



## Absorption study of deoxycholic acid-heparin conjugate as a new form of oral anti-coagulant

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### Abstract

The oral delivery of macromolecules is a topic of much interest as this would undoubtedly improve patient acceptance and compliance with chronic regimens. Heparin and insulin are perhaps among the first candidates that should be considered for oral macromolecule delivery systems. Heparin is the most potent anti-coagulant known for the prevention of deep vein thrombosis and pulmonary embolism, and an orally active heparin would undoubtedly effectively reduce chronic thrombotic events. Here, we report on the development of an orally administrable chemical conjugate of heparin and hydrophobic deoxycholic acid (DOCA), which we refer to as LHD. LHD was pre-formulated with dimethyl sulfoxide (DMSO) as solubilizer to further improve its oral bioavailability (9.1% in monkey). LHD was found to be absorbed mainly in the jejunum and ileum of the small intestine, although it is in the ileum that the absorption is most notable. From the mechanism studies of LHD absorption using Caco-2 cell monolayers for mimicking the intestine, we found that LHD highly permeated by passive diffusion through the transcellular route and its permeation was partially affected by bile acid transporters. This study demonstrates the feasibility of chemically modified heparin for long-term oral administration as an effective therapy for venous thromboembolism in clinical trials.

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**Keywords:** Oral absorption; Anti-coagulant; Low molecular weight heparin; Deoxycholic acid

### 1. Introduction

The oral route of delivery of macromolecular drugs, *i.e.*, heparin and insulin, is a topic of intense interest as it is likely to markedly improve patient acceptance and compliance. Moreover, heparin is the most potent anti-coagulant known in terms of deep vein thrombosis (DVT) and pulmonary embolism (PE) prevention. Venous thromboembolisms, *i.e.*, DVT and PE, are

diagnosed in ~250,000 hospitalized patients annually; and ~100,000 patients die each year of PE, [1,2] and without therapy, there is a 50% chance of a recurrent thromboembolism [3]. Currently 5 days of parenteral heparin injections followed by 3 months of oral warfarin therapy, the so-called ‘gold-standard’ treatment, successfully prevents PE in 95% of patients with proximal DVT [4]. However, although heparin and warfarin are effective anti-coagulants, individual patient response to oral warfarin therapy varies, and doses must be monitored closely to determine the duration of treatment, because warfarin has a slow onset, is pre-dominantly protein bound, and is subject to drug–drug interactions [5]. However, heparin does not have teratogenic effects, and has a rapid onset anti-coagulant effect with a half-life of <4 h, which allows its

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anti-coagulant effect to be readily reversed if necessary [6]. Therefore, chronic heparin administration is a highly attractive alternative therapy for the prevention of venous thromboembolism. Unfortunately, heparin cannot be absorbed orally because of its high charge density and its large molecular weight, and thus, can only be given parenterally. Thus, there is a need for an orally absorbable heparin, because currently the long-term use of intravenous heparin is not a practical option. In addition, oral heparin would facilitate venous thromboembolism treatment in an outpatient setting.

To increase heparin absorption in the intestine, various approaches, such as, liposomes, enteric coatings, and enhancers have been investigated [7–10]. Recently, we developed an orally active heparin derivative that enhances heparin absorption in the intestine [11–13]. This derivative is a chemical conjugate of low molecular weight heparin (LMWH) and deoxycholic acid (DOCA), which we refer to as LHD. This conjugated DOCA in LHD promotes intestinal absorption by enhancing the hydrophobic properties of LMWH and by increasing the interaction between heparin and the intestinal membrane [14,15]. Moreover, when we designed this conjugate system it was envisaged that DOCA would more easily interact with bile acid transporters expressed on the intestinal membrane, and that this would increase the absorption of macromolecules into the intestinal membrane. In the previous study, it was confirmed that LHD was highly absorbed in mice and did not induce any systemic toxicities as well as local toxicities in the GI tract at 10 mg/kg dose [16]. In the current study, we explored the oral absorption of LHD in a non-human primate model (cynomolgus monkeys) and investigated the absorption mechanism involved.

## 2. Methods

### 2.1. Materials

Low molecular weight heparin (LMWH; Fraxiparin®, 4500 Da) was obtained from GlaxoSmithKline (Brentford, Middlesex, UK). Deoxycholic acid (DOCA), dicyclohexylcarbodiimide (DCC), hydroxysuccinimide (HOSu), 1-ethyl-3-(3-dimethylamino propyl) carbodiimide hydrochloride (EDAC), ethylenediamine, dimethyl sulfoxide (DMSO), sodium taurocholate, ethylene glycol tetra acetic acid (EGTA), and colchicine were purchased from Sigma Chemical Co. (St. Louis, MO). Dimethylformamide (DMF) was obtained from Merck (Darmstadt, Germany). Fluorescein-5-isothiocyanate (FITC) and tetramethylrhodamine B isothiocyanate (TRITC)-labeled phalloidin were obtained from Fluka (Buchs, Switzerland). Coatest anti-Factor Xa assay kits were purchased from Chromogenix (Milano, Italy).

### 2.2. Synthesis of LHD

The chemical conjugate of LMWH and deoxycholic acid (DOCA) was synthesized by conjugating the carboxylic group of DOCA with the carboxylic group of LMWH. Briefly, the carboxylic group of DOCA (196 mg) was activated using DCC

(165 mg) and HOSu (92 mg) in 15 ml of DMF. Then the activated carboxylic group of DOCA was further reacted with ethylenediamine in DMF for 5 h at 25 °C, thereby forming deoxycholyethylamine. The feed mole ratio of activated DOCA to ethylenediamine was 3:1. The carboxylic groups of LMWH (100 mg) were also activated by using formamide (2 ml) and EDAC (11.49 mg), and then further reacted with an amine group of deoxycholyethylamine at 25 °C for 12 h. The feed mole ratio of deoxycholyethylamine to LMWH was 2:1. After the product was lyophilized and precipitated in acetone, the LMWH–DOCA conjugate, *i.e.*, LHD, was obtained as a white powder.

Fluorescein-labeled LMWH or LHD were synthesized to confirm the absorption of LMWH derivatives *in vitro* and *in vivo*. For fluorescein (FITC; fluorescein isothiocyanate) labeling LMWH derivatives a binding molar ratio of 1:1 (w/w) was used, *i.e.*, FITC to LMWH or LHD. The absence of an effect by fluorescein on the particle sizes of LMWH derivatives in water or in 10% DMSO solution was confirmed by dynamic light scattering.

### 2.3. Oral absorption of LMWH derivatives in monkeys

Cynomolgus male monkeys, each weighing about 2.5–3.0 kg, were individually housed at Korea Research Institute of Chemical Technology (KRICH; Daejeon, Korea). Animal care and all animal-related procedures were approved by KRICH. After fasting for 12 h, different doses (5, 10, 50 mg/kg) of LMWH or LHD were administered singly to monkeys by oral gavage. For pharmacokinetic analysis, 1 mg/kg of LMWH or LHD was intravenously injected into monkeys. After administering LMWH or LHD, monkey blood samples were collected from vein at each time and directly mixed with 50 µl of sodium citrate (3.8%). The plasma concentrations of LMWH or LHD were calculated from their anti-Factor Xa (FXa) activity, as determined by anti-FXa chromogenic assays [12]. In brief, the samples were diluted with human normal plasma at heparin concentrations above 0.7 IU/ml. LHD sample (100 µl) was mixed with 100 µl of anti-thrombin III (ATIII) solution, where ATIII concentration was in excess of the LHD concentration, followed by incubating at 37 °C for 3 min. FXa (100 µl) was added to the solution, and the resulting solution was then incubated for an additional 30 s. The concentration of FXa was also in excess of the LHD concentration. The substrate (200 µl, 0.8 µmol/ml) was then added and incubated at 37 °C for 3 min. The reaction was terminated by adding 300 µl of 20% acetic acid. The bioactivity and the concentration of LHD were calculated from the absorbance at 405 nm. The detection limit of anti-FXa chromogenic assay was 0.05 IU/ml, and the coefficient of variation was 2.6% in the range from 0.05 to 0.7 IU/ml.

In order to evaluate the absorption site of orally administered LHD, tissues from the duodenum, jejunum, and ileum were harvested and washed in lysis buffer for 30 min after fluorescein-labeled LHD (10 mg/kg) in 10% DMSO formulation was orally administered to mice. The tissue from each part was ground and homogenized with Ultra Turrax T 25 (JANKE

& KUNKEL, IKA Labortechnik). After centrifugation of tissue extract, their supernatants were filtered, and fluorescences of all samples were measured with synergy HT (BioTek Instruments, Inc, VT).

#### 2.4. Permeability study using a Caco-2 cell transport model

The human colon cancer cell line Caco-2 (American Type Culture Collection, Manassas, VA) were used as the transport model since they express bile acid transporters [17,18]. The cells were cultured in DMEM medium (pH 7.4) supplemented with 1% NEAA, 10% fetal bovine serum, 1% antibiotics at 37 °C in a 5% CO<sub>2</sub> atmosphere at 95% RH. Caco-2 cells ( $3 \times 10^5$  cells/ml) were seeded on the collagen-coated filter membranes of Transwell® cell culture inserts (3.0 µm pore diameter; Costar, Cambridge, MA) and further cultured for 4 weeks to reach confluence. The culture media were placed into the apical (0.5 ml) and basolateral (1.5 ml) chambers, and changed every other day for a week and then daily.

Caco-2 cell monolayers with a transendothelial electrical resistance (TEER) value of more than 400 Ω cm<sup>2</sup> were used for permeability studies. Briefly, Caco-2 cell monolayers were washed twice with cold phosphate buffered saline (PBS; Biowhittaker) and pre-incubated with pre-warmed transport media (pH 7.4), comprised of: hanks balanced salt solution (HBSS; Biowhittaker), 10 mM HEPES (*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid, Biowhittaker), 25 mM d-glucose, and metabolic inhibitor (sodium azide, 10 mM) for 30 min at 37 °C in a 5% CO<sub>2</sub> incubator. In addition, 4% bovine serum albumin (BSA; GIBCO) was pre-treated into the basolateral part as a cytoprotective agent. To evaluate the main absorption routes of LMWH or LHD, sodium taurocholate, colchicine, EGTA, or glucose free media were placed in the apical or basolateral region of transwell, respectively. Culture medium in the opposite part was collected at 10, 20, 30, 60, 90, 120 min after treating with LMWH derivatives (0.5 mg/ml) and the various blockers such as sodium taurocholate, sodium azide, colchicine and EGTA. The amounts of LMWH or LHD that permeated through the filter were measured indirectly using anti-FXa activity chromogenic assays. The apparent permeability ( $P_{app}$ , cm/s) was calculated from the slope at the steady state in the plot of the amount of permeated drug versus time.

To visualize LMWH or LHD permeation, 0.5 mg/kg of fluorescein-labeled LMWH or LHD with or without 10% DMSO was administered to Caco-2 cell monolayers. At 30 min after treatment, monolayers were washed and observed under a confocal laser scanning microscope. To confirm patent tight junctions between Caco-2 cells in the presence of 10% DMSO, RITC-phalloidin (a stain for actin filament) was treated and observed under a confocal laser scanning microscope.

#### 2.5. Statistical analysis

All data are expressed as means ± SEM. The paired *t*-test was used to compare before and after treatment data and ANOVA was used to compare groups. *P* values of ≤0.05 were considered statistically significant.

### 3. Results

#### 3.1. Oral absorption of LMWH derivatives in monkeys and mice

The anti-coagulant activity of LHD measured by Factor Xa chromogenic assay was 86 IU/mg, whereas that of LMWH was 97 IU/mg [16]. In addition, the partition coefficient of LHD in octanol/water was 15 folds higher than LMWH. To evaluate the oral absorption of LHD in a primate model, LHD was orally administered to fasted male cynomolgus monkeys. Furthermore, because LHD itself tended to form self-assembled particle in water due to the hydrophobic nature of the conjugated DOCA molecules, LHD was pre-formulated with 10% dimethyl sulfoxide (DMSO) as a solution. When LMWH (10 mg/kg) in 10% DMSO formulation, *i.e.*, DMSO–LMWH, was orally administered, plasma anti-Xa activity of LMWH remained below 0.1 IU/ml; the minimum effective concentration (MEC) required for treating venous thromboembolism (Fig. 1A). However, LHD (10 mg/kg) in an identical DMSO formulation, *i.e.*, DMSO–LHD, was rapidly absorbed with a maximum plasma anti-FXa activity at 4 h, and its plasma level

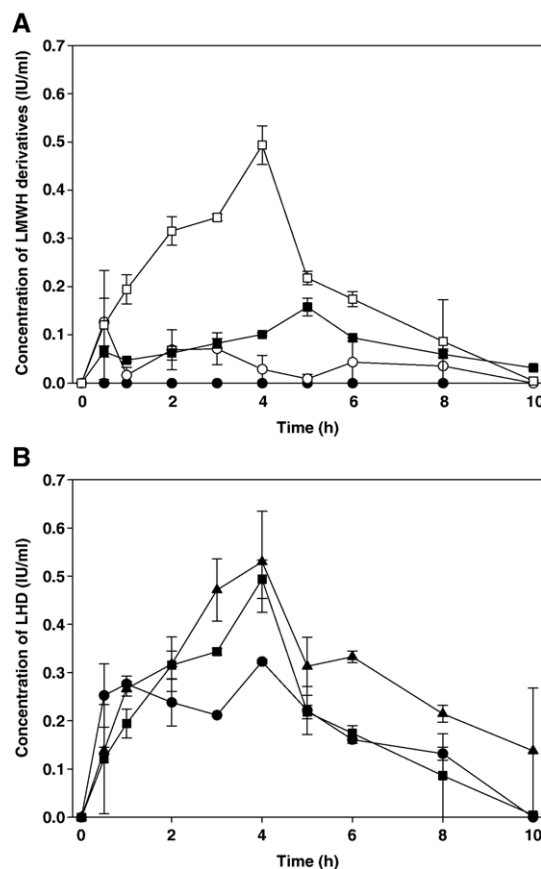


Fig. 1. Oral absorption profiles of LHD or LMWH in monkeys are shown. (A) Absorption profiles of LMWH or LHD alone or pre-formulated with 10% DMSO. After the oral administration of 10 mg/kg of LMWH (●), DMSO–LMWH (○), LHD (■) or DMSO–LHD (□), anti-FXa activity in plasma was measured (*n*=3) for each case. (B) Absorption profiles of 5 mg/kg (●), 10 mg/kg (■), and 50 mg/kg (▲) of DMSO–LHD in monkeys (*n*=3).

was maintained above the minimum effective anti-Xa activity of 0.1 IU/ml for 8 h. On the other hand, the AUC of LHD at 5, 10 and 50 mg/kg were  $94.6 \pm 9.7$ ,  $104.0 \pm 13.0$  and  $153.1 \pm 24.9$   $\mu\text{g}/\text{ml}/\text{min}$ , respectively (Fig. 1B). The absorption of LHD was not increased proportionally according to its dose. This result was related to the solubility of LD2 in 10% DMSO solution. For 5 mg/kg dosage, the concentration of LD2 in the dosage was 1 mg/ml, and it was completely dissolved. However, 50 mg/kg dosage (10 mg/ml) was administered in a dispersed state. Therefore, the solubility of LD2 might be important to the absorption in the intestine. Also, the specific interaction of LD2 with bile acid transporters might be an additional factor which affected the absorption of LD2 in the intestine. Moreover, when we pharmacokinetically analyzed the absorption profiles of LHD with/without DMSO in the formulation, we found that its bioavailabilities were 9.2 and 3.5%, respectively (Table 1).

To study the absorption site of LHD in the intestine, fluorescein-labeled DMSO–LHD was orally administered in mice (Fig. 2). Fluorescein-labeled DMSO–LHD was mostly absorbed in the jejunum (26%) and ileum (72%) parts of the small intestine, although it is most notable in the ileum; the absorption in the duodenum and the colon was negligible.

### 3.2. Permeation of LMWH derivatives in the Caco-2 cell transport model

Having found that LHD can be orally absorbed in monkeys, we sought to investigate the absorption pathway involved. First, we developed an *in vitro* model in which 10% DMSO formulation did not affect the integrity of confluent Caco-2 cell monolayer in the presence of bovine serum albumin (4%) as a cytoprotective agent. Then, to observe the absorption mechanism of DMSO–LHD, we determined the optimum concentration of LHD for transport by studying the correlation between the drug concentration and its absorption by Caco-2 cell monolayers formed in transwell. At 2 h after treating the

Table 1  
Pharmacokinetic parameters of low molecular weight heparin (LMWH) derivatives in Cynomolgus monkeys

LMWH derivatives	Dose (mg/kg)	AUC <sub>0–600 min</sub> ( $\mu\text{g}/\text{ml}/\text{min}$ )	$V_d$ (l/kg)	CL (ml/min/kg)	$T_{1/2}$ (h)
<i>Intravenous</i>					
LMWH	1	$118.9 \pm 1.0$	$0.11 \pm 0.0$	$0.3 \pm 0.0$	$4.3 \pm 0.0$
LHD	1	$113.6 \pm 6.0$	$0.25 \pm 0.0$	$0.8 \pm 0.0$	$3.4 \pm 0.1$
	Dose (mg/kg)	AUC <sub>0–600 min</sub> ( $\mu\text{g}/\text{ml}/\text{min}$ )	$C_{\text{max}}$ (IU/ml)	$T_{\text{max}}$ (h)	$F$ (%)
<i>Oral</i>					
DMSO–LHD	10	$104.0 \pm 13.0$	$0.49 \pm 0.0$	4	9.2
LHD	10	$45.9 \pm 3.0$	$0.16 \pm 0.0$	5	3.5
DMSO–LMWH	10	$13.4 \pm 10.0$	$0.13 \pm 0.10$	0.5	1.1
LMWH	10	ND	ND	ND	ND

LMWH, low molecular weight heparin; LHD, LMWH-deoxycholic acid (DOCA) conjugate; DMSO, 10% dimethyl sulfoxide (DMSO) formulation; AUC<sub>0–600 min</sub>, area under curve from 0 to 600 min (10 h) to plasma profile after LMWH derivative administration orally;  $V_d$ , volume of distribution; CL, clearance;  $T_{1/2}$ , half-life time;  $C_{\text{max}}$ , maximum concentration;  $T_{\text{max}}$ , the time at  $C_{\text{max}}$ ;  $F$ , bioavailability; ND, not detectable.

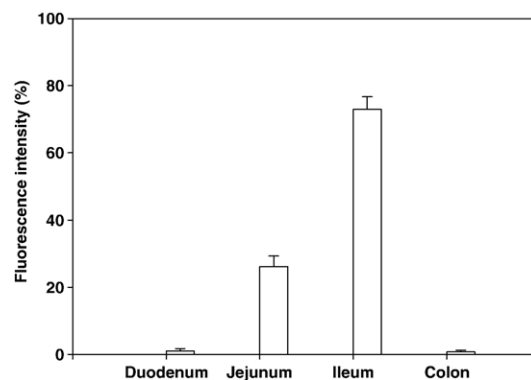


Fig. 2. The amount of fluorescein-labeled LHD in intestinal tissues.

apical region of Caco-2 cell monolayers with different concentrations of LHD or LMWH, the amounts of LHD or LMWH that permeated to the basolateral region was measured. The apparent permeability ( $P_{\text{app}} \times 10^{-7}$  cm/s) values of 0.1, 0.5, 1, and 5 mg/ml of LHD were  $45.6 \pm 10.9$ ,  $42.3 \pm 4.6$ ,  $26.0 \pm 2.3$ , and  $2.6 \pm 0.3$ , respectively (Fig. 3A, white bar). Moreover, the  $P_{\text{app}}$  values of 0.1 and 0.5 mg/ml of LHD, which were not statistically different, were significantly higher than those of 1

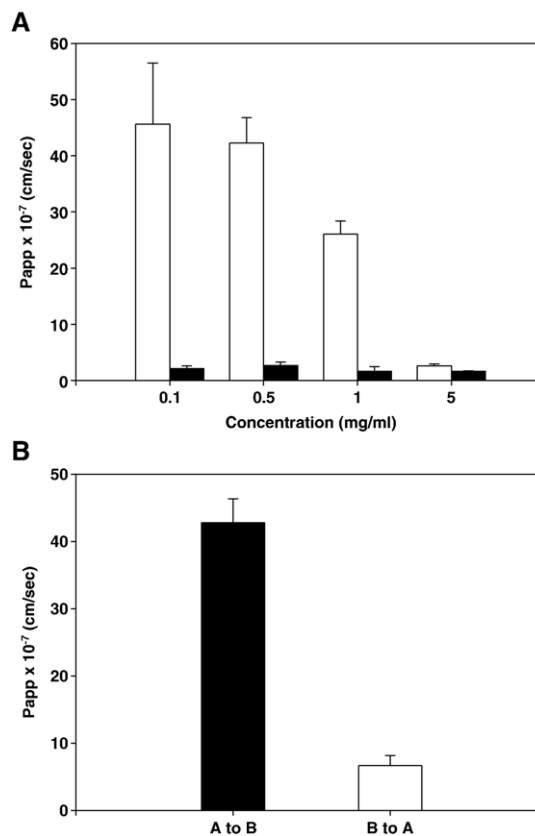


Fig. 3. Permeation of DMSO–LMWH or DMSO–LHD through a Caco-2 cell monolayer. (A) Apparent permeability ( $P_{\text{app}}$ ) at 0.1, 0.5, 1, and 5 mg/ml of DMSO–LMWH (black bar) or DMSO–LHD (white bar) from the apical (A) to basolateral (B) direction for 2 h. (B) The permeability of 0.5 mg/kg of DMSO–LHD from A to B direction (black bar) or from B to A direction (white bar) for 2 h. Data are expressed as means  $\pm$  SEM ( $n=5$ ).

and 5 mg/ml of LHD. On the other hand, the  $P_{app}$  values of the same concentrations of LMWH were below  $3.0 \times 10^{-7}$  cm/s (Fig. 3A, black bar). Fig. 3B shows that the permeability of

LHD (0.5 mg/ml) from apical (A) to basolateral (B) direction was six times greater than that from B to A.

### 3.3. Mechanism study to the LHD permeation through Caco-2 cell monolayer

To evaluate whether the oral absorption of LHD was aided by an adhesive effect between the conjugated DOCA molecule and intestinal membrane, Caco-2 cell monolayers were treated with sodium taurocholate. Sodium taurocholate is used as competitive inhibitor to bile acid transporter because of its high affinity for IBAT. Compared to the permeated amount of LHD in the absence of sodium taurocholate, the relative amounts of LHD that permeated for 120 min at 5, 10, 25, 50, 100, and 200  $\mu$ M of sodium taurocholate were  $94.3 \pm 2.5$ ,  $81.9 \pm 5.1$ ,  $64.9 \pm 7.7$ ,  $62.1 \pm 6.0$ ,  $65.4 \pm 10.3$  and  $64.2 \pm 4.7\%$ , respectively. We found that as the permeated amount of LHD was significantly decreased with the increase of the sodium taurocholate concentration; however, the permeation of LHD was not further reduced when the concentration of sodium taurocholate was above 25  $\mu$ M (Fig. 4A).

We evaluated the absorption of LHD in the presence of sodium azide to inhibit an active transport. As shown in Fig. 4B, the amounts of LHD that permeated from A to B and from B to A directions under glucose deprivation conditions with sodium azide were not significantly different with each other as compared with LHD permeation under the normal condition. Also, the permeated amount of LHD in the presence of colchicine (endocytosis inhibitor by blocking polymerization of microtubules) was not significantly different from that in the absence of colchicine, that is, the relative amounts of permeated LHD from A to B and from B to A directions in the presence of colchicine were  $110.3 \pm 11.6$  and  $96.9 \pm 15.1\%$ , respectively (Fig. 4C). This result indicated that LHD did not permeate Caco-2 cell monolayer by endocytosis.

To evaluate the involvement of paracellular route, in the permeation of LHD, we used ethylene glycol tetra acetic acid (EGTA; a calcium chelating agent) to open the tight junction in Caco-2 cell monolayer. The relative permeated amounts of LHD in the presence of EGTA from A to B and from B to A directions were  $127.0 \pm 1.8$  and  $158.7 \pm 1.6\%$ , respectively (Fig. 4D). Under the same condition, however, the relative amounts of

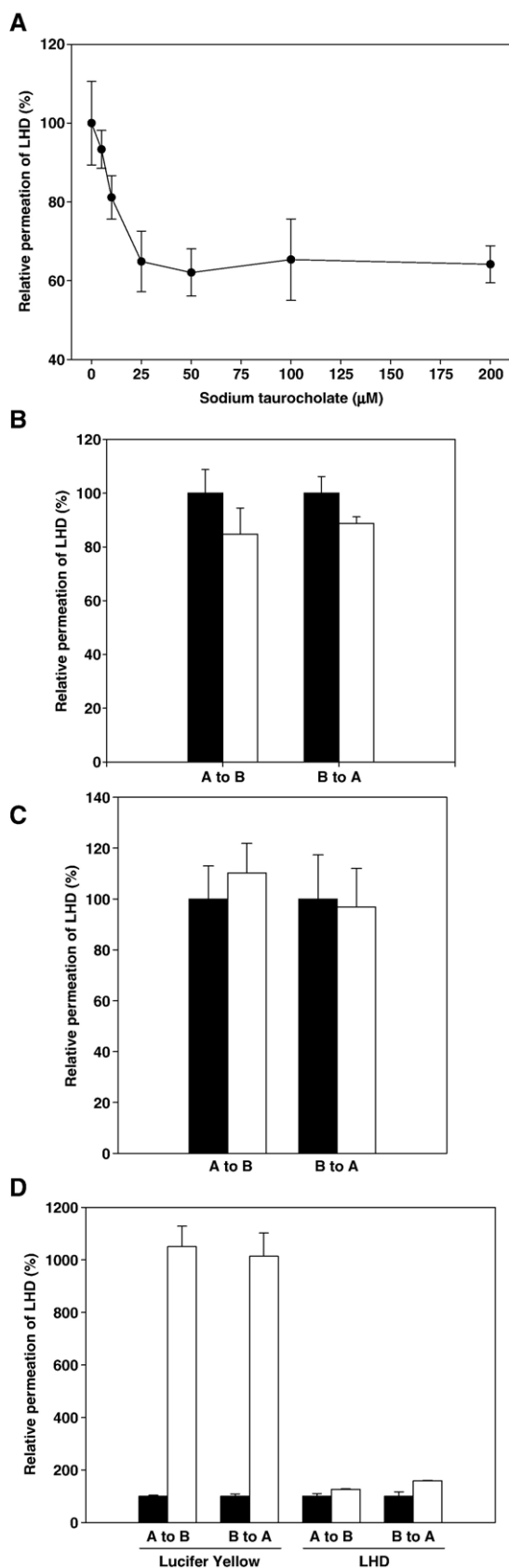


Fig. 4. The mechanism studies of DMSO–LHD permeation through a Caco-2 cell monolayer. (A) The relative permeation of 0.5 mg/ml of DMSO–LHD in the A to B direction in the presence of different concentrations (0, 5, 10, 25, 50, 100, and 200  $\mu$ M) of sodium taurocholate to inhibit active transport. (B) The relative permeability of 0.5 mg/ml of DMSO–LHD in the A to B and the B to A directions for 2 h in the glucose presence (black bar) or deprivation with 10 mM sodium azide (white bar). (C) The relative permeation of 0.5 mg/ml of DMSO–LHD for 2 h in the A to B and the B to A directions through Caco-2 cell monolayers (black bar) or Caco-2 cell monolayers pre-treated with 2.5  $\mu$ M colchicine for 30 min (white bar). (D) Relative permeation of 0.5 mg/ml of DMSO–LHD for 2 h in the A to B and the B to A directions through Caco-2 cell monolayer pre-treated with 2.5 mM EGTA. As a paracellular marker, Caco-2 cell monolayers were separately treated with 100  $\mu$ M of Lucifer yellow. Data are expressed as means  $\pm$  SEM ( $n=5$ ). Black bar: no treatment (control); white bar: absence of glucose or treatment with colchicine or EGTA.

permeated Lucifer yellow (a general paracellular transport marker) from A to B and from B to A directions were  $1050.1 \pm 78.0$  and  $1013.6 \pm 88.6\%$ , respectively. Therefore, the increase of permeated amount of LHD due to EGTA was negligible, compared to Lucifer yellow.

### 3.4. Visualization of LHD permeation through Caco-2 cell monolayer

To visualize LHD permeation through Caco-2 cell monolayers, fluorescein-labeled LHD or LMWH with or without DMSO pre-formulation were treated to Caco-2 cell monolayers for 2 h. The fluorescein-LMWH failed to completely permeate through monolayers, whereas fluorescein-labeled DMSO-LMWH was intermittently detected in Caco-2 cell monolayers (Fig. 5A, B). On the other hand, the fluorescein-labeled DMSO-LHD showed a typical image pattern of transcellular permeation (Fig. 5C). The fluorescent image intensity of the fluorescein-labeled LHD was reduced, compared to Fig. 5C, since LHD was not completely dissolved in buffer (Fig. 5D).

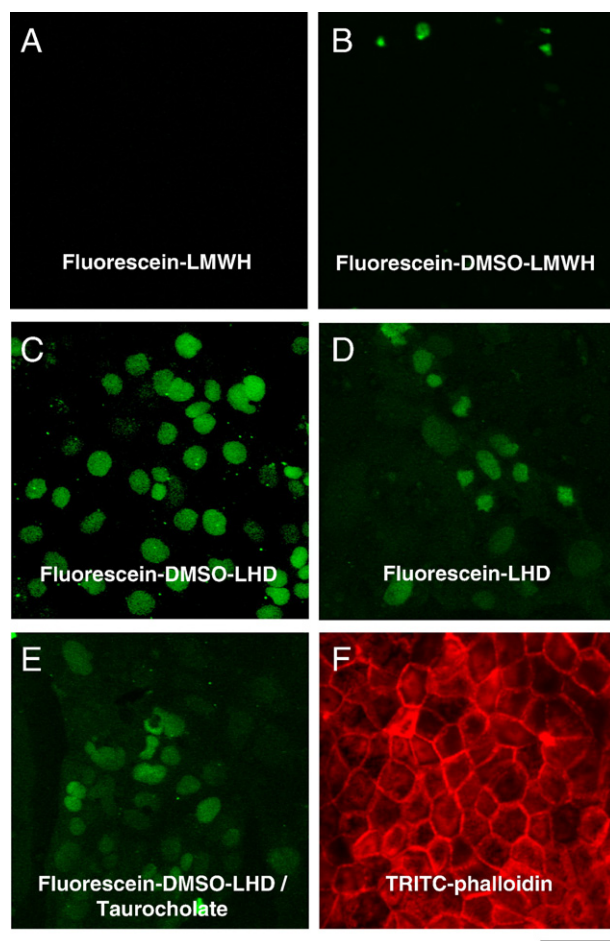


Fig. 5. Confocal laser scanning microscopic images of Caco-2 cell monolayers. Caco-2 cell monolayers treated with fluorescein-labeled LMWH for 30 min (A), DMSO-LMWH for 30 min (B), DMSO-LHD for 30 min (C), LHD for 30 min (D), DMSO-LHD for 30 min after pre-treatment with  $200 \mu\text{M}$  taurocholate for 30 min (E). Confirmation that DMSO caused no tight junction damage between Caco-2 cells by staining with TRITC-phalloidin after treatment of 10% DMSO for 30 min (F) (bar  $10 \mu\text{m}$ ).

Moreover, the fluorescein-labeled DMSO-LHD in the presence of  $200 \text{ mM}$  sodium taurocholate (Fig. 5E) also showed a reduced fluorescent image, compared to Fig. 5C. The actin filament (F-actin) in tight junction was stained using TRITC-labeled phalloidin after incubating Caco-2 cell monolayers for 2 h in 10% DMSO solution (Fig. 5F).

## 4. Discussion

We describe here the absorptive properties of an orally active low molecular weight heparin conjugate with DOCA, *i.e.*, LHD. The conjugated hydrophobic DOCA molecules increase the hydrophobicity of heparin and aid its ability to penetrate the intestinal membrane. In addition, DOCA can interact with bile acid transporters in the intestine and thus aid heparin absorption. LHD absorption occurs in mainly jejunum and ileum of the small intestine, especially, in the ileum because IBAT is highly expressed in this region. Moreover, the absorption of LHD was found to occur primarily via a transcellular route.

It was possible that LHD formed a self-assembled particle in aqueous solution because it has amphiphilic characteristics due to the presence of hydrophilic heparin and hydrophobic DOCA. Therefore, we used DMSO as a solubilizer, since it is a good solvent of DOCA. Compared to the DMSO free formulation, the bioavailability of LHD was 3 folds higher in DMSO formulation. On the other hand, the oral absorption of DMSO-LMWH, *i.e.*, without DOCA, in the monkey model was negligible. This result suggested that DMSO itself did not have any enhancing effect on the absorption of LMWH in the intestine.

After treating monkeys with  $10 \text{ mg/kg}$  DMSO-LHD, the plasma concentration of LHD was maintained at higher than  $0.1 \text{ IU/ml}$  for 8 h, which is a therapeutic concentration for treating DVT and PE. Therefore, this study suggested that the designed orally active heparin derivative, LHD, could be potentially administered for a long term to prevent venous thromboembolism in hospitalized patients and/or in outpatients.

In the mechanism study, the permeation of LHD in Caco-2 cell monolayer from A to B was higher than that from B to A, indicating the possibility of the interaction between LHD and the cell membrane. The dose dependency of LHD permeation, that the LHD permeability was decreased with the increase of LHD concentration, also showed the possibility of the interaction of LHD to the cell membrane. Such a low apparent permeability of LHD at high concentration would be induced by the saturation of interaction between LHD and the Caco-2 cell membrane. The result of inhibition test using sodium taurocholate showed that the interaction mentioned above was between LHD and ileal bile acid transporters. These results were also supported by the animal study that the amount of permeated fluorescein-labeled LHD in the ileum was higher than jejunum. However, the permeation of LHD was not further reduced above  $25 \mu\text{M}$  of sodium taurocholate and the relative permeated amount of LHD reached at a constant level about 65%. This result suggested that about 35% of LHD permeation was attributed to the bile acid transporter and about 65% LHD permeation was due to non-specific absorption. On the other

hand, the permeation result using sodium azide did not show any differences from that in the absence of sodium azide, that is, there was no active transport. Therefore, LHD did not permeate through the bile acid transporters even though LHD interacted with ileal bile acid transporters. However, the interaction between LHD and bile acid transporters would be important to enhance the concentration gradient of LHD across the intestinal wall, and LHD permeated through the transcellular pathway by a simple diffusion.

## 5. Conclusion

The proposed heparin derivative, LHD, in a DMSO formulation could be highly absorbable in the intestine, that is, 10 mg/kg of LHD could match the dose for the prevention of deep vein thrombosis. The conjugated DOCA could interact with ileal bile acid transporters, thereby increasing its concentration at the intestinal wall, and LHD permeate intestinal wall through the transcellular pathway by a simple diffusion. Therefore, LHD showed a high potential to be applied to prevent deep vein thrombosis for a long term, and it could provide a clinically effective option as a new strategy for oral macromolecule drug delivery.

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