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Improvement of low bioavailability of a novel factor Xa inhibitor through formulation of cationic additives in its oral dosage form

Yoshimine Fujii[a,*], Taro Kanamaru[b], Hiroshi Kikuchi[c], Hiroaki Nakagami[d], Shinji Yamashita[e], Mitsuru Akashi[f], Shinji Sakuma[e,**]

[a] Drug Metabolism & Pharmacokinetic Research Laboratories, R&D Division, Daiichi Sankyo Co. Ltd., 1-2-58, Hiromachi, Shinagawa-ku, Tokyo 140-8710, Japan
[b] Formulation Technology Research Laboratories, Pharmaceutical Technology Division, Daiichi Sankyo Co. Ltd., 1-2-58, Hiromachi, Shinagawa-ku, Tokyo 140-8710, Japan
[c] Pharmaceutical Science & Technology Unit, Eisai Product Creation Systems, Eisai Co. Ltd., 5-1-3, Tokodai, Tsukuba, Ibaraki 300-2635, Japan
[d] The Pharmaceutical Manufacturers' Association of Tokyo, 3-4-1, Nihonbashi Honcho, Chuo-ku, Tokyo 103-0023, Japan
[e] Faculty of Pharmaceutical Sciences, Setsunan University, 45-1, Nagaotoge-cho, Hirakata, Osaka 573-0101, Japan
[f] Department of Applied Chemistry, Graduate School of Engineering, Osaka University, 2-1, Yamadaoka, Suita, Osaka 565-0871, Japan

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ABSTRACT

A clinical trial of (2S)-2-[4-[[(3S)-1-acetimidoyl-3-pyrrolidinyl]oxy]phenyl]-3-(7-amidino-2-naphtyl) propanoic acid (DX-9065) revealed that its oral bioavailability was only 3% when it was administered as a conventional capsule formulation. The low bioavailability of DX-9065 was likely caused by both its poor membrane permeability and its electrostatic interaction with anionic bile acids. We hypothesized that DX-9065 absorption would be enhanced when the cationic drug was free from the complex through its replacement with other cationic substances. Polystyrene nanospheres coated with cationic poly(vinylamine) and cholestyramine, which is clinically used as a cholesterol-lowering agent, dramatically prevented DX-9065 from interacting with chenodeoxycholic acid *in vitro*. Successive animal experiments showed that bioavailability of DX-9065 administered with these cationic substances was 2–3 times that of DX-9065 administered solely. A dry syrup formulation with one-half of a minimal cholesterol-lowering equivalent dose of cholestyramine was designed, and the clinical trial was resumed. A 1.3-fold increase in bioavailability of DX-9065 was observed when the dry syrup was administered. We successfully demonstrated that DX-9065 absorption was enhanced when the drug was administered with cationic additives; however, it appeared that the absorption-enhancing function of cholestyramine largely depended on its dose. The dose escalation is probably prerequisite for the significant improvement of DX-9065 absorption in humans.

1. Introduction

Intestinal membranes, which are composed of a row of epithelial cells, possess diverse functions. Nutrients, which are essential for life, are recognized by influx transporters expressed in the apical membranes of intestinal epithelial cells. These nutrients are taken up into the cells via the transporters and delivered to the systemic circulation (Altmann et al., 2004; Fei et al., 1994; Hediger et al., 1987). Drugs whose chemical structures are analogous to those of nutrients are sometimes actively absorbed via this route (Ara et al., 2008; Sai and Tsuji, 2004; Tamai and Tsuji, 1996). However, most oral medicines are passively absorbed through the transcellular pathway of intestinal membranes on the basis of their lipophilicity and molecular weight (Lipinski et al., 2001), although some hydrophilic drugs may be absorbed through the paracellular pathway.

The oral bioavailability of drugs largely depends on their membrane permeability in the intestine (Amidon et al., 1995; Lipinski et al., 2001; Sakuma et al., 2007). Generally, there are few risks associated with the administration of drugs with high membrane permeability via the oral route. To the contrary, invasive routes such as injection are essential to obtain therapeutic effects of drugs with low membrane permeability. Since oral administration is the most convenient route of drugs (Sakuma et al., 2001), many researchers have taken up the challenge using medicinal chemistry-based and pharmaceutical technology-based approaches with the aim of improving the oral absorption of poorly membrane permeable drugs. The former approach (pro-drug) includes chemical modification with fatty acid to increase lipophilicity (Uchiyama et al., 2000), peptidyl derivation to enhance drug absorption through the H+/peptide cotransporter (Tamai and Tsuji, 1996), conjugation with specific peptides that can penetrate the cell membrane (Futaki,

* Corresponding author. Tel.: +81 3 5436 3131; fax: +81 3 5436 8587.
** Corresponding author. Tel.: +81 72 866 3124; fax: +81 72 866 3126.
E-mail addresses: fujii.yoshimine.r3@daiichisankyo.co.jp (Y. Fujii), sakuma@pharm.setsunan.ac.jp (S. Sakuma).

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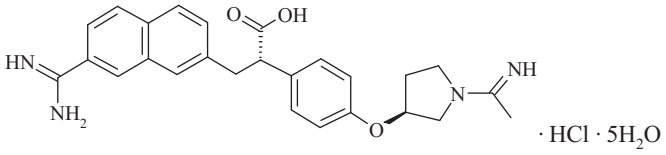


Fig. 1. Chemical structure of DX-9065a.

2005), etc., while the latter approach includes particulate drug delivery (Sakuma et al., 2001), colon-specific drug delivery (Saffran et al., 1986), mucoadhesive drug delivery (Morishita et al., 2006; Sakuma et al., 2001), the use of absorption enhancers (Muranishi, 1990), etc. However, successful cases are largely limited, and the development of technologies that enable poorly membrane-permeable drugs to effectively penetrate the biological membranes is still one of the greatest challenges in the pharmaceutical field.

(2S)-2-[4-[[(3S)-1-acetimidoyl-3-pyrrolidinyl]oxy]phenyl]-3-(7-amidino-2-naphtyl) propanoic acid (DX-9065), which was developed by Daiichi Pharmaceutical Co. (Tokyo, Japan), is an inhibitor of factor Xa that accelerates the conversion of prothrombin to thrombin (Nagahara et al., 1994). This drug candidate is a poorly lipophilic compound and is not recognized by intestinal transporters. As predicted from its physicochemical and physiological properties, the clinical trial using immediate-release gelatin capsules containing DX-9065a, which is a hydrochloride salt pentahydrate of DX-9065 (Fig. 1), showed that the oral bioavailability of DX-9065 was only 3% in humans. Mechanism studies on the intestinal absorption of DX-9065 indicated that the low absorption was caused by its poor membrane permeability and its electrostatic interaction with bile acids (Fujii et al., 2007). In our previous study (Fujii et al., 2011), enteric-coated tablets containing DX-9065a were designed with the aim of reducing the interaction that induced the formation of water-insoluble DX-9065–bile acid complex. Only DX-9065, which is free from the interaction with bile acids, can be absorbed from the intestine. We expected that the concentration of free DX-9065 will increase if the bulk of DX-9065 is immediately released and is exposed to bile acids in the proximal small intestine. A significant increase in AUC (the area under the plasma concentration–time curve) of DX-9065 after oral administration of its enteric-coated tablet was observed in monkey experiments when compared with the case of DX-9065a solution.

The reduction of DX-9065–bile acid interactions is obviously effective for improving the oral bioavailability of this drug candidate. However, the enteric coating seems unable to completely prevent DX-9065 from interacting with bile acids, which are detected through the entire small intestine and absorbed actively via the Niemann-Pick C1 Like 1 protein located on the distal small intestine (Altmann et al., 2004; Ara et al., 2008). DX-9065 with multiple dissociation groups (pK_a: 3.3 (acid); 11.2 and 13.3 (base)) is positively charged in the wide range of pH. The interaction is caused by intermolecular bindings of cationic DX-9065 to anionic bile acids (Fujii et al., 2007). Since the binding is generally an equilibrium reaction, DX-9065 that interacts with bile acids may be replaced with other cationic substances. The oral bioavailability of DX-9065 will elevate if free DX-9065 is released through the replacement.

Sakuma and Akashi have investigated the potential of nanospheres composed of graft copolymers having a hydrophobic polystyrene backbone and hydrophilic polyvinyl branches as particulate carriers for oral peptide delivery (Sakuma et al., 1997a,b, 1999, 2001, 2002a,b). The submicron-sized nanosphere is shaped like the sun, and the core made of hydrophobic polystyrene is decorated with coronae made of hydrophilic polyvinyl chains (Akashi et al., 1985, 1989). Diverse properties of nanospheres are primarily attributed to surface polyvinyl chains; Akashi et al. have already developed nanospheres having cationic poly(vinylamine) (PVAm), anionic poly(methacrylic) acid (PMAA), and nonionic poly(N-vinylacetamide) (PNVA). In this study, the effect of these nanospheres on DX-9065–bile acid interactions was first examined. Furthermore, cholestyramine, which is a cationic ion exchange resin that is being clinically used (Ast and Frishman, 1990), was substituted for nanospheres, and its potential as an additive that enhances DX-9065 absorption was evaluated beyond species.

2. Materials and methods

2.1. Materials

(2S)-2-[4-[[(3S)-1-acetimidoyl-3-pyrrolidinyl]oxy]phenyl]-3-(7-amidino-2-naphtyl) propanoic acid hydrochloride pentahydrate, DX-9065a (a salt form of DX-9065), was synthesized in Daiichi Pharmaceutical Co. 3-(7-amidino-2-naphthyl)-2-[4-[(1-propanimidoyl-4-piperidinyl)oxy]phenyl] propanoic acid and tritiated DX-9065 ([^{3}H]-DX-9065, 138 GBq/mmol, dissolved in sterilized water), which were used for the assay of DX-9065 in rat plasma and monkey plasma, respectively, were synthesized in Daiichi Pure Chemicals Co. (Tokyo, Japan). In-house rabbit anti-DX-9065 antiserum was used (Murayama et al., 1996). N-vinylacetamide (NVA) monomers were donated by Showa Denko Co. (Tokyo, Japan). t-Butyl methacrylate (BMA) monomers were obtained from Kohjin Co. (Tokyo, Japan). Sodium chenodeoxycholate was purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Cholestyramine was obtained from Muromachi Technos Co. (Tokyo, Japan). All other chemicals were commercial products of analytical or reagent grade and were used without further purification.

2.2. Preparation of nanospheres with different surface charges

Preparation procedures of nanospheres have been described previously (Sakuma et al., 1997a,b, 1999, 2001, 2002a,b). Briefly, PNVA and PBMA were prepared by the free radical polymerization of NVA and BMA monomers, respectively, using 2, 2′-azobisisobutyronitrile (AIBN) as an initiator in the presence of 2-mercaptoethanol as a chain transfer agent in ethanol. The resulting hydroxyl group-terminated PNVA and PBMA were reacted with *p*-chloromethyl styrene to introduce a polymerizable vinylbenzyl group in an alkaline solution with tetrabutylphosphonium bromide as a phase transfer catalyst. Vinylbenzyl group-terminated PBMA and PNVA were hydrolyzed in an acidic solution with hydroquinone as a polymerization inhibitor to obtain vinylbenzyl group-terminated PMAA and PVAm. Each of vinylbenzyl group-terminated polymers was copolymerized with styrene at a weight ratio of 1:1 in an ethanol/water mixture containing AIBN to obtain polystyrene nanospheres having the corresponding surface chains. The resulting nanosphere dispersion was centrifuged to remove unreacted substances. After purification, nanospheres with cationic PVAm, anionic PMAA, or nonionic PNVA chains were dispersed in purified water and lyophilized.

2.3. Characterization of nanospheres

Nanospheres were characterized as described in our previous articles (Sakuma et al., 1997a,b, 1999, 2001, 2002a,b). Briefly, weight-average molecular weights (M_w) of the surface polymeric chains were determined by gel permeation chromatography (HLC-8220GPC, Tosoh Co., Tokyo, Japan). The nanosphere size was measured by dynamic light scattering spectrophotometry (DLS-700, Otsuka Electronics Co., Osaka, Japan). The zeta potential of nanospheres was measured by electrophoretic light scattering

spectrophotometry in PBS (pH 7.4; ionic strength: 0.15) at 25 °C (ELS-800, Otsuka Electronics Co., Osaka, Japan).

2.4. DX-9065–bile acid interactions in the presence of nanospheres and cholestyramine

A phosphate buffered solution (PBS, 0.025 M, pH 6.8) was used as a medium. DX-9065a was dissolved in the medium at a concentration of 4 mg/mL as a DX-9065 equivalent. Sodium chenodeoxycholate was separately dissolved in the medium at a concentration of 5.6 mg/mL. Nanosphere dispersion (4 and 40 mg/mL) were also prepared. After the drug solution (0.5 mL) was mixed with sodium chenodeoxycholate solution (1 mL), nanosphere dispersion (0.5 mL) was immediately added to the mixture. As a reference, the dispersion was substituted with an equivalent volume of PBS. The mixture was incubated at 37 °C for 30 min, and then centrifuged (10,000 × *g*, 30 min, 37 °C). The concentration of DX-9065 in the supernatant was determined by the HPLC method reported in our previous study (Fujii et al., 2007). Briefly, phosphate-buffered solution (0.05 M, pH 6.8) was mixed with acetonitrile at a ratio of 100/14 (aqueous solution/acetonitrile, v/v). Octylamine was dissolved in the mixture at a concentration of 5 mM to prepare a mobile phase. A column of 150 mm × 4.6 mm filled with octadecylsilyl silica gel of 5 μm mean particle size was used (L-column ODS, Chemicals Inspection and Testing Institute, Tokyo, Japan). The injection volume was 0.01 mL, the flow rate was 1.0 mL/min, and the column temperature was 50 °C. DX-9065 was detected by measuring the UV absorption at 240 nm. The recovery of DX-9065 in the presence of nanospheres was compared with that in the absence of nanospheres. The same test was performed for cholestyramine.

2.5. Animal experiments

2.5.1. Experiments in rats

All animal experiments were approved by the Animal Experiment Ethics Committee of Daiichi Pharmaceutical Co. Seven-week-old male Sprague-Dawley strain rats (weight: 200–240 g) were fasted for 16 h with free access to water before experiments (n = 4–5). The abdomen of rats was opened under ether anesthesia, and the duodenum was observed. A mixture of DX-9065 (0.2 mg/mL) and nanospheres (2 mg/mL) was prepared using purified water as a solvent. The mixture was injected intraduodenally at a dose of 0.5 mg of DX-9065 and 5 mg of nanospheres in 2.5 mL of the mixture/kg of body weight. The abdomen was immediately sutured, and the rats were allowed to recover from anesthesia. Blood samples (0.6 mL) were taken from the jugular vein at predetermined time points under ether anesthesia. The blood was mixed with heparin and centrifuged at 3000 × *g* for 5 min at 4 °C to obtain plasma samples, which were stored frozen at −20 °C until analysis. Separately, aqueous solution of DX-9065 (0.2 mg/mL) was given intraduodenally at the same dose as that in the case of the mixture administration. Intravenous administration of DX-9065 was also conducted to provide a bioavailability reference. Saline solution of DX-9065 (0.5 mg/mL) was injected intravenously through the jugular vein under ether anesthesia at a dose of 0.5 mg of DX-9065 in 1 mL of the solution/kg. Plasma samples were obtained from the blood by means of the same procedure as described above.

Bioavailability of DX-9065 administered with cholestyramine was examined by means of the same procedure as described above, with exception of following conditions: a mixture of DX-9065 and cholestyramine with concentrations at 1 mg/mL and 33.5 mg/mL, respectively, was used; the dose was set as 2 mg of DX-9065 and 67 mg of cholestyramine in 2 mL of the mixture/kg; the intraduodenal administration was substituted with oral administration.

DX-9065 concentration in plasma was determined by the HPLC method reported in our previous study (Fujii et al., 2007). Plasma samples (0.1 mL) were mixed with an equivalent volume of purified water containing (2RS)-(3-(7-amidinonaphth-2-yl)-2-(4-(1-ethyl-carbon-imidoylpiperidin-4-yloxy)phenyl)) propionic acid as an internal standard at a concentration of 10 μg/mL. After the mixture was diluted with purified water (×30), it was loaded onto Sep-Pak plus C18 cartridge (Waters Co, Milford, MO, USA) that was preconditioned by passing 10 mL of methanol followed by 10 mL of purified water through the cartridge, at a rate of less than 1 mL/min. Cartridges were washed with 10 mL of purified water, and DX-9065 remaining in the cartridges was eluted with 6 mL of methanol containing 1% of acetic acid at the same rate. After removal of the solvent under vacuum at 40 °C, residues were dissolved in the mobile phase (0.2 mL). DX-9065 was assayed in a manner same as that described above.

2.5.2. Experiments in monkeys

The same set of 6 female cynomolgus monkeys (weight: 2.8–3.1 kg) was used in all experiments after a washout period of at least 7 days between trials. We confirmed that this time interval assured non-accumulation of DX-9065 in plasma (data not shown). Monkeys were fasted for 16 h with free access to water before experiments. Aqueous solution of DX-9065 (2 mg/mL) with or without cholestyramine (67 mg/mL) was administered orally at a dose of 2 mg of DX-9065 and 67 mg of cholestyramine in 1 mL of the solution/kg via a feeding tube. The drug solution remaining in the feeding tube was flashed with 2 mL of purified water. Blood samples (ca. 1 mL) were taken from the femoral vein at predetermined time points without anesthesia. Plasma samples were obtained from the blood by means of the same procedure as described above.

The concentration of DX-9065 in plasma was determined by the radioimmunoassay reported by Murayama et al. (1996). The plasma sample (0.1 mL) was mixed with 0.1 mL of [^{3}H]-DX-9065 solution (1.66 ng/mL, ca. 500 Bq), 0.1 mL of anti-DX-9065 antiserum solution (2000-fold dilution), and 0.5 mL of an assay buffer (phosphate buffered saline of pH 7.4 containing 0.1% BSA). The mixture was incubated at 4 °C for 16 h, and then 0.5 mL of phosphate buffered saline containing dextran T70 (0.1%, w/v) and charcoal (0.4%, w/v) was added. After incubation at 4 °C for 1 h, the mixture was centrifuged at 3000 × *g* for 15 min. The supernatant was mixed with 10 mL of scintillation fluid and the radioactivity was measured with a liquid scintillation counter (LS-6000TA, Beckman Coulter Inc., Brea, CA, USA). The concentration of DX-9065 in plasma was calculated using IMMUNOFIT EIA/RIA ver. 3.0 (Beckman Coulter Inc., Brea, CA, USA).

2.6. Clinical trial

A clinical trial was performed at Tokyo Research Center of Clinical Pharmacology (Tokyo, Japan) in accordance with the Declaration of Helsinki and the ICH guidelines on Good Clinical Practice (CPMP/ICH/135/95). Protocols were approved by Independent Ethics Review Committees. Nine healthy subjects (Japanese, male, 23.6 ± 2.6 years, 168.6 ± 4.8 cm, 63.1 ± 5.9 kg (mean ± s.d.)) with informed consents participated in the trial. They were selected from a panel of volunteers registered at Tokyo Research Center of Clinical Pharmacology on the basis of clinical remarks, medication histories, etc. Subjects were not allowed to take any drugs for at least 1 week before the trial. As shown in Table 1, a couple of oral dosage forms containing DX-9065a were used in this trial: one is an immediate-release gelatin capsule and the other is a cholestyramine-based dry syrup. Either the capsule or the dry syrup was administered orally to subjects with 150 mL of water in an open-labeled crossover design with a washout interval of 14 days. When the dry syrup was administered, its dispersion was

Table 1
Formulations of an immediate-release conventional gelatin capsule and a cholestyramine-based dry syrup.

Components		Conventional gelatin capsule (size: 3)	Cholestyramine-based dry syrup
DX-9065a	Active ingredient	128.5 mg[a]	128.5 mg[a]
Cholestyramine	Enhancer	–	2000 mg
Microcrystalline cellulose	Diluent	35 mg	–
Corn starch	Diluent	4.3 mg	–
Erythritol	Diluent	–	294 mg
Magnesium stearate	Lubricant	2 mg	–
Colloidal silicon dioxide	Glidant	0.2 mg	10 mg
Aspartame	Sweetening agent	–	65 mg
Orange flavor	Flavor	–	2.5 mg
Total weight		170 mg	2500 mg

[a] 100 mg as a DX-9065 equivalent.

Table 2
Characterization of nanospheres used in this study.

Characterization	PVAm nanospheres	PMAA nanospheres	PNVA nanospheres
Weight-average molecular weight of macromonomer[a]	25,000	15,000	25,000
Particle size (nm)[b]	220	200	150
Zeta potential (mV)[c]	12.7	−27.8	−1.6

[a] Weight-average molecular weights (M_w) of PVAm, PMAA, and PNVA chains on the nanosphere surface determined by gel permeation chromatography.
[b] Weight-average diameter measured by dynamic light-scattering spectrophotometry.
[c] Zeta potential measured by electrophoretic light-scattering spectrophotometry in PBS.

prepared using one-third of water. Subjects were fasted for 12 h before drug administration and meals were served at 4 h after drug administration. Venous blood samples (ca. 4 mL) were collected at the protocol-specified time points, mixed with lithium/heparin, and then centrifuged (2000 × *g*, 15 min). Plasma samples were stored frozen at −20 °C until analysis. The radioimmunoassay described in the previous section was used to determine the DX-9065 concentration in plasma.

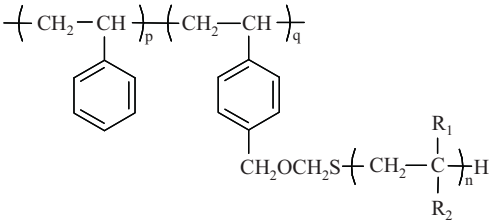
2.7. Data analysis

AUC from 0 to infinity was calculated by noncompartmental analysis using WinNonline Ver. 5.2. (Pharsight, St. Louis, MO, USA). Data were presented as means with standard deviation (s.d.) for individual groups. Statistical significance was assessed with the unpaired Student's *t*-test (rat experiment) and the paired Student's *t*-test (monkey and human experiments), and *p* values of 0.05 were considered significant.

3. Results

3.1. Characterization of nanospheres

Three types of nanospheres with different surface charges were prepared in this study: cationic PVAm, anionic PMAA, and nonionic PNVA nanospheres. Fig. 2 and Table 2 show the chemical structure and characterization, respectively, of them. Cationic PVAm with an M_w of 25,000, anionic PMAA with an M_w of 15,000, and nonionic PNVA with an M_w of 25,000 were used. All nanospheres possessed good water-dispersibility. Nanospheres with an average diameter of ca. 200 nm were obtained, irrespective of their chemical compositions, when they were synthesized according to procedures described in the experimental section. The zeta potential of nanospheres was consistent with the charge of the corresponding vinylbenzyl group-terminated polymers immobilized on the nanosphere surface.



	R1	R2
PVAm nanospheres	H	NH_2
PMAA nanospheres	CH_3	COOH
PNVA nanospheres	H	$NHCOCH_3$

Fig. 2. Chemical structure of nanospheres used in this study.

3.2. In vitro drug recovery and in vivo enhancement of drug absorption in the presence of nanospheres

Table 3 shows the *in vitro* recovery of DX-9065 incubated with bile acids in the presence and absence of nanospheres. Sodium chenodeoxycholate was used as a model for bile acids and its final concentration was adjusted to 6.75 mM (2.8 mg/mL) which was 3 times that of DX-9065 at a molar quantity. When DX-9065 was incubated with sodium chenodeoxycholate in neutral PBS at 37 °C for 30 min in the absence of nanospheres, 9.2% of DX-9065 applied was recovered on an average. An increase in the recovery of DX-9065 was observed when the drug was incubated with sodium chenodeoxycholate in the presence of cationic PVAm nanospheres. DX-9065 concentration in the supernatant became higher with an increase in the nanosphere concentration, and 76% of DX-9065 applied was free from chenodeoxycholate-induced interactions when the concentration of PVAm nanospheres was adjusted to

Table 3
Recovery (%) of DX-9065 incubated with sodium chenodeoxycholate in the presence and absence of additives (mean of 3 experiments).[a]

Additives	Concentration of additives		
	0 mg/mL[b]	1 mg/mL	10 mg/mL
PVAm nanospheres	9.2	37	76
PMAA nanospheres	–	10	7.9
PNVA nanospheres	–	11	11
Cholestyramine	–	27	94

[a] Concentrations of DX-9065 and sodium chenodeoxycholate were adjusted to 1 and 2.8 mg/mL, respectively.
[b] Control experiment (without additives).

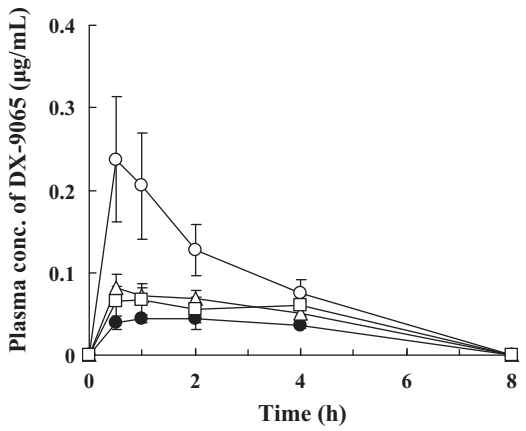


Fig. 3. Plasma concentration–time profiles of DX-9065 after intraduodenal administration of its aqueous solution (●), a mixture of DX-9065 and PVAm nanospheres (○), a mixture of DX-9065 and PMAA nanospheres (△), and a mixture of DX-9065 and PNVA nanospheres (□) in rats (0.5 mg of DX-9065 with or without 5 mg of nanospheres/2.5 mL/kg). Each value represents the mean ± s.d. (n = 4–5).

Table 4
Bioavailability of DX-9065 after its intraduodenal administration at a dose of 0.5 mg/kg with or without nanospheres in rats (mean ± s.d., n = 4–5).

Dosage forms	Bioavailability (%)
DX-9065 alone	5.9 ± 0.8
A mixture of DX-9065 with PVAm nanospheres	17.3 ± 4.2[a]
A mixture of DX-9065 with PMAA nanospheres	9.0 ± 1.9
A mixture of DX-9065 with PNVA nanospheres	9.0 ± 1.3

[a] Statistically significant difference from DX-9065 alone (P < 0.05).

10 times that of DX-9065. Neither PMAA nanospheres nor PNVA nanospheres affected the DX-9065 recovery.

Fig. 3 shows plasma concentration–time profiles of DX-9065 after intraduodenal administration of its aqueous solution and a mixture of DX-9065 and nanospheres in rats. Bioavailability in each of conditions is summarized in Table 4. When DX-9065 was given solely to fasted rats at a dose of 0.5 mg/kg, its plasma concentration was constantly low and the average bioavailability was estimated to be 5.9%. The intestinal absorption of DX-9065 was clearly improved when the drug was administered with PVAm nanospheres whose dose was 10 times that of DX-9065, and the average bioavailability was 17.3%, which was about 3 times that after drug administration without nanospheres. A 1.5-fold increase in bioavailability of DX-9065 was observed when the drug was given intraduodenally with either PMAA nanospheres or PNVA nanospheres; however, the difference was statistically insignificant.

3.3. *In vitro* drug recovery and *in vivo* enhancement of drug absorption in the presence of cholestyramine

Nanospheres were replaced with cholestyramine (Fig. 4), and *in vitro* experiments were first performed, as done for PVAm

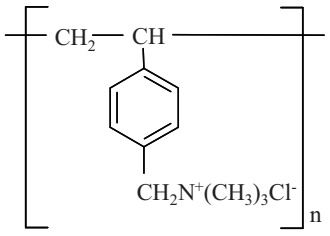


Fig. 4. Chemical structure of cholestyramine.

Table 5
Bioavailability of DX-9065 after its oral administration at a dose of 2 mg/kg with or without cholestyramine in rats and monkeys (mean ± s.d., n = 5 for the rat experiment, n = 6 for the monkey experiment).

Dosage forms	Bioavailability (%)	
	Rats	Monkeys
DX-9065 alone	4.6 ± 1.2	6.5 ± 3.1
A mixture of DX-9065 with cholestyramine	9.1 ± 3.3[a]	14 ± 4.4[a]

[a] Statistically significant difference from DX-9065 alone (P < 0.05).

nanospheres (Table 3). When cholestyramine was applied at a concentration equivalent to DX-9065, the average recovery of DX-9065 was 27%, which was about 3 times that in the absence of cholestyramine. Cholestyramine prevented more than 90% of DX-9065 from interacting with chenodeoxycholic acid when its concentration was adjusted to 10 times that of DX-9065.

In vivo animal experiments were next performed to evaluate the absorption-enhancing function of cholestyramine. The dose of cholestyramine in animals was set as 67 mg/kg. Fig. 5 shows plasma concentration–time profiles of DX-9065 after its oral administration with or without cholestyramine in rats and monkeys. Bioavailability in each of conditions is summarized in Table 5. Irrespective of species, DX-9065 absorption was enhanced by cholestyramine with a statistical significance, and the average bioavailability was about 2 times that after drug administration without cholestyramine.

A human trial was carried out using a couple of oral dosage forms containing DX-9065a: the immediate-release gelatin capsule without cholestyramine and the cholestyramine-based dry syrup (Table 1). Two grams of powdered cholestyramine was formulated into the dry syrup. Table 6 summarizes pharmacokinetic parameters of DX-9065 after oral administration of each dosage form. A 1.3-fold increase in bioavailability was observed when the dry syrup was used. Co-administration of cholestyramine clearly reduced the variation of pharmacokinetic parameters such as bioavailability, AUC, and C_{max}.

4. Discussion

Nanotechnologies are currently the undoubted driving force for innovative progress in sciences. Particularly, successful development of nanomaterials has largely contributed to the medical innovation. Akashi et al. have thoroughly investigated characteristics of nanospheres composed of graft copolymers having a hydrophobic polystyrene backbone and hydrophilic polyvinyl branches (Akashi et al., 1985, 1989, 1998; Riza et al., 1994, 1995; Chen et al., 1996). The core-corona type structure of nanospheres with the magnitude of 10^2 nm is formed when hydrophilic polyvinyl compounds with terminal polymerizable groups are copolymerized with hydrophobic styrene in a polar solvent. By changing the chemical structure of polyvinyl compounds, nanospheres with diverse surface properties can be obtained.

Table 6
Pharmacokinetic parameters of DX-9065 after its oral administration at a dose of 100 mg/man with or without cholestyramine in humans (mean ± s.d., n = 9).

Parameters	Dosage forms	
	Conventional gelatin capsule	Cholestyramine-based dry syrup
Bioavailability (%)	2.8 ± 1.5 (48)[a]	3.5 ± 1.1 (30)[a]
$AUC_{0-\infty}$ (ng h/mL)	446 ± 212 (48)[a]	549 ± 162 (30)[a]
C_{max} (ng/mL)	74.5 ± 61.9 (84)[a]	55.5 ± 16.8 (30)[a]
T_{max} (h)	1.4 ± 0.8	2.2 ± 1.7
$MRT_{0-48\,h}$ (h)	11.2 ± 1.2	11.4 ± 0.9

[a] Coefficient of variation (%).

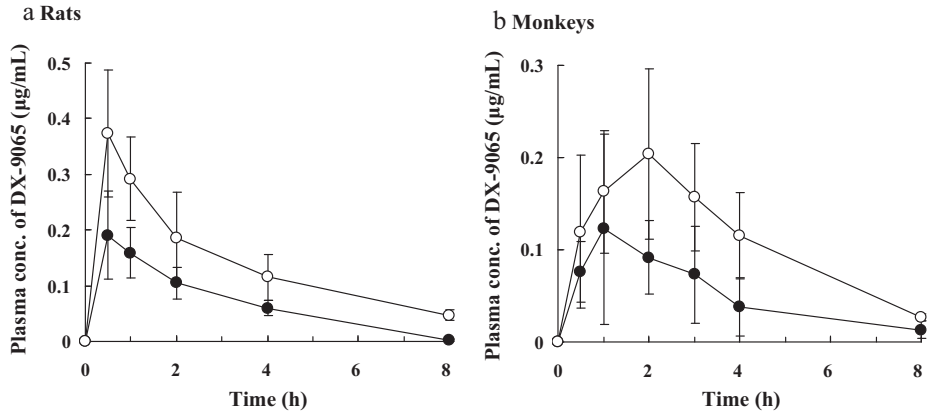


Fig. 5. Plasma concentration–time profiles of DX-9065 after oral administration of its aqueous solution (●) and a mixture of DX-9065 and cholestyramine (○) in rats (2 mg of DX-9065 with or without 67 mg of cholestyramine/2 mL/kg) and in monkeys (2 mg of DX-9065 with or without 67 mg of cholestyramine/1 mL/kg). Each value represents the mean ± s.d. (n = 5 for the rat experiment, n = 6 for the monkey experiment).

Macromolecules such as lectins immobilized on the nanosphere surface through chemical reactions with functional groups of polyvinyl chains also confer unique properties that nanospheres do not originally possess. Sakuma et al. are currently investigating the potential of peanut agglutinin-immobilized nanospheres with surface PNVA chains encapsulating fluorescent coumarin 6 as a colonoscopic imaging agent (Hiwatari et al., 2008; Sakuma et al., 2009, 2010, 2011a,b). Three types of nanospheres with different surface charges: cationic PVAm, anionic PMAA, and nonionic PNVA nanospheres, were prepared in this study (Fig. 2 and Table 2). All characteristics were similar to those described in our previous researches (Sakuma et al., 1997a,b, 1999, 2001, 2002a,b).

The enhancement effect of DX-9065 absorption by nanospheres was first examined. Low bioavailability of DX-9065 is likely caused by both its poor membrane permeability and its electrostatic interaction with bile acids that induce the formation of water-insoluble drug–bile acid complex (Fujii et al., 2007). We attempted to remove the latter cause due to the lack of promising commercial-based technologies that improve the membrane permeability. Our hypothesis is that DX-9065 absorption will be enhanced when free drug is released from the complex through the replacement of the drug with other cationic substances. Sodium chenodeoxycholate was used as a model for bile acids because our previous study demonstrated that this salt strongly interacted with DX-9065 (Fujii et al., 2007). Since the strongest interaction was observed when the molar quantity of sodium chenodeoxycholate was 3 times that of DX-9065, final concentrations of sodium chenodeoxycholate and DX-9065 were adjusted to 6.75 mM (2.8 mg/mL) and 2.25 mM (1.0 mg/mL), respectively. Clarysse et al. (2009) reported that the concentration of bile acids in the small intestine of fasted humans was 2–3 mM, which are comparable to our experimental conditions. Net charge of DX-9065 in the neutral solution is positive, while chenodeoxycholic acid is negatively charged in the solution. As shown in Table 3, more than 90% of DX-9065 precipitated through its electrostatic interaction with chenodeoxycholic acid. Cationic PVAm nanospheres clearly prevented DX-9065 from interacting with chenodeoxycholic acid.

Since an increase in free DX-9065 concentration was observed in the presence of PVAm nanospheres, *in vivo* experiments were subsequently performed. Our past researches revealed that nanospheres other than those with surface PNVA chains adhered to the mucous membranes of the gastrointestinal tract (Sakuma et al., 1999, 2001). This finding points out that the transit of DX-9065 is faster than that of PVAm nanospheres. When a mixture of DX-9065 and PVAm nanospheres are administered orally, nanospheres will be left behind from the drug because of their mucoadhesion to the gastric mucosa. Due to the difference in the gastric emptying time between DX-9065 and PVAm nanospheres, the former which is not concomitant with the latter will reach the small intestine where it is mainly absorbed. Enteric-coated dosage forms containing both DX-9065 and PVAm nanospheres are likely to remove the above-mentioned problem; however, extra time is required to design the formulation. Therefore, we alternatively employed intraduodenal administration of the mixture. Based on *in vitro* data (Table 3), the dose of nanospheres was set as 10 times that of DX-9065. As shown in Fig. 3 and Table 4, DX-9065 absorption was clearly improved when the drug was administered with cationic PVAm nanospheres. *In vitro* data suggested that this improvement resulted from an increase in free DX-9065 through the replacement of the drug with nanospheres in bile acid-countered complex.

We were strongly interested in a potential of cationic substances as an additive that enhances DX-9065 absorption in humans. There was a reasonable likelihood that PVAm nanospheres would be developed as a safe pharmaceutical additive (Sakuma et al., 2001, 2002a); however, the regulation-specified safety tests of them have not been initiated yet. Then, other cationic substances whose safety tests had been completed were listed with the aim of proving our hypothesis and simultaneously acquiring the oral dosage form of DX-9065a with developable bioavailability. Cholestyramine, which is widely used as an oral medicine that lowers cholesterol concentration in blood (Ast and Frishman, 1990), is a cationic ion exchange resin with quaternary amines (Fig. 4). Bile acids in the intestine are adsorbed on cholestyramine administered orally, and their excretion into feces is enhanced. The intestinal absorption of exogenous cholesterol is consequently reduced; it is also considered that the conversion of endogenous cholesterol in the liver to bile acids is accelerated to compensate for their reduction. Here, cholestyramine was replaced for PVAm nanospheres, and we evaluated its potential as an additive that enhances DX-9065 absorption.

Since *in vitro* data revealed that cholestyramine prevented DX-9065 from interacting with bile acids (Table 3), as did PVAm nanospheres, *in vivo* animal experiments were performed. A minimal cholesterol-lowering equivalent dose of cholestyramine in humans is 4 g, and patients usually take the dose twice a day (Ast and Frishman, 1990). The dose is escalated unless a sufficient effect is observed. Since human body weight is generally estimated to be 60 kg as an average, the dose of cholestyramine in animals was set as 67 mg/kg. A mixture of DX-9065 and cholestyramine was given orally because the mucoadhesive property of cholestyramine has not been reported. Irrespective of species, DX-9065 absorption was improved with a statistical significance when the drug was administered with cholestyramine (Fig. 5 and Table 5). The

elevation was probably due to an increase in free DX-9065 through the replacement of the drug with cationic cholestyramine in bile acid-countered complex. A reduction of bile acids in the intestine may also influence the improvement of DX-9065 absorption.

Through animal experiments, we successfully demonstrated that the oral bioavailability of DX-9065 was elevated when the drug was administered with cationic additives. Based on our evidence for the above-mentioned hypothesis, Daiichi Pharmaceutical Co. resumed the clinical trial using a couple of oral dosage forms containing DX-9065a: the immediate-release gelatin capsule without cholestyramine and the cholestyramine-based dry syrup (Table 1). There was a guideline for pharmaceutical additives stated by the then Japanese authorities. Even if chemical substances possess pharmacological activities, they can be used as a pharmaceutical additive when the formulated amount is less than one-half of the minimal dose. Based on this guideline, 2 g of cholestyramine was formulated as a pharmaceutical additive (Table 1). Patient-friendly dry syrup, which is used as aqueous suspension when patients take, was designed for a dosage form containing cholestyramine, because the cholestyramine dose was too large to take once as solids. As shown in Table 6, a slight increase in bioavailability of DX-9065 was observed when the dry syrup was used; co-administration of cholestyramine reduced the variation of pharmacokinetic parameters.

The observed reduction of the variation was an advantage of the cholestyramine-containing dry syrup to the capsule without this cationic additive. However, based on the insignificant improvement of DX-9065 absorption, Daiichi Pharmaceutical Co. decided to discontinue the clinical trial using the dry syrup. The absorption-enhancing function of cholestyramine was reduced presumably because the dose/kg was halved in human studies. In this clinical trial, ca. 1.6 mg of DX-9065 was given orally with ca. 32 mg of cholestyramine per kg of subjects (the average body weight of subjects: 63.1 kg). The ratio of cholestyramine to DX-9065 was calculated to be 20. On the other hand, this ratio in animal experiments was 33.5. A dose reduction of cholestyramine possibly resulted in a difference in its absorption-enhancing function between animal and human experiments. An increase in the dose of cholestyramine was probably prerequisite for the significant improvement of DX-9065 absorption in humans. The development of PVAm nanospheres should be also accelerated because *in vivo* data suggest that the effective dose of this cationic additive is lower than that of cholestyramine.

5. Conclusions

The electrostatic interaction between DX-9065 and bile acids was one cause of poor drug absorption. We hypothesized that oral absorption of DX-9065 would be enhanced when free DX-9065 was released from the water-insoluble DX-9065–bile acid complex through the replacement of the drug with other cationic substances. When DX-9065 was incubated with sodium chenodeoxycholate, less than 10% of the drug was free from the interaction. Cationic PVAm nanospheres and cholestyramine dramatically prevented DX-9065 from interacting with chenodeoxycholic acid *in vitro*. Successive animal experiments showed that both cationic additives significantly enhanced DX-9065 absorption *in vivo*. Based on our evidence, the clinical trial of DX-9065 was resumed. A 1.3-fold increase in bioavailability of DX-9065 was observed when the drug was administered with cholestyramine. We successfully demonstrated that DX-9065 absorption was enhanced when the drug was administered with cationic additives; however, an increase in the dose of cholestyramine is probably prerequisite for the significant improvement of DX-9065 absorption in humans.

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