compounds, respectively, since they could be assayed easily and are widely used for evaluating the efficiency of absorption enhancers in the intestinal absorption of drugs. It was found that SLG-30 could

significantly enhance the intestinal absorption of hydrophilic compounds, CF and FDs, in a dose-dependent manner. However, the extent of absorption of FDs was lesser compared to CF, indicating that SLG-30 enhanced the absorption of hydrophilic poorly absorbed compounds depending on their molecular weights. As previously reported, the absorption enhancing effects of the second generation- polyamidoamine (PAMAM) dendrimers, *in vivo* [34], and *N*-ethyl-2-(1-ethyl-hydroxy-2-nitrosohydrazino)-ethaneamine (NOC12), one of the typical nitric oxide donors, *in vitro* [53] decreased, as the molecular weight of the drugs increased. Therefore, SLG-30 could easily and significantly improve the permeability of low- and high-molecular weight hydrophilic molecules, consistent with the previous findings. However, the reason for the molecular weight-dependent, absorption enhancing action of SLG-30 has not been fully understood. It may be possible that SLG-30 loosens TJs of the epithelium to a limited extent, in which the enlargement of the pore radius between the adjacent cells may not be sufficient to enhance the intestinal absorption of macromolecular compounds. It was noticed that the $AUC_{0\rightarrow240}$ values of FDs control were bigger than that of CF control, as shown in the Table. 2. It is possible because of the difference in the administrated dose between CF and FDs. However, the differences of the $AUC_{0\rightarrow240}$ values among FDs themselves were not fully understood. These differences were also shown in previously published manuscript [34]. The renal elimination of dextrans in rats is reported to take place only through glomerular filtration; no tubular secretion or reabsorption process is involved [54]. A possible explanation for these differences might be the substantial differences between the pore sizes of the capillary walls of the kidneys. Therefore, the elimination of absorbed FD70 was lower in speed than that of FD10 and FD4, for that, and as shown in Fig.8, FD10 and FD70 were still in the absorption phase at the 240 min. In this study, the AUC values were calculated only for limited period (i.e., up to 4h) not calculated as $AUC_{0\rightarrow\infty}$ due to experimental difficulties in taking blood samples in periods longer than the designated time. Therefore, larger $AUC_{0\rightarrow240}$ for FD70 can be seen in this case. However, the point of study was to compare the effects of SLG-30 on enhancing the absorption of FDs with their control. SLG-30 was able to enhance the intestinal absorption of FDs comparing to the control, therefore, it can be said that the point of the study was partially achieved.

SLG-30 could also decrease the plasma levels of calcium when co-administered with calcitonin, in the absence of any protease inhibitor in the rat small intestine. For the absorption enhancing effects of SLG-30 0.5% (v/v), the area above the curve was about 5.5 times higher, the calcium levels in the plasma were 29% lower (D%) and the PA value was 0.44%, when compare to the control. These results suggest that SLG-30 was effective in improving the intestinal absorption of calcitonin from the rat small intestine. PA% was calculated depending on D% of i.v calcitonin with dose of 0.1 μg/Rat. Unfortunately, SLG-30 did not increase the intestinal absorption of insulin from the small intestine. In the case of co-administration of insulin with SLG-30 in the large intestine, a significant decrease in the glucose concentrations in the plasma for 4 h post-administration was observed, with recovery observed during the next 2 h. The D% value was

63%, and PA value was 7.18% for SLG-30 1% (v/v). The large intestine shows lower activity of the degradation enzymes and this can partly explain the higher pharmacological action of insulin when co-administered with SLG-30 in the large intestines, as compared to the small intestine. The results are also depended on the dose-dependent effect of SLG-30. The pretreatment of the large intestine with SLG-30 had negligible effects on the plasma levels of glucose; however, the co-administration of insulin with SLG-30 enhanced the absorption of insulin in the large intestine. The plasma levels of calcium decreased when calcitonin was co-administered with SLG-30 in the large intestine, and the D value was about 46% for SLG-30 0.5% (v/v). It appears that the pharmacological action of calcitonin was influenced by the saturation when calcitonin was administered into the large intestine at a dose of 80 μg/kg. Therefore, a dose-response curve of calcitonin is required to confirm this assumption.

SLG-30 is a gemini type surfactant which is different from conventional absorption enhancers with better surface-active properties than the conventional surfactants with the same chain length [55]. In this study, the efficacy of SLG-30 in improving the intestinal absorption was compared with that of a conventional absorption enhancer. NaGC, a typical bile salt, was used as a conventional absorption enhancer at a concentration of 20 mM, which is equal to 1% (w/v). On one hand, it is suggested that bile acids interact with cell membranes to form a reverse micelle, which acts as a channel to increase the permeation, as previously reported [56]. On the other hand, previous reports [57] demonstrated the hydrolysis of insulin in the various mucosal homogenates of rats was inhibited by 20 mM NaGC; i.e. a protease inhibitor. As shown in Fig. 11, 20 mM NaGC enhanced the absorption of CF from the small intestines, which was much less than SLG-30 absorption enhancement effect. As shown in Table 4, the $AUC_{0\rightarrow240}$ of CF in the case of using SLG-30 0.5% (v/v) was 3.5 times greater than that in case of using 20 mM NaGC. This suggest that SLG-30 as a gemini surfactant was much better than a conventional absorption enhancer in the enhancing effect.

The absorption enhancing effect of sodium laurate was also studied as a conventional absorption enhancer and as a major component of SLG-30 molecule. From Fig. 11, it can be concluded that the effect of sodium laurate on the absorption of CF was quite small and fast, probably because the sodium laurate itself was absorbed into bloodstream due to its small molecular weight, about 288 Da, leading to decrease the concentration of sodium laurate at the site of absorption. That could explain the small value of T_{max} compared with SLG-30 case. Table 4 also shown the great difference in the $AUC_{0\rightarrow240}$ of CF between SLG-30 and sodium laurate. Therefore, SLG-30 by itself could improve the intestinal absorption of poorly absorbable drugs rather than sodium laurate, a degradation product of SLG-30. These results declared three outcomes, firstly, the superiority of SLG-30 on conventional absorption enhancer in their intestinal absorption enhancing effects. Secondly, SLG-30 was effective as a whole compound, not because of lauric

acid moieties contained in SLG-30 molecule. Finally, SLG-30 was stable in the intestines and was not degraded to its basic components.

When absorption enhancers are used in clinical practice, reversibility, local irritation and membrane toxicity of these compounds should be considered and evaluated. As shown in Fig. 12, the intestinal absorption of CF was partially enhanced by pretreatment with SLG-30 0.5% (v/v), although the co-administration with SLG-30 significantly increased the intestinal absorption of CF. These findings suggest that the absorption enhancing effect of SLG-30 was partially reversible, at least during the duration of the experiment and it might not cause the irreversible membrane toxicity in the intestines. This reversibility can also explain the lack of absorption enhancement of insulin in the large intestine, after pretreatment with SLG-30 for 2 h.

The membrane damage caused by SLG-30 was estimated by measuring the biological membrane damage markers, namely, LDH activity and the protein release from the intestinal epithelial cells. Proteins are one of the components of biological membrane and are released if the bio-membrane gets damaged. Hence, they are generally considered to be an index of membrane damage. The presence of LDH, a cytosolic enzyme, in the luminal fluid is generally regarded as an evidence of cellular membrane damage. As shown in Fig. 13A and 13B, SLG-30 did not increase the LDH activity or protein release from the rat intestinal membranes, suggesting that this adjuvant had no toxic effect on the intestinal membranes even at the highest concentration, when compared with the positive control. These outcomes were confirmed by the morphological studies. As shown in Fig. 13C, SLG-30 at various concentrations, did not cause any morphological changes, although the morphology of the intestinal membrane was changed in the positive control arm. These findings suggest that SLG-30 could increase the intestinal absorption of poorly absorbed drugs without causing serious membrane damage.

At present, the underlying mechanisms of surfactant-biological membrane interaction are not fully understood. Labeling the cell membrane with fluorescent compounds allows the tracking of membrane fluidity changes by Fluorescence polarization techniques [43,45]. Intestinal BBMVs are used to study membrane lipid fluidity in the presence of SLG-30 by measuring fluorescence intensity and calculating fluorescence anisotropy [58,59] by using DPH, tma-DPH and DNS-Cl. As shown in Fig. 14A, SLG-30 caused a little decrease in the anisotropy of DPH, while it deceased the anisotropy of DNS-Cl in dose-dependent manner, as shown in Fig. 14C. These results suggest that SLG-30 has a great effect on the protein portion of the cell membrane, with mild effect on the inner portion between the phospholipids bilayers, but not on the outside of phospholipids bilayers as indicated in Fig. 14B. These results suggest that SLG-30 might enhance the absorption through transcellular route.

Caco-2 cells monolayers were used in this study to understand the mechanism whereby SLG-30 could enhance the absorption of chemical and peptide compounds. Caco-2 monolayers are more susceptible to the cytotoxic influences of permeation enhancers than whole intestinal tissue [60,61]. For that reason I used diluted concentrations of SLG-30 i.e., 0.0001% (v/v), 0.001% (v/v), 0.025% (v/v), 0.05% (v/v) and 0.1% (v/v), and a low concentration of CF (10 µM). When SLG-30 was added at concentrations used in the *in vivo* studies, the cells were detached and badly damaged. TEER values usually decrease when the tight junctions between the adjacent cells are opened. In this study, all SLG-30 concentrations significantly decreased the TEER values, which suggest loosening in the tight junctions which led to increase the permeability of CF. The TEER values did not recover to the baseline when the SLG-30 was used at high concentrations, while in the *in vivo* absorption studies [62], it was known that the concentrations of drugs in the plasma were decreased. That might be due to the nature of Caco-2 cells which are more sensitive than the intestinal cells and have less abilities to remove the substances away from the cell surface comparing with *in vivo* intestinal cells [60,61].

In this study, the effects of conventional absorption enhancers were also compared with the effects of SLG-30 in Caco-2 cells. As shown in Fig. 15B, the effects of NaGC were consist with the results of previous report [18], and with the *in vivo* studies. NaGC at a concentration of 20 mM might also open the tight junctions in an extent lesser than SLG-30. The cumulative amounts of CF (Fig. 16) after using 20 mM NaGC was also less than that of SLG-30 at a concentration of 0.001% (v/v), and these results confirmed the superiority of SLG-30 effects. Fig. 15B also indicates that sodium laurate decreased TEER values significantly without recovering, and with a significant cumulative amount of CF (Fig. 16) even more than that of SLG-30 at a concentration of 0.1% (v/v), while in the *in vivo* studies the absorption enhancement ratio for SLG-30 was better than that of sodium laurate. This discrepancy can be partially explained by the highly toxicity effects of sodium laurate on Caco-2 cells. As shown in Fig. 17A, the amount of LDH released from Caco-2 cells were significant, and sodium laurate might cause a disintegration of Caco-2 cells monolayer, and cells membrane ruptures which led to a high permeability of CF to the basal side.

The results of toxicity studies on Caco-2 cells (Fig. 17A) also confirm the safety of SLG-30 on the intestinal epithelium. Apparently, SLG-30 might loosen the tight junctions to an extent enough for drug permeability but not enough to cause cell detachment as in the case of sodium laurate, or to cause damage to the cell membrane, as the release of LDH was normal.

Many previous reports indicated the importance of members of the claudin family, which have molecular masses of ~23 k.Da, in the function of tight junctions [10,63]. Occludin family are also important discovery as tight junction components. However, deletion of occludin does not result in disruption of the TJ barrier function [64], and whether occludin is a major constituent of TJs remains uncertain. Claudins, which have molecular weight of ~23 kDa, comprise a multigene family consisting of more than 20 members. In this

study, the expression of claudin-1 and claudin-4 was studied after the treatment with SLG-30 0.5% (v/v). The expression levels of both claudin-1 and claudin-4 were decreased (Fig. 18A), suggesting that the loosening in tight junctions might be related to the decrease in the levels of these proteins, and that SLG-30 increased the intestinal absorption of drugs via a paracellular route. The pretreatment studies indicated that the expression of these proteins started to recover, but it might be in a different speed of recovery, and that could explain the partially reversibility effects of SLG-30 [62]. It seems that the tight junctions might need more than 4 h for recovery. However, more protein involved in the tight junctions should be studied including occludin and ZO-1, and more studies are required to understand the underlying mechanism of the decrease in these proteins levels.

These results suggest that SLG-30 might enhance the absorption through not only transcellular route but also through paracellular route. Although there is no direct evidence, it is considered that the paracellular route may be more predominant because CF and FDs are generally used for markers of the paracellular route. As shown in this study, there was a logical relation between the enhancement of these markers and the concentrations of SLG-30.

Chapter II:

Enhanced oral delivery of alendronate by sucrose fatty acids esters in rats and their absorption-enhancing mechanisms

In chapter I, the ability of the absorption enhancer SLG-30 was examined in enhancing the low- and high-molecular-weight hydrophilic model drugs. In chapter II, the answer of the question of "Are absorption enhancers capable of improving the intestinal absorption of clinically used drugs?" will be provided.

Chapter II describes absorption enhancers different from SLG-30. The reason to select sucrose fatty acids esters is that these compounds are already used in food as additives and quite safe. On the other hand, SLG-30 was not used as pharmaceutical excipient for oral administration, although SLG-30 had great absorption enhancing effects.

Bisphosphonates, carbon-substituted pyrophosphate analogs, are useful in reducing the hazard of future fractures in osteoporosis patients who have already sustained a fracture due to the disease [65]. Alendronate (4-amino-1-hydroxybutylidene-1, 1-bisphosphonate trihydrate) (Fig. 19A), which belongs to a nitrogen-containing bisphosphonates, is the most used bisphosphonate for the prevention and treatment of osteoporosis, particularly in women after menopause [66,67], corticosteroid-induced osteoporosis [68], and the treatment of Paget's disease [69]. However, the oral absorption of alendronate in animals is limited under fasting conditions and negligible in the existence of food. Therefore, the oral bioavailability (BA) of alendronate is approximately 0.9% to 1.8% [70], because alendronate is highly polar and charged at physiological pH (i.e., belongs to BCSIII). Furthermore, we can reduce the side effect of alendronate in the gastrointestinal tract, because the dose of alendronate can be reduced by some absorption improving method [70].

Sucrose fatty acid esters are non-ionic surfactants that possess a sugar substituent as hydrophilic head and fatty acids as lipophilic groups and are called sugar esters. Fig. 19B shows the common structure of sucrose fatty acids esters. Since sucrose has eight hydroxyl groups, sucrose fatty acids esters ranging from sucrose mono- to octa- fatty acid esters can be synthesized. Stearic, palmitic, myristic and lauric acid can be used to obtain sucrose fatty acids esters. The type of fatty acid and the grade of esterification define the hydrophilic lipophilic balance (HLB) value and the melting point of these substances. Sucrose fatty acids esters are tasteless, odorless and biodegradable components with HLB values from 1 to 16 and are usually used in pharmaceutical industries as solubilizing agents, lubricants and emulsifiers [71]. They are therefore expected to have applications in drug delivery systems as promising absorption enhancers with low toxicity to the intestinal membrane, biocompatibility, and biodegradability properties [72].

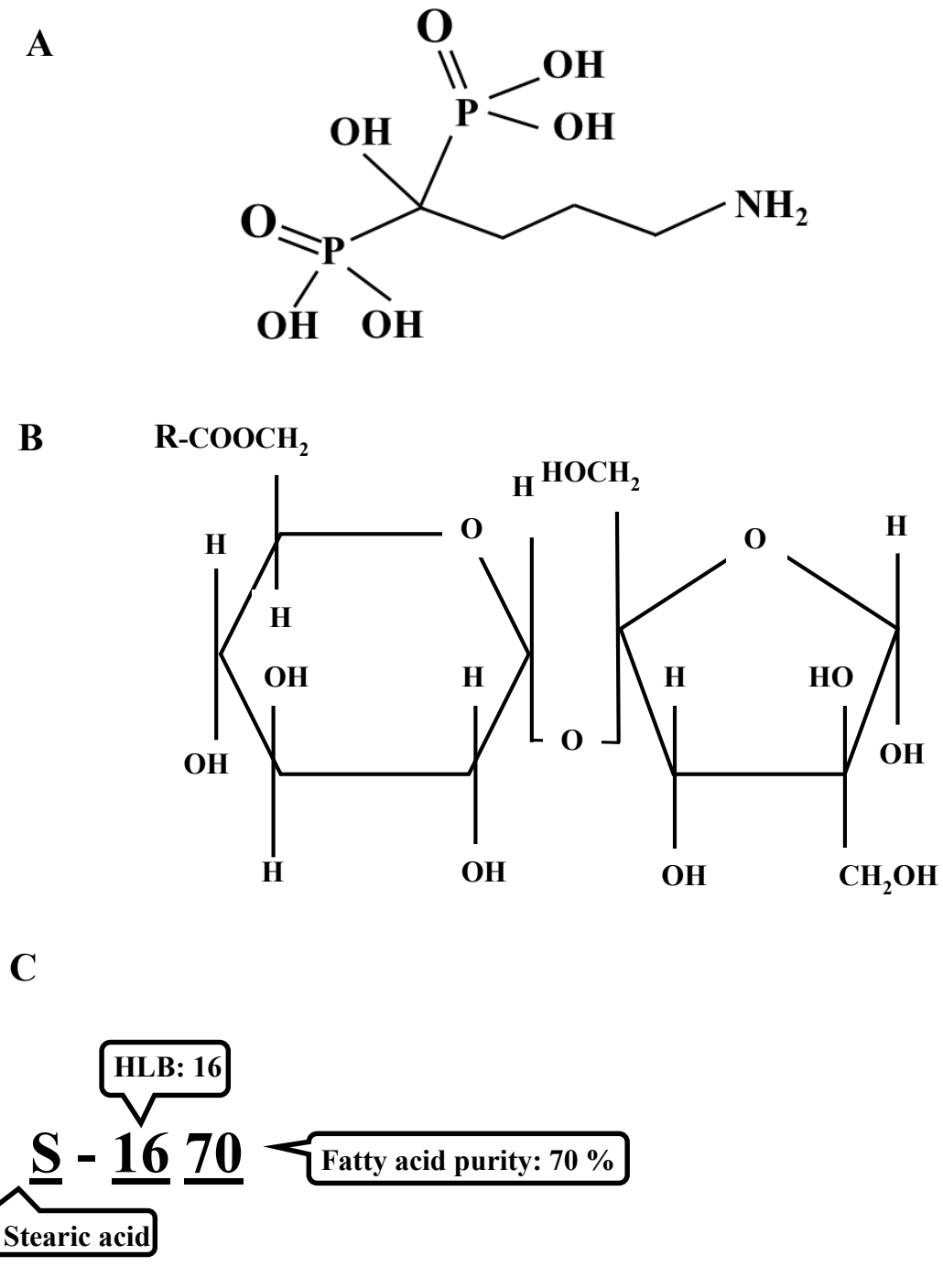


Fig. 19. (A) Chemical structure of alendronate. (B) The general chemical structure of sucrose fatty acid esters. The hydrophilic moiety of these agents is sucrose linked to the hydrophobic moiety, a fatty acid. (C) An example of sucrose fatty acid esters coding. (*Int. J. Pharm*, **515**, 476–489 (2016). Fig. 1).

1- Materials and methods

1-1- Materials

Male Wistar rats, weighing 250-300 g, were purchased from SLC, Inc. (Hamamatsu, Shizuoka, Japan). Sucrose fatty acids esters (L-1695, M-1695, P-1670, S-1670, and O-1570) were kindly provided by Mitsubishi-Kagaku Foods Corporation (Tokyo, Japan). Alendronate was purchased from Teikoku Pharma USA, Inc. (San Jose, California, USA). LDH-Cytotoxicity Test Wako and tma-DPH (1-(4-(trimethylamino)phenyl)-6-phenylhexa-1,3,5-hexatriene-p-toluenesulfonate) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). DPH (1,6-diphenyl-1,3,5-hexatriene) and Hank's balanced salt solution (HBSS) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Dansyl chloride (DNS-Cl) was purchased from Santa Cruz Biotechnology, Inc. (Texas, USA). Anti-claudin-1 and anti-β-actin (rabbit monoclonal antibodies) and goat anti-rabbit IgG HRP-linked antibodies were purchased from Cell Signaling Technology® (Danvers, MA, USA). Anti-claudin-4 (mouse monoclonal antibodies) and rabbit anti-mouse IgG HRP-linked antibodies were purchased from Invitrogen™ (Carlsbad, CA, USA). Chemi-Lumi One Ultra kit, Dulbecco's modified Eagle medium (DMEM) with 4500 mg/L glucose, nonessential amino acids (MEM–NEAA), antibiotic-antimycotic mixture stock (10,000 U/mL penicillin, 10,000 µg/mL streptomycin, and 25 µg/ml amphotericin B in 0.85% sodium chloride), and 0.25% trypsin-1mM EDTA solutions were purchased from Nacalai Tesuque (Kyoto, Japan). Human colon adenocarcinoma-derived Caco-2 cell line was purchased from Dainippon Sumitomo Pharma Co., Ltd. (Osaka, Japan). Fetal bovine serum (FBS) was purchased from Gibco® Life Technologies (Grand Island, USA). The bicinchoninic acid (BCA) protein assay kit was obtained from Pierce Biotechnology Inc. (Rockford, USA). All other reagents used in the experiments were of analytical grade.

1-2- Animal studies

Intestinal absorption of alendronate was examined by an *in situ* closed-loop method, as reported previously [18]. The experiments were carried out in accordance with the guidelines of the Animal Ethics Committee at Kyoto Pharmaceutical University. The rats were starved overnight for ~ 16 h pre-dosing, but water was freely available. After inducing anesthesia with sodium pentobarbital administered intraperitoneally at a dose of 32 mg/kg body weight, the rats were placed under a heating lamp to maintain body temperature at around 37 °C, and the intestines were exposed using a midline-abdominal incision. After the bile duct was ligated, the intestines were washed with phosphate buffered saline (PBS, pH 7.4) and the remaining buffer solution was expelled with air. Intestinal cannulation was performed at both ends using polyethylene tubing, and the distal parts of the small or large intestines were clipped by forceps. The dosing solutions were prepared in a phosphate-buffered saline (PBS, pH 7.4) at a concentration of 0.83 mg/ml. The volume of administration was 3 ml/rat in order to achieve the doses of 10 mg/kg. The dosing

solutions (3 ml for the small intestine and 1 ml for the large intestine), with or without absorption enhancers, kept at 37 °C, were directly introduced into the lumen of the intestinal loop through a cannulated opening in the proximal part of the small or large intestinal loop, which was then closed by clipping with another forceps. The jugular vein was exposed and ~0.3 ml of blood samples were collected via a direct puncture into heparinized syringes at predetermined time intervals up to 240 min. Rats were kept under anesthesia by injection of sodium pentobarbital at a dose of 5 mg/kg intraperitoneally every 2h. The samples were immediately centrifuged at 12,000 rpm (15,000 × *g*) for a period of 5 min to obtain the plasma fraction, which was stored on ice for further analysis. The concentrations of alendronate in these plasma samples were determined by reverse-phase high-performance liquid chromatography (RP-HPLC), as reported previously [73,74]. The RP-HPLC system consisted of a (LC-10AS; Shimadzu, Kyoto, Japan) pump equipped with a fluorescence (RF-10AXL; Shimadzu, Kyoto, Japan) detector. The excitation and emission wavelength were 395 nm and 480 nm, respectively. The mobile phase was composed of an aqueous solution of 1 mM disodium EDTA and methanol at a ratio of 97:3 (v/v) and adjusted to pH 6.5. The mobile phase was injected in a separation column (4.6 mm × 150 mm inside diameter) packed with 5C18-PAQ (Nacalai Tesque, Kyoto, Japan) at a flow rate of 1 mL/min and temperature of 40 °C. The samples were injected at volume of 50 µl.

The peak drug concentrations (C_{max}) and the time to reach the peak drug concentrations (T_{max}) in plasma were directly determined from the plasma concentration–time profiles. The area under the curve (AUC) was calculated by the trapezoidal method, from pre-dose (time zero) to the final sample. The absorption enhancement ratios of the drugs, with or without sucrose fatty acid esters, were calculated as follows:

Absorption enhancement ratio = $AUC_{\text{with enhancer}} / AUC_{\text{control without enhancer}}$

1-3- Estimation of intestinal membrane damage

To estimate the damage of small intestinal membrane in the presence of sucrose fatty acids esters, the LDH activity and the protein release from the small intestinal membrane were measured by an *in situ* closed-loop method, as reported previously [40,41]. Various concentrations of sucrose fatty acids esters were administered into the small intestinal loop, as described above, and the results were compared to the results obtained with 3.0% Triton X-100, which was used as a positive control. Four hours post-administration, the rat small intestine was washed thrice with 10 ml PBS. The PBS solution was thereafter, centrifuged for 7 min at 200 × *g* at a temperature of 4 °C. One ml of the supernatant was used for the determination of the LDH activity and protein release from the small intestinal membrane. LDH activity was determined using the LDH-Cytotoxicity Test Wako kit and the protein release was measured using a BCA Protein Assay Kit, with bovine serum albumin as the standard.

1-4- Morphological observations

L-1695, S-1670, and P-1670 at a concentration of 0.5% (w/v), 20 mM sodium glycocholate (NaGC), or 3% Triton X-100 (as a positive control) were administered into the small intestinal loop in a similar manner to that used for the absorption experiments. Rats were left for 4 h after administration and at the end of the experiments, the entire small intestine was removed and cut into three segments (proximal, middle, and distal) of equal lengths. A 5-cm long piece from the middle (which is expected to comprise the jejunum) was dissected, rinsed in ice-cold PBS (pH 7.4), and fixed in 4% buffered formaldehyde. The intestinal segments were paraffin embedded, conventionally sectioned, and stained with hematoxylin-eosin (H&E). Then, these stained segments were examined by light microscopy (BZ-8000 Fluorescence Microscope, KEYENCE Corp., Osaka, Japan).

1-5- Preparation of brush border membrane vesicles (BBMVs)

BBMVs were prepared by the method reported previously [42–44] with a slight modification. Briefly, an *in situ* small intestinal loop was prepared in each rat, as mentioned above, after which PBS washing solution (pH 7.4) was administered into the loop, and the fat was trimmed off the small intestine and mesentery. Then, the whole small intestine was soaked with ice-cold PBS (pH 7.4). The small intestine was divided into 10-cm segments. Mucosa was scraped out with a slide glass from each of those segments and used for subsequent experiments. BBMVs were prepared by the divalent cation precipitation method using $MgCl_2$ in the presence of ethylenebis (oxyethylenenitrilo) tetraacetic acid (EGTA) [42–44]. Briefly, the collected mucosa was homogenized in a buffer (containing mannitol 300 mM, EGTA 5 mM, Tris [pH 7.4] 12 mM) using a tissue homogenizer. An aqueous solution of 10 mM magnesium chloride was added to the homogenate. The homogenate was centrifuged at 3000 × *g* for 15 min. The supernatant was then centrifuged at 32,000 × *g* for 30 min. The pellet was re-suspended in a buffer (containing mannitol 300 mM, EGTA 5 mM, Tris [pH 7.4] 12 mM) by using a 26G needle. The protein concentration was determined by the BCA method using bovine serum albumin as a standard, and the final concentration was adjusted to 1 mg/ml in each Eppendorf tube. The samples were frozen in liquid nitrogen and maintained at -80 °C for further studies.

1-6- Measurement of membrane fluidity by fluorescence polarization

BBM vesicles (100 μg protein) were incubated with 1 μM DPH, 0.5 μM tma-DPH, or 5 μM DNS-Cl in HEPES-Tris buffer (HEPES 25 mM, KCl 5.4 mM, $CaCl_2$ 1.8 mM, $MgSO_4$ 0.8 mM, NaCl 140 mM, glucose 5 mM, pH 7.4 modified by 1 M Tris) in the dark at 37 °C for 30 min [43,45]. Then, various concentrations (0.25% w/v, 0.5% w/v and 1.0% w/v) of sucrose fatty acid esters (L-1695, M-1695, P-1670, S-1670, and O-1570) were added. The samples were then incubated in the dark for 1 min at 37 °C. For the control group,

the same procedures were carried out with the addition of HEPES-Tris buffer only. The fluorescence intensities and the steady state polarization of fluorescence expressed as the fluorescence anisotropy, r, of the labeled membrane vesicles were measured at 37 °C. The excitation and emission wavelengths used during the measurements were λ_{ex} = 360 nm, λ_{em} = 430 nm for DPH and tma-DPH, and λ_{ex} = 380 nm, λ_{em} = 480 nm for DNS-Cl. The measurements were carried out using a Hitachi Spectrofluorometer (F-2000 Spectrofluorometer, Hitachi Seisakusho Corp, Yokohama, Japan) equipped with a polarizer set.
Fluorescence anisotropy (r) was calculated using the following equation:

$$r = \frac{I_V - I_H}{I_V + 2I_H}$$

where I_V and I_H represent the fluorescence intensities perpendicular and parallel, respectively, to the polarized excitation plane [46].

1-7- Measurement of TEER and the transport of CF using Caco-2 cell monolayers

Caco-2 cells (passage 49) were cultured in 175-cm^2 culture flasks (Thermo Fisher Scientific™, Massachusetts, USA). The culture medium consisted of DMEM containing 10% FBS, 0.1 mM MEM–NEAA, 2 mM glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin [47]. Cells were cultured in a humidified atmosphere of 5% CO_2 at 37 °C. The culture medium was changed every two days. When the cultured Caco-2 cells became sub-confluent, Caco-2 cells were seeded onto 12 mm Transwell® with 0.4 µm Pore Polycarbonate Membrane Insert (Corning Inc., New York, USA) at a density of 1×10^5 cells/insert. The transepithelial transport studies were performed when the transepithelial electrical resistance (TEER) values were more than 500 Ω/cm^2 (i.e., after 21 days) [48]. Briefly, after removing the incubation medium by aspiration, the apical and basal sides were washed thrice using Hank's balanced salt (HBSS) solution (pH 7.4). The cells were incubated with HBSS (pH 6.0) at the apical side and HBSS (pH 7.4) at the basal side for 20 min at 37 °C. HBSS supplemented with 1 mg/ml glucose was used in all cell experiments. After removing the washing solution by aspiration, 500 µl of 500 µM alendronate in HBSS (pH 6.0) solution with or without sucrose fatty acid esters were added to the apical side at the zero-time point, whereas precisely 1500 µl of HBSS (pH 7.4) were added to the basal side at 37 °C. The cells were kept at 37 °C and 5% CO_2 and were continuously agitated on a shaker during the transport experiments.

TEER values were measured using a Millicell® (ERS-2 Volt-Ohm Meter, Massachusetts, USA) system at predetermined times up to 24 h, and the initial values were considered as 100%. Samples from the basal side were withdrawn at predetermined times up to 24 h. The samples were replaced with an equal volume of HBSS (pH 7.4). Alendronate was determined by HPLC, as previously mentioned. The apparent permeability coefficients P_{app} (cm/s) for the transported alendronate was determined by the following equation: $P_{app} = \frac{dX_R}{dt} \cdot \frac{1}{A} \cdot \frac{1}{C_0}$, where P_{app} is the apparent permeability coefficient in centimeters per second,

X_R is the amount of the drugs in moles in the receptor side, $\frac{dX_R}{dt}$ is the flux across the monolayer, A is the diffusion area in square centimeters, and C_0 is the initial concentration of drugs in the donor side in moles per milliliter.

1-8- Western Blotting

Western blotting was evaluated by the methods described previously with slight modification [51,52]. Three male Wistar rats weighing 250 g were treated to enable the use of an *in situ* closed-loop method, as mentioned above, on the small intestine. A control study, a treatment study, and a pre-treatment study were conducted on the first, second, and third rat, respectively. For the control study, only PBS solution (pH 7.4) was administered into the small intestine and then the rat was sacrificed, while for the treatment study, L-1695 was administered, without washing it out, for 1 h, and then the rat was sacrificed. The pretreatment study was performed by administrating L-1695 for 1 h and then washing it out with PBS solution (pH 7.4), after which the rat was sacrificed after 4 h. The small intestine (60 cm) of three rats was taken and treated, as mentioned above, for the extraction of the brush border membrane vesicles. The total protein amount of each sample was adjusted to 30 μg.

The protein expression levels of the claudin family in homogenate of the small intestine membrane were evaluated by western blotting. Briefly, equal amounts of protein samples (30 μg protein) were mixed with SDS buffer solution and separated onto 15% SDS-polyacrylamide gels electrophoretically. The separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking in 5% skim milk in Tris-buffered saline (pH 7.4) for 1 h at room temperature, the PVDF membrane was incubated overnight in blocking buffer with diluted (1:1000) monoclonal antibodies for claudin-1, claudin-4, and β-actin at 4 °C. Subsequently, the PVDF membrane was washed three times using Tris-buffered saline containing 0.05% Tween-20 (TTBS), followed by incubation with peroxidase-conjugated anti-rabbit IgG antibody for claudin-1 and β-actin, and with peroxidase-conjugated anti-rabbit IgG antibody for claudin-4 for 1 h at room temperature. The signals were visualized by luminescence imaging (Fujifilm Luminescent Image Analyzer LAS4000 System, Tokyo, Japan). The intensity of each signal was corrected using the values obtained from the β-actin bands, and the relative protein intensity was expressed as fold-change of the content in the control group.

1-9- Statistical analyses

Results are expressed as the mean ± S.E. of at least three experiments. Statistical significances between groups were analyzed using Dennett's test; differences were considered significant at $P < 0.05$. Significance levels are denoted (**) $p < 0.01$, (*) $p < 0.05$ and (n.s.) not significantly different. The number of

experiments is indicated by n.

2- Results

2-1- The effects of sucrose fatty acid esters on the intestinal absorption of alendronate

The effect of two concentrations (i.e., 0.5% w/v and 1.0% w/v) of varied types of sucrose fatty acid esters (i.e., L-1695, M-1695, P-1670, S1670 and O-1570) on the intestinal absorption of alendronate was evaluated using an *in situ* closed-loop method. As shown in Fig. 20, all sucrose fatty acid esters significantly enhanced the intestinal absorption of alendronate at all the concentrations studied in a concentration-dependent pattern. All sucrose fatty acid esters showed a higher rate of alendronate absorption at concentrations higher than 0.5% (w/v), and the best absorption-enhancing effect was observed in the presence of L-1695 1.0% (w/v), with AUC $_{0\rightarrow240}$ being 4.7 (** $p < 0.01$) times higher than with the control, as shown in Table 5. Additionally, L-1695 at both concentrations showed a similar C_{max} (Table 5). Therefore, L-1695 was considered as the most effective sucrose fatty acid ester for enhancing the intestinal alendronate absorption and was selected for further studies.

Table 5 Summary of the pharmacokinetic parameters of alendronate after its co-administration with sucrose fatty acid esters at concentrations of 0.5% (w/v) and 1.0% (w/v) into rat small intestines by an *in situ* closed-loop method.

	Conc. (w/v)	C_{max} (ng/mL)	T_{max} (min)	$AUC_{0\rightarrow240}$ (ng · min/mL)	Enhancement Ratio
Control		———	———	**740 ± 58**	———
+ L-1695	**0.5 %**	**2830 ± 262**	**25 ± 5**	**2920 ± 288 ***	**3.9**
	1.0 %	**2500 ± 122**	**30 ± 0**	**3460 ± 454 ****	**4.7**
+ M-1695	**0.5 %**	**1570 ± 140**	**30 ± 0**	**1790 ± 172**	**2.3**
	1.0 %	**3260 ± 492**	**30 ± 0**	**3050 ± 377 ***	**4.1**
+ P-1670	**0.5 %**	**701 ± 320**	**50 ± 0**	**1400 ± 619**	**1.9**
	1.0 %	**2380 ± 503**	**30 ± 0**	**3120 ± 756 ***	**4.2**
+ S-1670	**0.5 %**	**1150 ± 229**	**40 ± 10**	**1610 ± 241**	**2.2**
	1.0 %	**1990 ± 425**	**60 ± 0**	**3020 ± 883 ***	**4.1**
+ O-1570	**0.5 %**	**1940 ± 496**	**60 ± 0**	**2940 ± 382**	**2.4**
	1.0 %	**1510 ± 283**	**40 ± 10**	**2040 ± 962 ***	**4.0**

The results are expressed as the mean ± S.E. (n = 3). * $p < 0.05$, when compared with the control. ** $p < 0.01$, when compared with the control. (*Int. J. Pharm*, **515**, 476–489 (2016). Table 1).

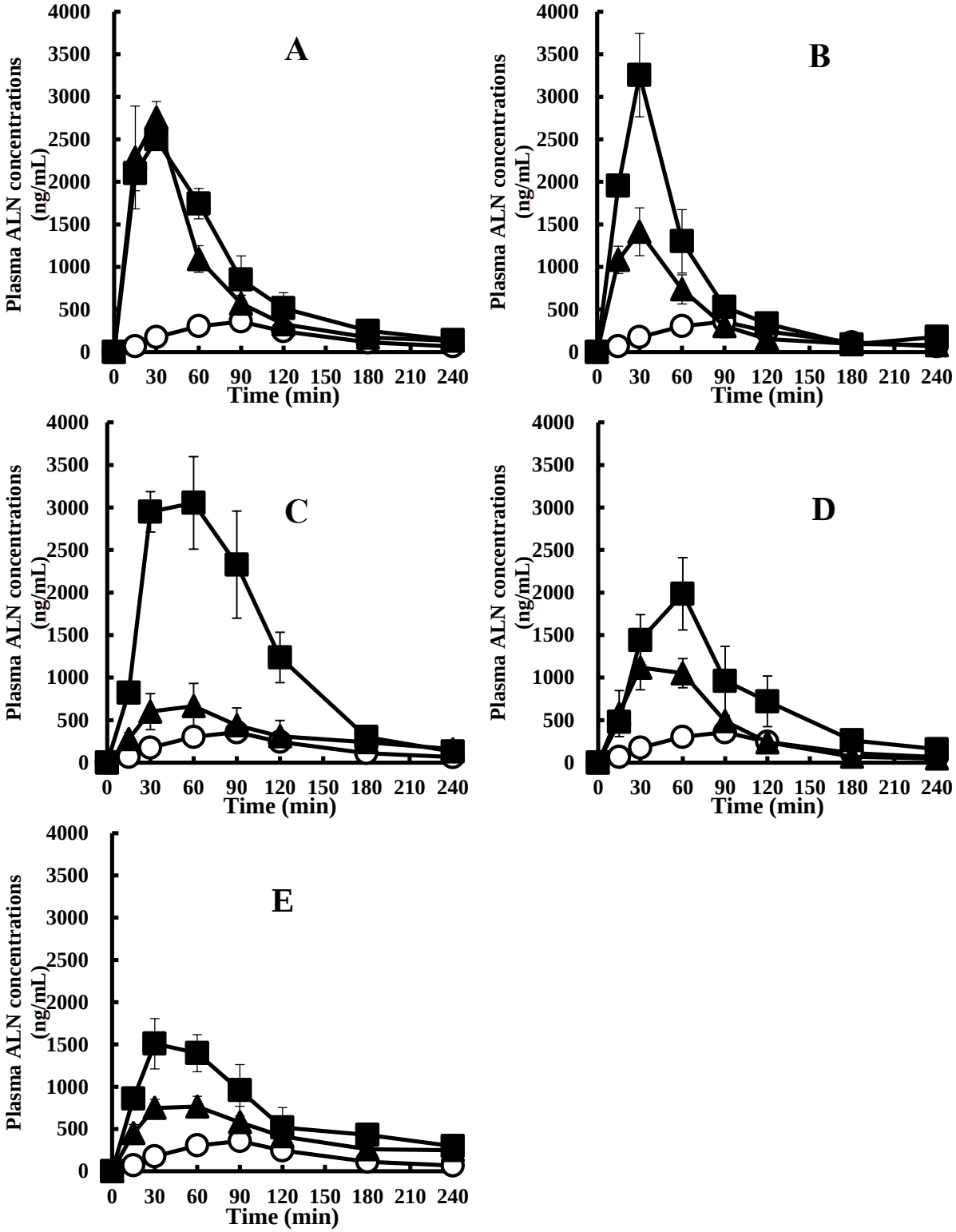


Fig. 20. Effects of various sucrose fatty acid esters (A) L-1695, (B) M-1695, (C) P-1695, (D) S-1670 and (E) O-1570 on the absorption of alendronate (10 mg/kg) from the rat small intestine evaluated using an *in situ* closed-loop method. The results are expressed as the mean ± S.E. (n = 3). Keys: (◯) Control, (▲) sucrose fatty acid esters at a concentration of 0.5% (w/v), (■) sucrose fatty acid esters at a concentration of 1.0% (w/v). (*Int. J. Pharm*, **515**, 476–489 (2016). Fig. 2).

2-2- Effects of different concentrations of the sucrose fatty acid ester L-1695 on the intestinal absorption of alendronate

L-1695 with various concentrations (i.e., 0.5% w/v, 1% w/v, 2% w/v, 3% w/v and 5% w/v) was co-administered with alendronate into rat small intestine using *in situ* closed-loop method. Fig. 21 shows that the intestinal absorption of alendronate was significantly improved by treatment with all L-1695 concentrations in a dose-dependent manner. The absorption enhancement ratio of alendronate in the presence of L-1695 5% (w/v) was 11.2 (** $p < 0.01$) times higher than for the control group with a C_{max} of 5470 ng/ml at T_{max} of 20 min (Table 6). It can be also seen in Table 6 that the T_{max} values of all L-1695 concentrations ranged between 15 to 30 min, after which the plasma concentrations of alendronate started to linearly decrease.

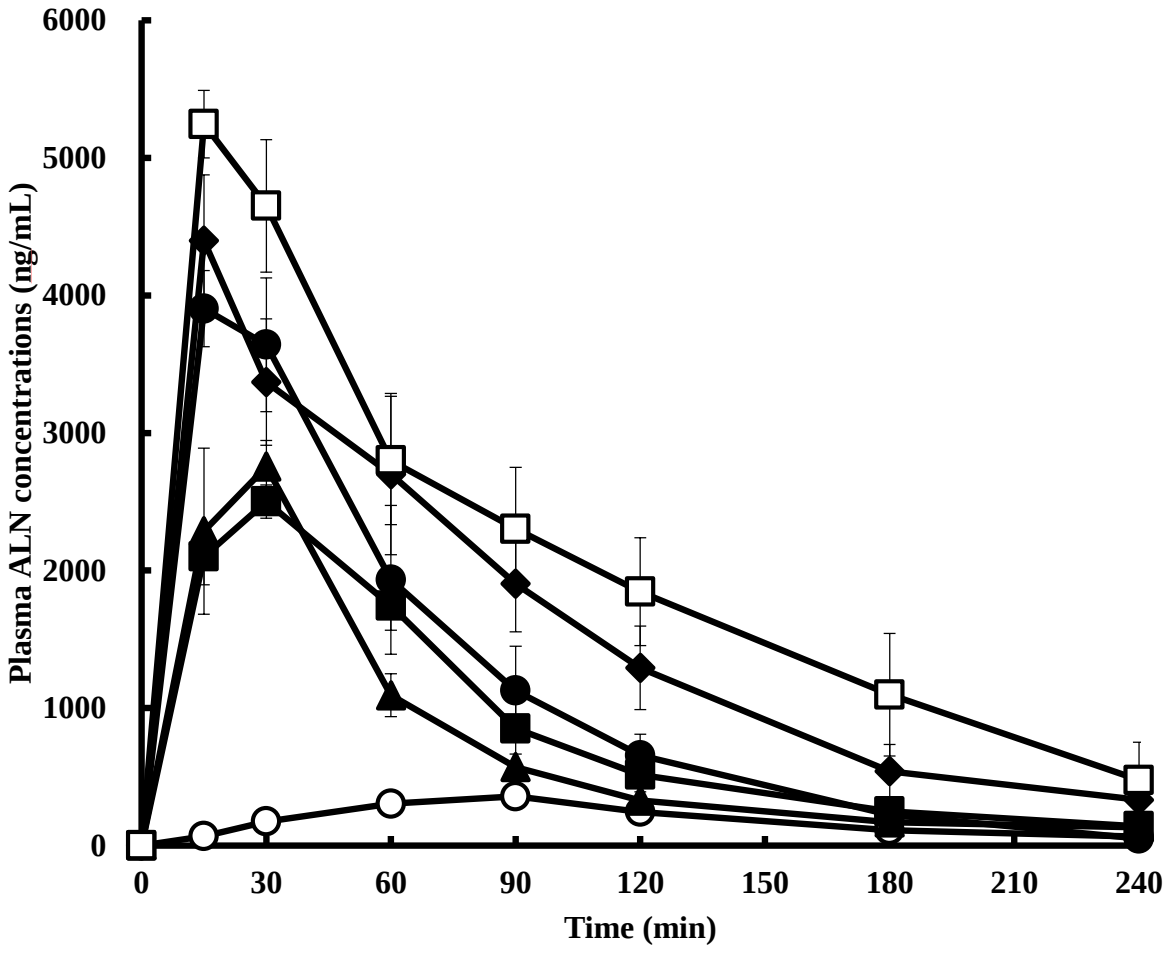


Fig. 21. Effects of various concentration of the sucrose fatty acid ester L-1695 on the absorption of alendronate (10 mg/kg) from the rat small intestine evaluated using an *in situ* closed-loop method. The results are expressed as the mean ± S.E. (n = 3). Keys: (◯) Control, (▲) L-1695 0.5% (w/v), (■) L-1695 1.0% (w/v), (●) L-1695 2.0% (w/v), (◆) L-1695 3.0% (w/v), and (□) L-1695 5.0% (w/v). (*Int. J. Pharm,* **515**, 476–489 (2016). Fig. 3).

Table 6 Summary of the pharmacokinetic parameters of alendronate after its co-administration with various concentrations of the sucrose fatty acid ester L-1695 into rat small intestines by an *in situ* closed-loop method.

	Conc. (w/v)	C_{max} (ng/mL)	T_{max} (min)	$AUC_{0\rightarrow240}$ (ng · min/mL)	Enhancement Ratio
Control		———	———	740 ± 58	———
+ L-1695	0.5 %	2830 ± 262	25 ± 5	2920 ± 288 *	3.9
	1.0 %	2500 ± 122	30 ± 0	3460 ± 454 **	4.7
	2.0 %	3960 ± 78	26 ± 4	4610 ± 345 **	6.2
	3.0 %	4400 ± 414	15 ± 0	6340 ± 957 **	7.6
	5.0 %	5470 ± 205	20 ± 5	8330 ± 1460 **	11.2

The results are expressed as the mean ± S.E. (n = 3). * $p < 0.05$, when compared with the control. ** $p < 0.01$, when compared with the control. (*Int. J. Pharm*, **515**, 476–489 (2016). Table 2).

2-3- Regional differences in the effects of the sucrose fatty acid ester L-1695 on the intestinal absorption of alendronate in small and large intestines

To study the regional differences in the effects of L-1695 on the small and large intestines, L-1695 2.0% (w/v) was co-administered with alendronate into small and large intestines in separated study groups (n = 3) by *in situ* closed-loop method. Fig. 22 shows that L-1695 2.0% (w/v) enhanced the absorption of alendronate in both the small and large intestines with $AUC_{0\rightarrow240}$ being 6.2 (** $p < 0.01$) times and 5.1 (** $p < 0.01$) times higher than the control group, respectively, as shown in Table 7.

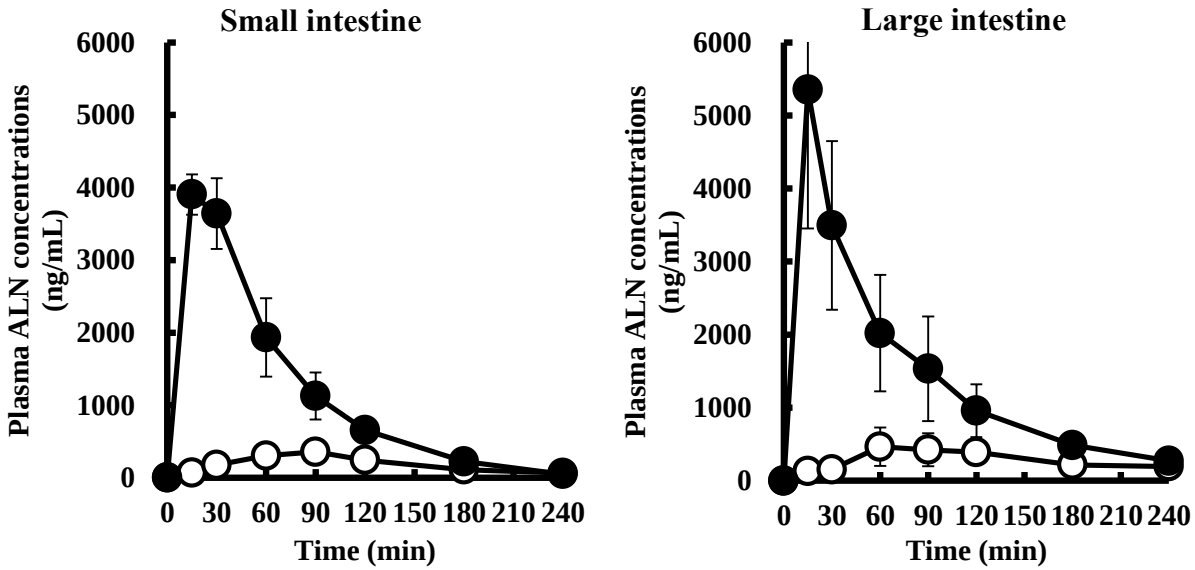


Fig. 22. Regional difference between (A) the small intestine and (B) the large intestine when alendronate (10 mg/kg) was co-administrated with the sucrose fatty acid ester L-1695 2.0% (w/v) into the rat small and large intestines, evaluated using an *in situ* closed-loop method. The results are expressed as the mean ± S.E. (n = 3). Keys: (◯) Control and (●) L-1695 2.0% (w/v). (*Int. J. Pharm*, **515**, 476–489 (2016). Fig. 4).

Table 7 Summary of the pharmacokinetic parameters of alendronate after its co-administration with the sucrose fatty acid ester L-1695 at a concentration of 2.0% (w/v) into rat small and large intestines by an *in situ* closed-loop method.

	C_{max} (ng/mL)	T_{max} (min)	$AUC_{0\rightarrow240}$ (ng · min/mL)	Enhancement Ratio
Small intestine				
Control	———	———	**740 ± 58**	———
L-1695 2.0 % (w/v)	**3959 ± 78**	**26 ± 4**	**4611 ± 345 ****	**6.2**
Large intestine				
Control	———	———	**1140 ± 427**	———
L-1695 2.0 % (w/v)	**5353 ± 20**	**15 ± 0**	**5762 ± 2101 ****	**5.1**

The results are expressed as the mean ± S.E. (n = 3). * $p < 0.05$, when compared with the control. ** $p < 0.01$, when compared with the control. (*Int. J. Pharm*, **515**, 476–489 (2016). Table 3).

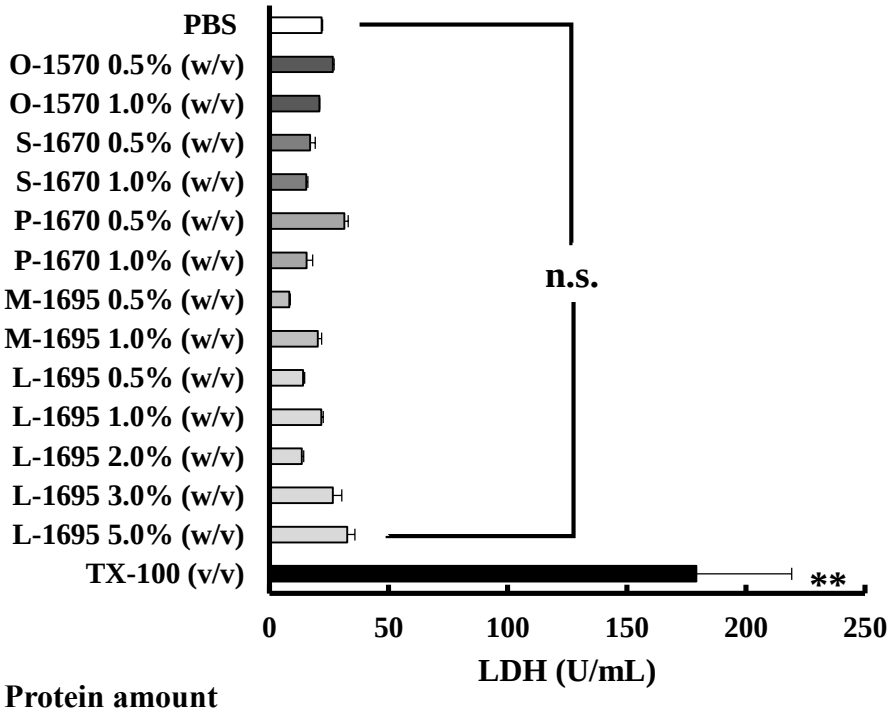
2-4- Estimation of membrane damage

To evaluate the extent of small intestinal membrane injury in the presence of the sucrose fatty acid esters at both concentrations 0.5% (w/v) and 1.0% (w/v), the LDH activity and the protein release from the small intestinal membrane were measured using an *in situ* closed-loop method. Apart from L-1695 at concentrations of 2.0% (w/v), 3.0% (w/v), and 5.0% (w/v) and P-1670 at a concentration of 1.0% (w/v), almost no significant increase, either in the LDH activity or protein release, was found with respect to the positive control, Triton X-100 3% (v/v) and to the PBS only control, as shown in Fig. 23A and B. These findings suggest that sucrose fatty acid esters at concentrations equal to or lower than 1.0% (w/v) might not cause serious damage to the small intestinal membrane.

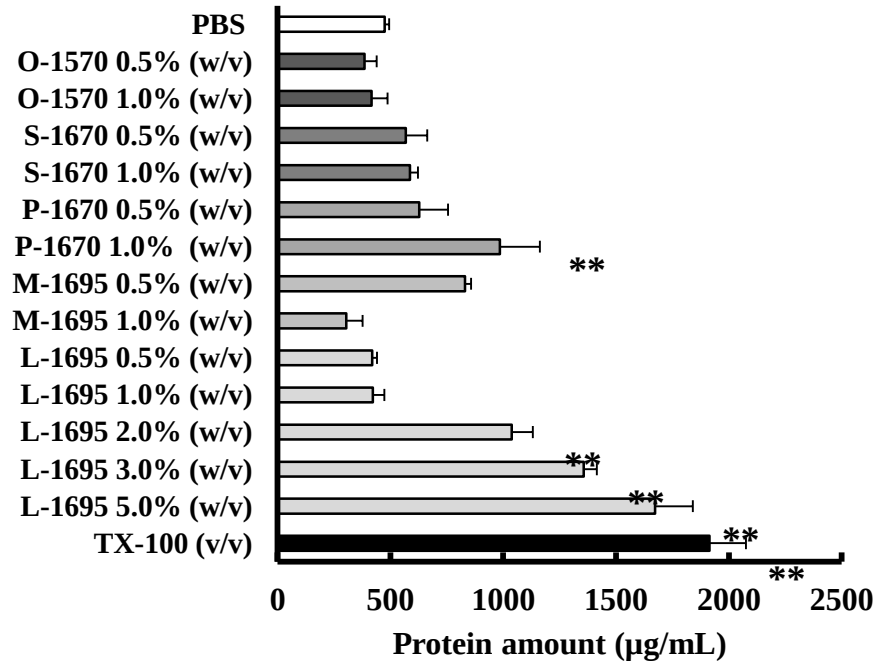
2-5- Morphological observations

The intestinal membrane toxicity of these sucrose fatty acid esters was also evaluated by morphological observations. As shown in Fig. 23C, no morphological changes were observed in the presence of sucrose fatty acid esters L-1695 0.5% (w/v), S-1670 0.5% (w/v), or P-1670 0.5% (w/v) when compared with control in the non-treated jejunum and the conventional absorption enhancer sodium glycocholate (20 mM) and Triton X-100 3% (v/v) as a positive control. These morphological studies confirm the safety of these sucrose fatty acid esters on the intestinal membrane at concentrations of 0.5% (w/v).

A. LDH



B. Protein amount



C. Morphological observations

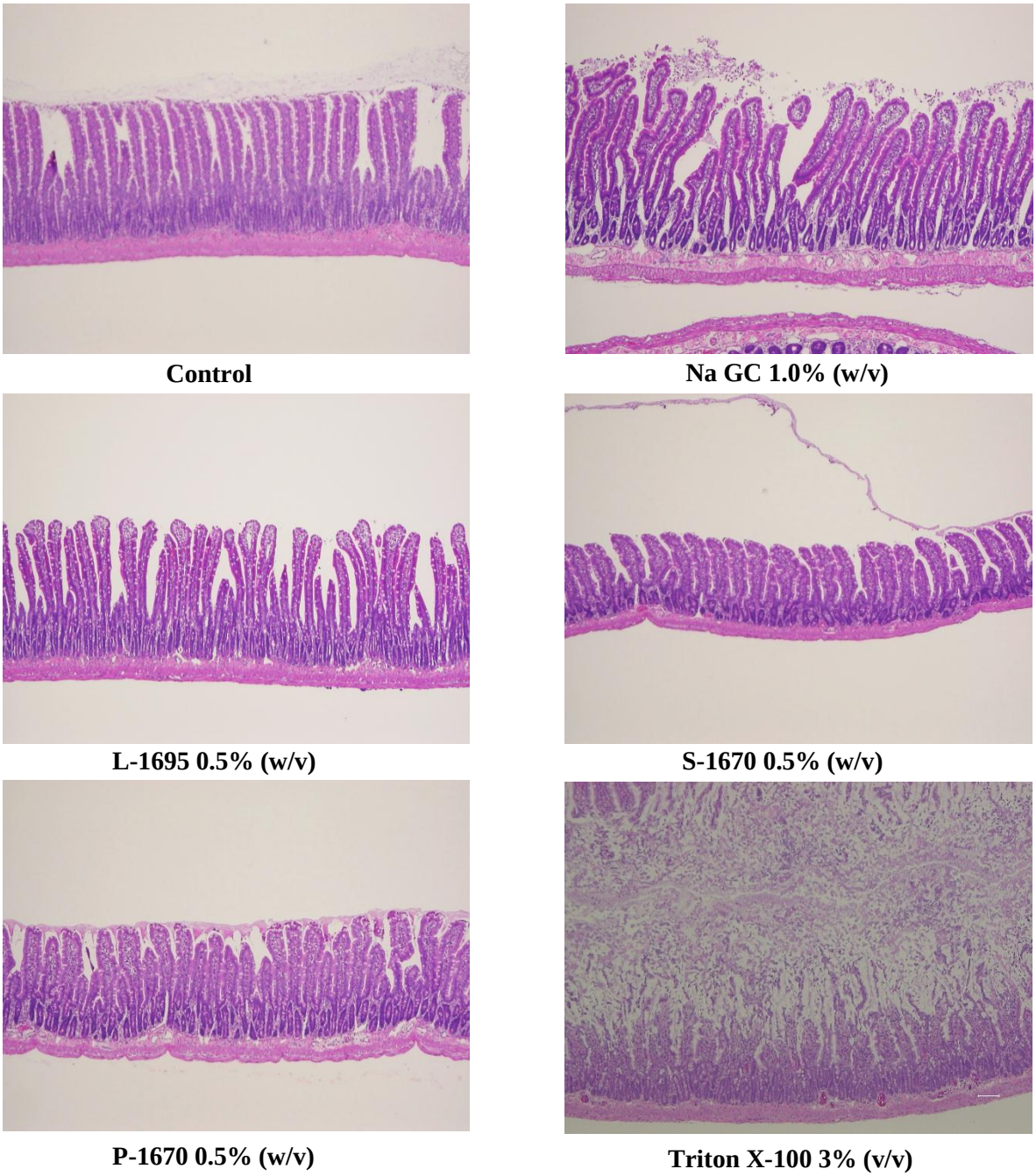
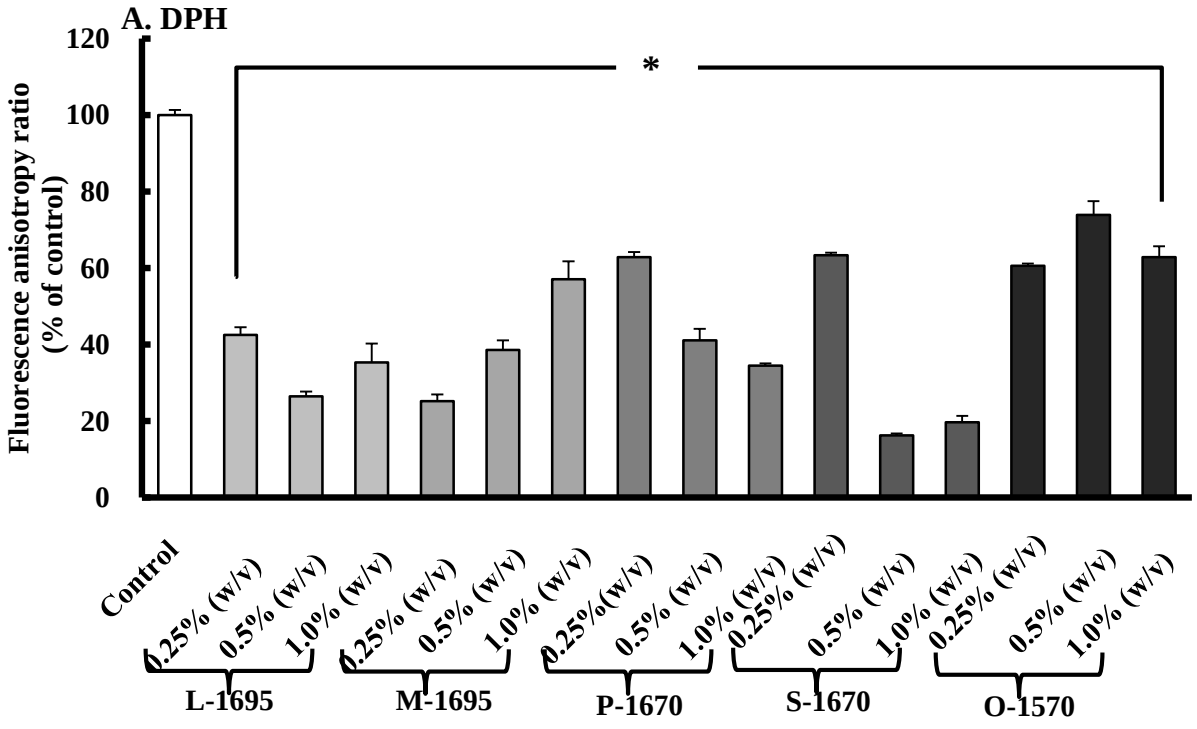


Fig. 23. Evaluation of rat small intestinal membrane damage in the presence of various sucrose fatty acid esters at multiple concentrations 0.5% (w/v), 1.0% (w/v), 2.0% (w/v), 3.0% (w/v), and 5.0% (w/v) for L-1695 and two concentrations of 0.5% (w/v) and 1.0% (w/v) for the other sucrose fatty acid esters. The membrane damage was determined by measuring (A) the activity of LDH, (B) the amount of protein released from the intestinal membranes, and (C) the morphological observations of the rat jejunum. The results are expressed as the mean ± S.E. (n = 6). ** $p < 0.01$, n.s. not significantly different when compared with the control. (*Int. J. Pharm*, **515**, 476–489 (2016). Fig. 5).

2-6- Effects of sucrose fatty acid esters on the membrane fluidity of rat small intestines

Alterations in the membrane fluidity of the intestinal epithelium cells was measured by using DPH, tma-DPH, and DNS-Cl, which can bind and label specific parts of the membrane bilayers. As shown in Fig. 24A, the sucrose fatty acid esters (L-1695, M-1695, P-1670, S-1670 and O-1570) at concentrations of 0.25% (w/v), 0.5% (w/v), and 1.0% (w/v) significantly reduced in the fluorescence anisotropy of DPH compared to the control group. The reduction in the anisotropy of DPH was related to the concentration of sucrose fatty acid esters. When tma-DPH was used as a marker, as shown in Fig. 24B, L-1695 at a concentration of 0.5% (w/v) significantly reduced the fluorescence anisotropy of tma-DPH, but this effect was not observed at a concentration of 1.0% (w/v), while at a concentration of 0.25% (w/v), the anisotropy was slightly reduced. M-1695 at concentrations of 0.25% (w/v), and 0.5% (w/v) reduced the fluorescence anisotropy of tma-DPH significantly in a concentration-dependent manner, but not at a concentration of 1.0% (w/v). P-1670 at concentrations of 0.5% (w/v) and 1.0% (w/v) also significantly reduced the fluorescence anisotropy of tma-DPH in a concentration-dependent manner. S-1670 at concentrations of 0.25% (w/v), 0.5% (w/v), and 1.0% (w/v) significantly reduced the fluorescence anisotropy of tma-DPH in a concentration-dependent manner. However, O-1570 at all concentrations showed no effect on the fluorescence anisotropy of tma-DPH. All studied sucrose fatty acid esters at all studied concentrations did not reduce the fluorescence anisotropy of DNS-Cl with respect to that of the control group; on the contrary, some sucrose fatty acid esters increased the fluorescence anisotropy of DNS-Cl (Fig. 24C).



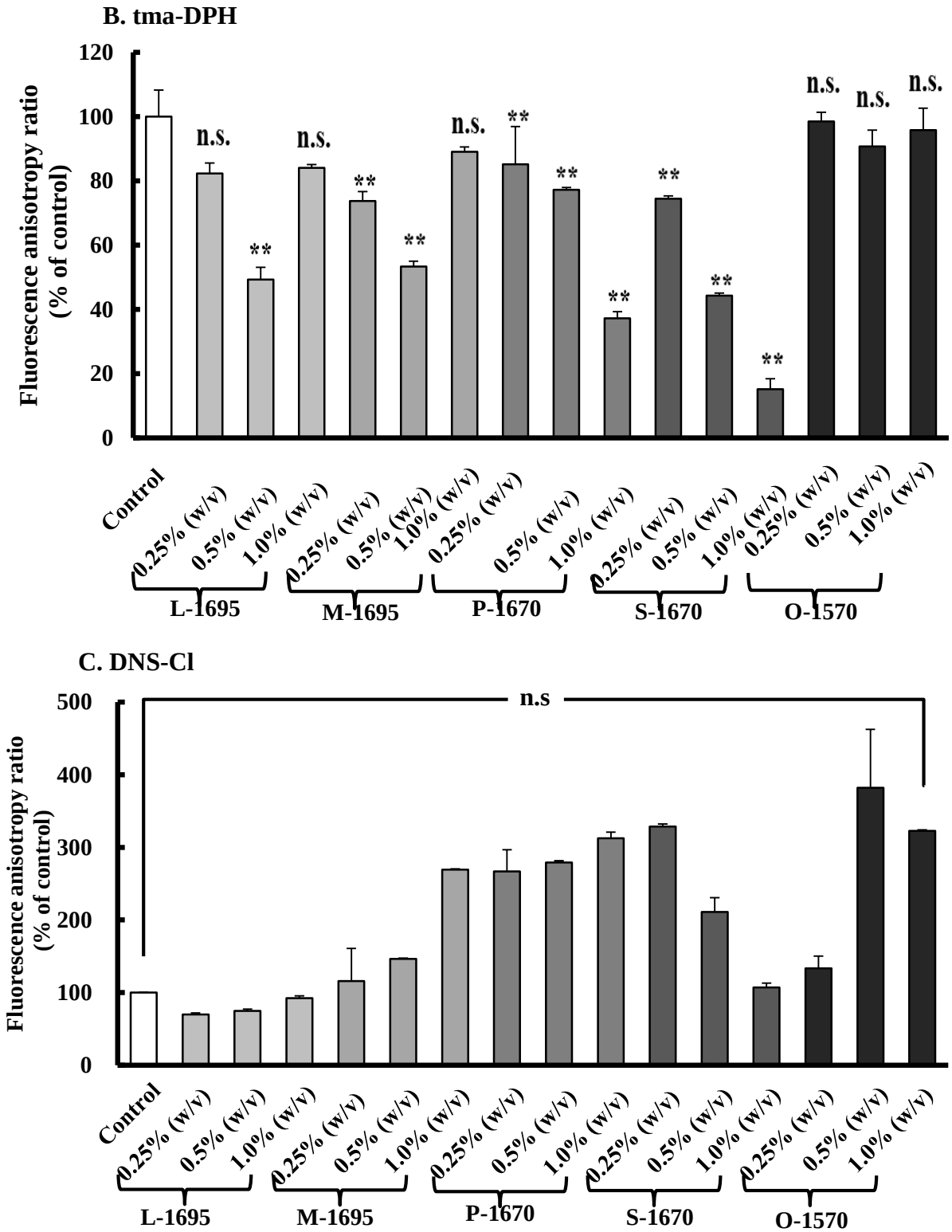
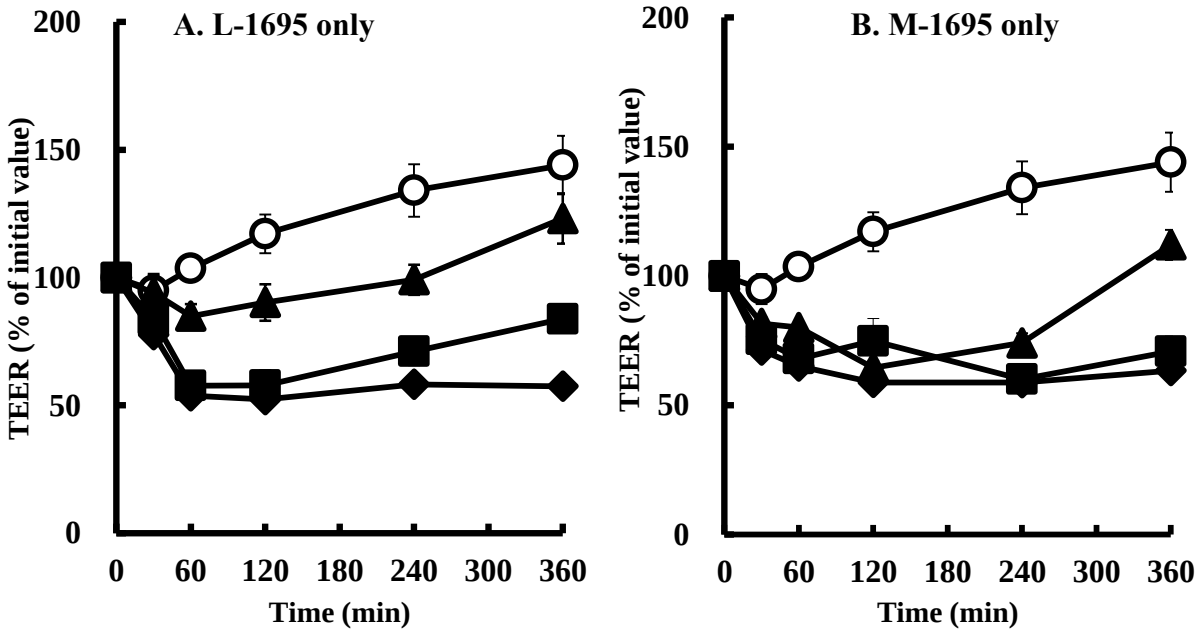


Fig. 24. Effects of various sucrose fatty acid esters on the fluorescence intensity of DPH, tma-DPH, and DNS-Cl. All samples contained DPH, tma-DPH, and DNS-Cl at a final concentration of 1 μM, 0.5 μM and 0.5 μM, respectively. The results are expressed as the mean ± S.E. (n = 4). ** $p < 0.01$, n.s. not significantly different when compared with the control. (*Int. J. Pharm*, **515**, 476–489 (2016). Fig. 6).

2-7- Effect of sucrose fatty acid esters on TEER values and the transport of alendronate in Caco-2 cell monolayers

Sucrose fatty acid esters at concentrations of 0.001% (w/v), 0.025% (w/v), and 0.05% (w/v) without alendronate were added to the apical side at 37 °C. As shown in Fig. 25, a significant decrease in the TEER values (represented as a percentage of the initial value) was observed in the presence of all sucrose fatty acid esters at all concentrations. Sucrose fatty acid esters at a concentration of 0.001% (w/v) decreased TEER values at the beginning with a quick recovery to the baseline. Sucrose fatty acid esters at concentrations of 0.025% (w/v) decreased TEER values to an extent higher than that observed at a concentration of 0.001% (w/v) and less than that observed at a concentration of 0.05% (w/v) with a little recovery to the baseline, while at a concentration of 0.05% (w/v), TEER values decreased to approximately 50% of the initial value without recovering to the baseline during the experiment. Fig. 25G shows the permeability of alendronate after 24 h of experiment in Caco-2 cells, at a concentration 0.05% (w/v) of sucrose fatty acid esters. All sucrose fatty acid esters at a concentration of 0.05% (w/v) increased the transport of alendronate, as the P_{app} values of alendronate in the presence of sucrose fatty acid esters were 10 times higher than with the control, and the highest value was for SE O-1570 with about 14 times higher than control. TEER values recovered to the baseline at the end of the experiment (i.e., after 24 h) after removing sucrose fatty acid esters and alendronate from the apical side at time 360 min, as shown in Fig. 25F.



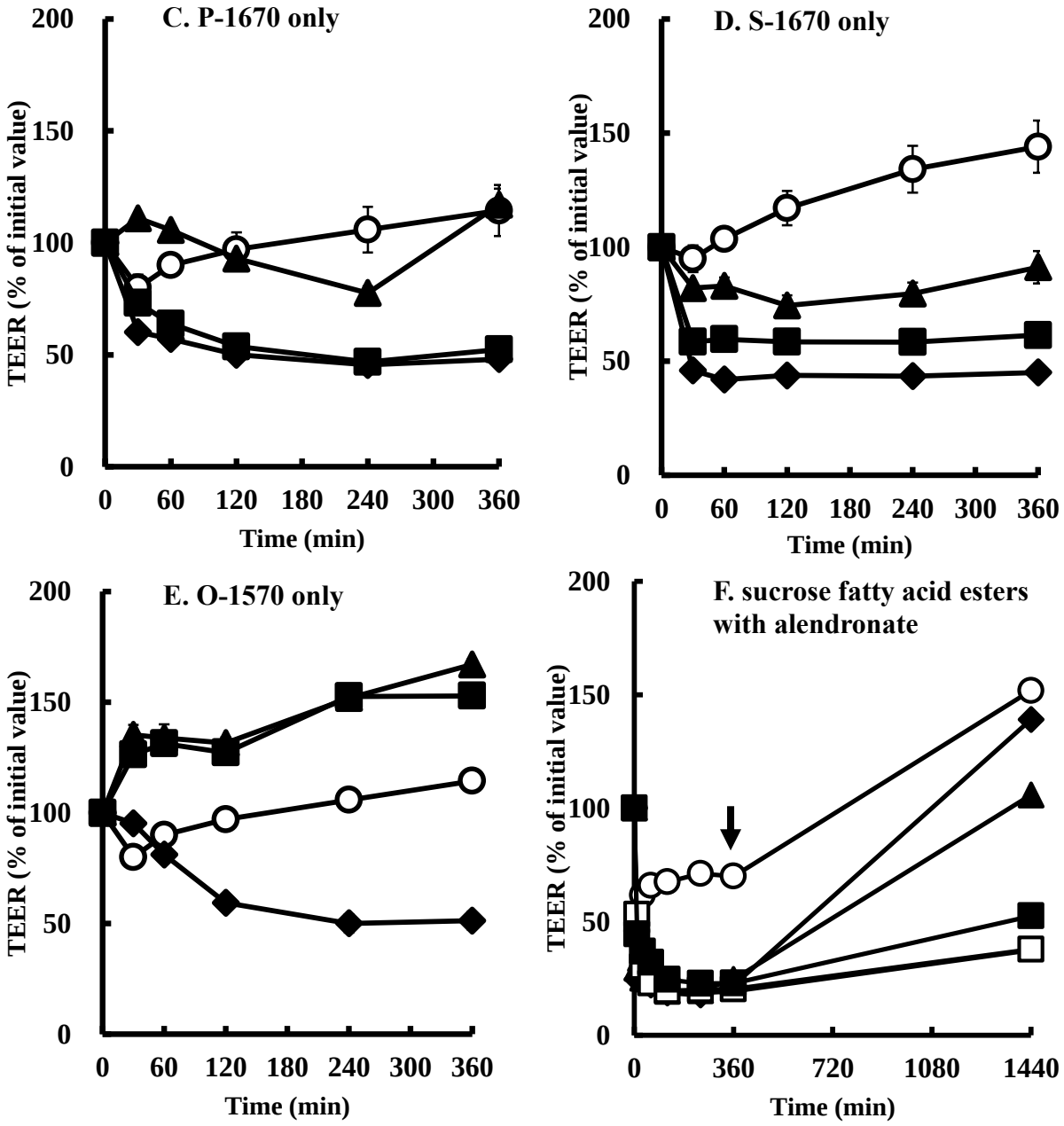


Fig. 25. Effects of various sucrose fatty acid esters on the permeation of alendronate through Caco-2 cell monolayers. (A) to (F) are the measurements of transepithelial electrical resistance (TEER % of initial value) with or without sucrose fatty acid esters. (A) L-1695 only, (B) M-1695 only, (C) P-1695 only, (D) S-1670 only, and (E) O-1570 only. Keys: (○) Control, (▲) 0.001% (w/v), (■) 0.025% (w/v) and (◆) 0.05% (w/v). (F) Transepithelial electrical resistance (TEER % of initial value) with or without sucrose fatty acid esters in the presence of alendronate (500 μM). The black arrow refers to the time point when alendronate and sucrose fatty acid esters were washed out. Keys: (○) Control, (◆) L-1695 0.05% (w/v), (▲) M-1695 0.05% (w/v), (△) P-1670 0.05% (w/v), (■) S-1670 0.05% (w/v), and (□) O-1570 0.05% (w/v). (G) The permeability of alendronate with or without sucrose fatty acid esters 0.05% (w/v) across Caco-2 cell monolayers, expressed as P_{app}. The results are expressed as the mean ± S.E. (n = 4). ** $p < 0.01$, n.s. not significantly different when compared with the control. (*Int. J. Pharm*, **515**, 476–489 (2016). Fig. 7).

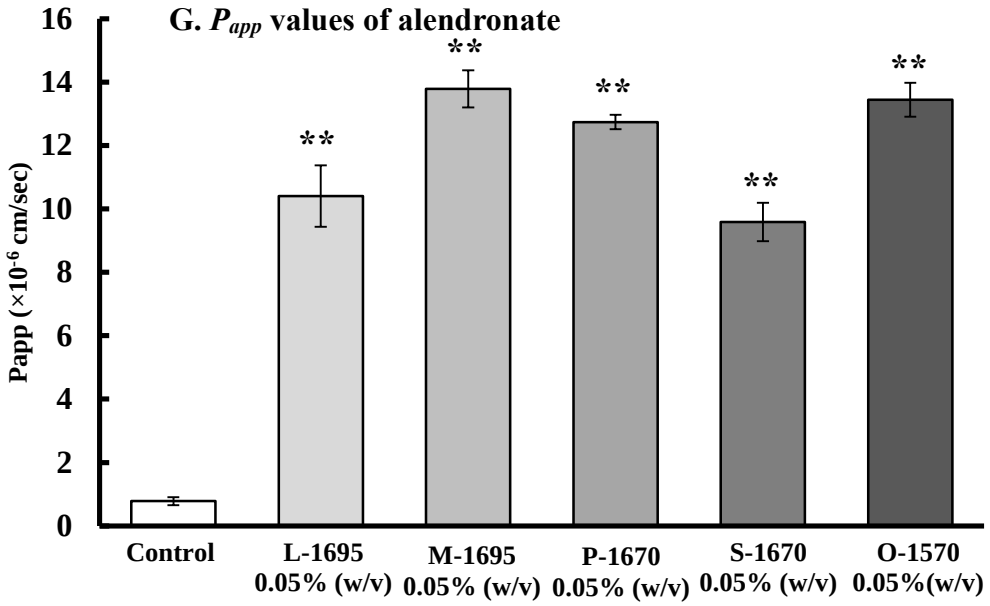
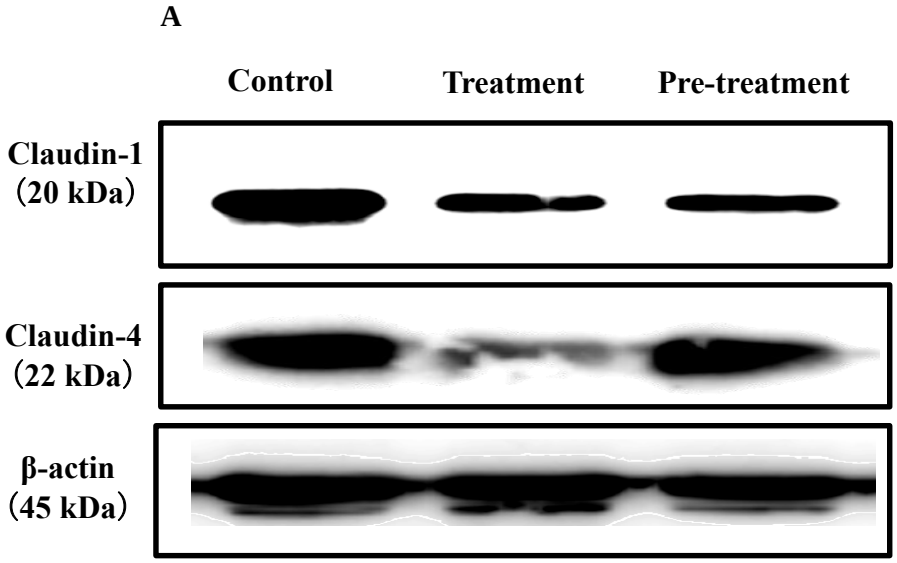


Fig. 25. (Continued)

2-8- Western blotting

One possible mechanism for the effect of sucrose fatty acid esters on intestinal absorption is the enhancement of intestinal membrane permeability through the paracellular pathway by loosening the tight junctions. Fig. 26A shows the western blotting bands for the decreased levels of claudin-1 and claudin-4, and that pre-treatment with L-1695 2.0% (w/v) led to a decrease in the intensity of claudin-1 and claudin-4, respectively.



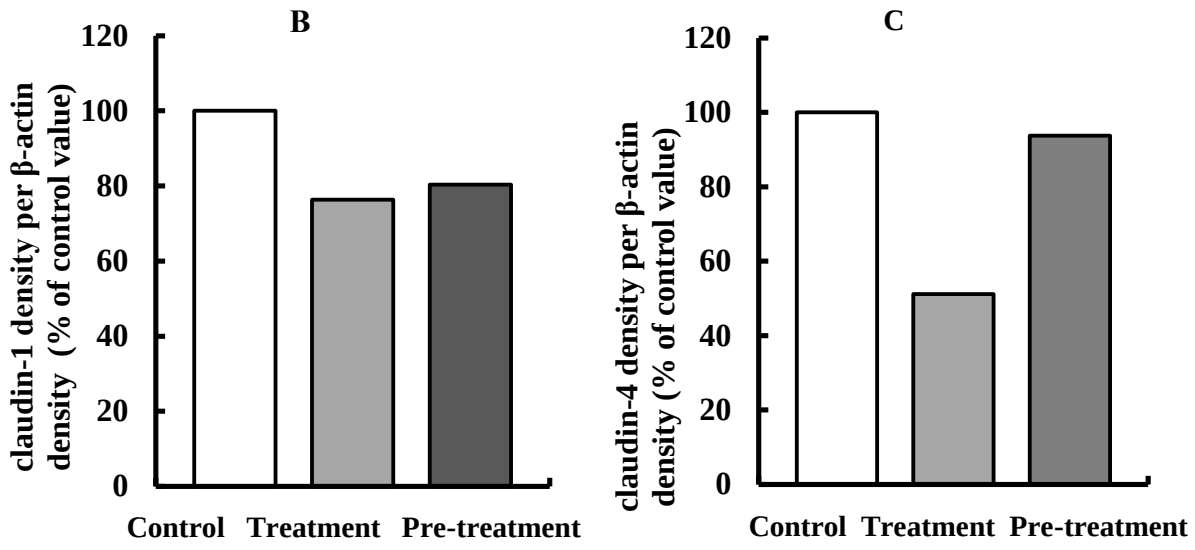


Fig. 26. Expression levels of claudin-1 and claudin-4 in the rat small intestine after treatment with L-1695 2.0% (w/v) for 1 h. Control; without treatment, treatment; treatment with L-1695 2.0% (w/v) for 1 h, pre-treatment; pre-treatment for 1 h. Proteins were extracted after 4 h. (*Int. J. Pharm*, **515**, 476–489 (2016). Fig. 8).

3- Discussion

In spite of the fact that researchers are keen to find other routes to deliver alendronate, e.g., parenteral injections [75], transdermal [76], and intrapulmonary delivery systems [77], the oral route still the most preferred by patients and pharmaceutical companies. It is the easiest and cheapest pharmaceutical form to formulate and produce with a long shelf life.

In the present study, the effects of sucrose fatty acid esters (L-1695, M-1695, P-1670, S-1670, and O-1570) on the intestinal absorption of a bisphosphonate drug, alendronate were examined. The possible mechanisms that sucrose fatty acid esters may affect the intestinal membrane to exert their actions were also studied.

Each agent of the sucrose fatty acid esters is coded with a symbol composed of a letter and a four-digit number. The letter refers to the fatty acid type in the structure (i.e., L = lauric; C12, M = myristic; C14, P = palmitic; C16, S = stearic; C18 and O = oleic; C18:1), whereas the first two-digits of the number refers to the HLB value and the last digit refers to the purity percentage of fatty acids, as shown in Fig. 19.

It was found that all sucrose fatty acid esters enhance the intestinal absorption of alendronate in a dose-dependent manner, as shown in Fig. 20. Table 5 shows the pharmacokinetic parameters of alendronate after its co-administration with sucrose fatty acid esters into rat small intestines by an *in situ* closed-loop method. Depending on the $AUC_{0\rightarrow240}$ values of alendronate, the C_{max} values and the corresponding T_{max} values, the rank order of the effectiveness of absorption enhancers in the small intestine is as follows; L-1695 > M-

1695 > P-1670 > S-1670 > O-1570, as shown in Table 5. It is not fully understood why there are differences among sucrose fatty acid esters in the effectiveness in enhancing the intestinal absorption of alendronate, but it seems that effectiveness is somehow related to the length of the carbon chain of fatty acids. It is well known that the medium-chain fatty acids are very effective in causing turbulence in the intestinal membrane and the tight junctions (TJs) located between the intestinal epithelial cells [78]. Regarding its best effect (** $p < 0.01$) in the preliminary studies, L-1695 was chosen among all the sucrose fatty acid esters to conduct additional studies in order to understand more about sucrose fatty acid esters actions and mechanisms. Fig. 21 and Table 6 shows that L-1695 enhanced the intestinal absorption of alendronate in a concentration-dependent manner, with the highest effect obtained at concentration of 5.0% (w/v) (** $p < 0.01$). Table 6 also shows that the enhancement effects of L-1695 at both concentrations 0.5% (w/v) and 1.0 % (w/v) were similar. It is possible that the lower concentration 0.5% (w/v) was enough to loosen the TJs to a certain extent to increase the absorption of alendronate via a paracellular route without increasing the toxicity effects of this action. The 1.0% (w/v) concentration may loosen the TJs to a certain extent more than with the concentration 0.5% (w/v) and increase the absorption of alendronate more without toxicity effects. The concentrations 2.0% (w/v), 3.0% (w/v), and 5.0% (w/v), however, may loosen the TJs to a certain extent enough to rupture the cells integrity, thus increasing the absorption of alendronate and increasing the membrane toxicity, as shown in Fig. 21 and Fig. 23.

T_{max} values of all concentrations of sucrose fatty acid esters ranged between 15 min to 30 min, as shown in Table 6. That is plausible because a part of the sucrose fatty acid ester L-1695 itself might be absorbed through the paracellular pathway, since it is a hydrophilic agent with a molecular weight less than 500 Da (~ 430 Da). Previously, it was reported that when fatty acids are used as absorption enhancers, they could be absorbed from the intestine even more quickly than the drug itself [61].

I selected the 2.0% (w/v) concentration of L-1695 for studying the regional differences between the small and large intestine and for conducting western blotting experiments. Although L-1695 2.0% (w/v) showed some toxic effects, as shown in Fig. 23B, the toxic effect was lower than that with L-1695 3% (w/v) and 5% (w/v). Besides, it did not show any increase in LDH activity, as shown in Fig. 23A. Moreover, the absorption enhancement of alendronate from small intestines with L-1695 at a concentration of 2.0% (w/v) was more pronounced than that in the control group, with values 6.2 (** $p < 0.01$) times higher, and also more pronounced than that observed with the 1.0% (w/v) concentration group, with values 4.7 (** $p < 0.01$) times higher.

Regional differences were observed, using *in situ* absorption studies, in the enhancement by L-1695 at a concentration of 2.0% (w/v) on the intestinal absorption of alendronate. However, this regional difference was not very pronounced, as shown in Fig. 22. Besides, alendronate itself is better absorbed from the large intestine than from the small intestine. L-1695 2.0% (w/v) significantly enhances the absorption of

alendronate from small and large intestine. Numata and co-workers [79] reported no regional difference in the absorption-enhancing effect of NOC7 on the absorption of FD4 by the *in situ* loop method. They found that FD4 absorption was significantly augmented after co-administration with NOC7 in all the regions of intestine (jejunum, colon, and rectum). Moreover, it is previously reported that NO donors improved the intestinal absorption of insulin and [$Asu^{1,7}$]-eel calcitonin from all the intestinal regions [33]. Therefore, the present findings are consistent with the previous results of absorption enhancers applied in the different intestinal regions and indicate that the absorption enhancing effect of sucrose fatty acid ester L-1695 is still significant in the large intestine as well as the small intestine when compared with those of conventional absorption enhancers.

However, further experiments are needed for studying different doses of ALN in the presence of sucrose fatty acid esters, in order to figure out the relationship between the dose of ALN and the concentration of sucrose fatty acid esters, i.e., the best ratio between ALN and sucrose fatty acid esters.

When absorption enhancers are used in clinical practice, local irritation and membrane toxicity of these enhancers should be considered and evaluated. The membrane injury caused by sucrose fatty acid esters was estimated by measuring the biological membrane damage markers, namely, LDH activity and protein release from the intestinal epithelial cells. The presence of LDH, a cytosolic enzyme, in the luminal fluid is commonly considered as evidence of cellular membrane damage. Proteins are one of the components of biological membranes and are released if the bio-membrane gets damaged. Hence, they are generally considered to be an index of membrane damage. As shown in Fig. 23A, no treatment with sucrose fatty acid ester led to increased LDH activity. However, as shown in Fig. 23B, L-1695 at concentrations of 2.0% (w/v), 3.0% (w/v), and 5.0% (w/v) and P-1670 at a concentration of 1.0% (w/v) increased the protein release from the rat intestinal membranes. L-1695, M-1695, P-1670, S-1670, and O-1570 at concentrations of 0.5% (w/v) and 1.0% (w/v) did not increase protein release from the rat intestinal membranes, suggesting that these absorption enhancers are not toxic to the intestinal membranes at concentrations equal to or lower than 1.0% (w/v) when compared with the positive control. These outcomes were confirmed by morphological studies. As shown in Fig. 23C, L-1695, S-1670, and P-1670, at a concentration of 0.5% (w/v), did not cause any morphological changes, although the morphology of the intestinal membrane was changed in the positive control group. These observations were also comparable to the conventional absorption enhancer NaGC 20 mM (~ 1% w/v) with a molecular weight of 487.60 Da. As shown in chapter I, SLG-30 could enhance the intestinal absorption of poorly absorbed drugs without causing serious membrane damage when SLG-30 was used at concentrations equal to or lower than 1.0% (v/v) [62]. Bearing in mind that SLG-30 also possess two lauric chains in the structure as shown in Fig. 6, these findings are consist with the observations of SLG-30 and suggest that sucrose fatty acid esters, especially L-1695 at concentrations lower or equal to 1.0% (w/v), could achieve the required balance between the intestinal

absorption of alendronate and the intestinal membrane safety. These findings are also consistent with the previous findings of Lindmark and colleagues [80]. They reported that epithelial cells exposed to C12, which is a very effective absorption enhancer, exhibited no clear morphological changes even at a concentration that enhanced the transport of [^{14}C] mannitol up to 100-fold. Such similarities with conventional enhancers suggest superior potency of sucrose fatty acid esters as intestinal absorption enhancers for alendronate.

Fluorescence polarization techniques [43,45] were applied to identify the effects of sucrose fatty acid esters on small intestinal membrane fluidity using common fluorescent probes,1,6-diphenyl-1,3,5-hexatriene (DPH), 1-(4-(trimethylamino)phenyl)-6-phenylhexa-1,3,5-hexatriene-p-toluenesulfonate (tma-DPH), and dansyl chloride (DNS-Cl). Intestinal BBMVs are used to study membrane lipid fluidity in the existence of sucrose fatty acid esters by measuring fluorescence intensity and calculating fluorescence anisotropy [58,59]. On the contrary, cholesterol increased the rigidity of membranes and the fluorescein anisotropy of DPH, tma-DPH, and DNS-Cl [45]. Sucrose fatty acid esters decreased fluorescein anisotropy of those markers and increased the membrane fluidity. Fig. 24A shows that sucrose fatty acid esters decreased fluorescein anisotropy of DPH (** $p < 0.01$), which suggests that sucrose fatty acid esters have an effect on the inner portion between the phospholipids bilayers of the membrane. This effect and the variations of effects among sucrose fatty acid esters at different concentrations are still not fully understood. Fig. 24B shows that sucrose fatty acid esters M-1695, P-1670, and S-1670 decreased fluorescein anisotropy of tma-DPH (** $p < 0.01$), suggesting that those sucrose fatty acid esters have an effect on the extracellular faces of the phospholipids bilayers of the phospholipids bilayers of the membrane. This effect and the variations of effects among sucrose fatty acid esters at different concentrations are also still not fully understood. Sucrose fatty acid esters did not decrease the fluorescein anisotropy of DNS-Cl, as shown in Fig.24C, suggesting that sucrose fatty acid esters have no effect on the protein portion of the bio-membrane. In summary, sucrose fatty acid esters affected the membrane by increasing membrane fluidity, suggesting that sucrose fatty acid esters might enhance the intestinal absorption of alendronate via a transcellular pathway. However, more studies are required to understand these phenomena.

Caco-2 cells monolayers were used in this study to understand the mechanism behind the enhancement effects of sucrose fatty acid esters. Caco-2 monolayers are often more susceptible to the cytotoxic influences of permeation enhancers than whole intestinal tissue [60,61]. For that reason, diluted concentrations of sucrose fatty acid esters i.e., 0.001% (w/v), 0.025% (w/v), and 0.05% (w/v) and a low concentration of alendronate (500 μM) were used, because when sucrose fatty acid esters were added at concentrations used in the *in vivo* studies, the cells were detached and badly damaged. In our study, two features of Caco-2 cells, which are the transepithelial electrical resistance (TEER) and the partition coefficient P_{app} by calculating the cumulative amount of alendronate in the basal side, were measured. Fig. 25 shows the TEER values (as

a percentage of the initial value at time zero before adding the solution of sucrose fatty acid esters and/or alendronate, i.e., TEER values were higher than 500 Ω/cm^2). Sucrose fatty acid esters alone decreased the TEER values in a concentration-dependent manner, suggesting the loosening of TJs between the adjacent cells. When the experiments were done in the presence of alendronate, the sucrose fatty acid esters at a concentration of 0.05% (w/v), which corresponds to the lowest effective concentration in the *in vivo* studies in improving the intestinal absorption of alendronate, also decreased TEER values significantly when compared to the control group (alendronate only) as shown in Fig. 25. Additionally, they increased the P_{app} of alendronate with values at least 10 (** $p < 0.01$) times higher than with the control, as shown in Fig. 25G, suggesting that sucrose fatty acid esters might increase the intestinal absorption of alendronate via a paracellular pathway. Fig. 25F also shows that when sucrose fatty acid esters were removed from the apical side by aspirating and washing, TEER values started to recover to the base-line, especially in the case of L-1695, in which TEERs value completely recovered.

Many previous reports indicated the importance of members of the claudin family, which have molecular masses of ~23 k.Da, in the function of tight junctions [10]. It is interesting that the localization and barrier function of each member of the claudin varies among tissues. In this study, claudin-1 and claudin-4 were chosen to study their expression levels after the treatment with L-1695 2.0% (w/v). Fig. 26 shows that the expression levels of both claudin-1 and claudin-4 were decreased, suggesting that the loosening in TJs observed in Caco-2 cells experiment might be related to the decrease in the levels of these proteins and confirms that L-1695 and sucrose fatty acid esters increased the intestinal absorption of alendronate via a paracellular route. The pretreatment study indicated that the expression of these proteins started to recover, but it might be in a different speed of recovery, and that could partially explain the recovering in TEER values to the base line. These findings suggest that the absorption enhancing effect of L-1695 is reversible. However, more proteins are involved in tight junctions, such as occluding family and ZO-1, and more studies are required. It is also suggested that sucrose fatty acid esters might re-distribute and re-localize claudin-1 and claudin-4 inside the cytoplasm. Further studies are required to understand the underlying mechanism of the decrease in these proteins levels.

Conclusions

Chapter I shows that the gemini surfactant, SLG-30, significantly improved the intestinal absorption of low- and high-molecular-weight hydrophilic molecules, in a concentration-dependent manner. The intestinal absorption of macromolecules such as peptides, including insulin and calcitonin, was enhanced in the presence of SLG-30, especially from the large intestine. Moreover, the morphological observations indicated the safety of SLG-30 on the intestinal membranes. *In vivo* and *in vitro* comparing studies showed that gemini surfactant, SLG-30, was superior to the conventional absorption enhancers including NaGC and sodium laurate in improving the intestinal absorption. Furthermore, It also suggested the stability of SLG-30 in the intestines and its effect might be related to the whole compound. *In vitro* evaluation of membrane damage results confirmed the safety of SLG-30 on the enteric membrane. In addition, *In vitro* mechanistic studies suggested that SLG-30 might improve the absorption through the transcelullar pathway through changing the membrane fluidity. SLG-30 might also affect the tight junctions by decreasing the expression of claudin-1 and claudin-4, thus disrupting and loosening the tight junctions and increasing the permeability through the paracellular pathway.

In chapter II, sucrose fatty acid esters, especially L-1695, significantly improved the intestinal absorption of a poorly absorbed hydrophilic drug, alendronate, in a concentration-dependent manner. Moreover, the toxicity observations indicated the safety of sucrose fatty acid esters at concentrations equal to or lower than 1.0% (w/v) on the intestinal membranes. In addition, mechanistic studies indicated that sucrose fatty acid esters might enhance the intestinal absorption of drugs through paracellular and transcellular pathways. Additional mechanistic and pharmacokinetic studies are required to understand the detailed mechanisms responsible for the improved intestinal absorption of drugs in the presence of SLG-30 and sucrose fatty acid esters, such as studying the expression levels of other TJs proteins (i.e., occuldins and ZO-1).

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