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Characteristics of Gastrointestinal Absorption of DX-9065a, a New Synthetic Anticoagulant

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Summary: DX-9065a, a newly synthesized anticoagulant that selectively inhibits factor Xa, is a zwitterion and has characteristics of high water solubility and low lipophilicity. We predicted the fraction absorbed (Fa) of DX-9065a to be approximately 15–35% in humans, based on the boundary layer theory using the intestinal perfusion method in rats. However, human oral bioavailability was 2–3% in clinical trials, and the result of actual human bioavailability was lower than that of the predicted Fa. Thus, in this report, the reason for low oral bioavailability of DX-9065a was examined by in vitro and in vivo experiments. The factors affecting oral bioavailability of DX-9065a were not the hepatic first-pass effect, degradation of the drug in intestinal fluid, nor the interaction of the drug with the intestinal mucin. Furthermore, no effect of P-gp efflux was observed. Oral absorption of the drug in rats with bile duct ligation was significantly higher than that in normal rats with bioavailability of 17 and 3%, respectively. It was confirmed that bile acids inhibited DX-9065a absorption because DX-9065a interacted with bile acids to form insoluble complexes. The results suggest that the complex formation of DX-9065a with bile acids in the intestinal tract is an important factor affecting absorption of DX-9065a.

Key words: DX-9065a; anticoagulant; factor Xa inhibitor; membrane permeability; rat; bioavailability; bile acid

Introduction

Warfarin and heparin are widely used as clinical antithrombotic therapy, however, their adverse effects limit the clinical use of these agents. When low molecular weight heparin was tested as an oral anticoagulant, no detectable anticoagulant activity was observed following oral administration in humans.1) Argatroban,2) a direct thrombin inhibitor can also only be administered by injection. DX-9065a (Fig. 1), a newly synthesized anticoagulant, selectively inhibits factor Xa3) and shows an anticoagulant effect following oral administration to rats.4,5)

DX-9065a has three polar groups in its molecule, that is, an amidino group, an acetoimidoyl group and a carboxyl group. DX-9065a has high water solubility (above 600 mg/mL) and low lipophilicity (log D6.8 = -3.0). DX-9065a might only be absorbed via the paracellular pathway, since DX-9065a exists in the ionized form under intestinal pH conditions. Furthermore, it must have low affinity for lipid membranes because of its low lipophilicity. Based on these characteristics, high oral bioavailability is not expected for DX-9065a.6) However, some types of cephalosporins, such as cephalaxin, exhibit high bioavailability despite their being zwitterion compounds, because their absorp-

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

M.W.: 571.1
tion might involve carrier-mediated transport. 7

With drug development, it is important to predict human oral drug absorption before starting clinical trials. Amidon et al. established a useful method to predict the human fraction absorbed based on the boundary layer theory using rat intestinal perfusion. 8 In the present study, we predicted human oral bioavailability of DX-9065a using this method, and elucidated the DX-9065a absorption mechanism by in vitro and in vivo experiments.

Materials and Methods

Materials: DX-9065a ((+)-(2S)-2-[4-[[3S]-1-acetimidoyl-3-pyrrolidinyl]oxy]phenyl]-3-[7-amidino-2-naphthyl]propanoic acid hydrochloride pentahydrate), and (2S)-(3-(7-amidinonaphth-2-yl)-2-(4-(1-ethyl-carbon-imidoyl)piperidin-4-xyloxy) phenyl) propionic acid were synthesized at Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan), and the latter was used as the internal standard for high-performance liquid chromatography (HPLC) analysis. [14C] PEG-4000 was purchased from DuPont NEN (Boston, MA, USA). Bile salts (cholate, deoxycholate, taurocholate, glycocholate, chenodeoxycholate, taurodeoxycholate) were purchased as sodium salts from Sigma Chemical Co. (St. Louis, MO, USA). HIONIC-FLUOR, a scintillation cocktail, was purchased from Packard Instrument Company, Inc. (Meriden, CT, USA). All other materials and solvents were of analytical or the highest grade available.

Animals: Male Sprague-Dawley rats weighing 230–400 g were fasted for 16 h prior to experiments, but had free access to water. All animal experiments were conducted with the approval of the Animal Experiment Ethics Committee of Daiichi Pharmaceutical Co., Ltd. and the University Committee on the Use and Care of animals of the University of Michigan.

Rat intestinal single-pass perfusion: Rats intestinal perfusion was performed according to the method of Hu et al. 10 Briefly, after washing the cannulated jejunum (15 cm) and ileum (15 cm) with 15 mL of saline, the jejunum and ileum segments were perfused with 0.1 and 1 mM of DX-9065a dissolved in 10 mM MES buffer solution of pH 6.5 or 10 mM phosphate buffer solution of pH 7.5, containing 140 mM sodium chloride, 5 mM potassium chloride and 0.01% PEG-4000 with a tracer amount of [14C] PEG-4000, at a flow rate of 0.123 mL/min using the infusion pump. Following perfusion for 30 min to attain to the steady state, the perfused solution was collected in 10 min intervals for a total of 60 min. The DX-9065a concentration in the perfusate was measured by the HPLC method, and the [14C] PEG-4000 radioactivity was measured with a liquid scintillation counter (Model LS 6000TA, Beckman, Fullerton, CA, USA).

Prediction of human Fa: Unbiased membrane parameters are estimated using a boundary layer model developed by Amidon et al. 8,9 The regular permeability parameters are converted to dimensionless ones by multiplying R/D, where R is the radius of the intestine, and D is an estimate of the aqueous diffusion coefficient of a solute in the perfusate. The dimensionless effective permeability, P*eff, and intrinsic wall permeability, P*w, were calculated based on their theory. Briefly, P*eff is calculated based on the results of the rat intestinal perfusion experiment:

\[ P_{\text{eff}}^* = \frac{1 - C_m/C_0}{4Gz} \]  

where C0 and Cm are inlet and outlet perfusate concentration, respectively, and Cm was compensated by [14C] PEG-4000 concentration. Graetz number, Gz, is

\[ Gz = \frac{\pi DL}{2Q} \]  

where L is the length of the intestine, and Q is the fluid flow rate.

The aqueous permeability, P*aq, can be estimated from the film model approximation to the boundary layer result:

\[ P_{\text{aq}}^* = \frac{1}{1 - e^{-2P_{\text{eff}}^*}} \]

The solution for the ratio of outlet (Cm) to inlet (C0) concentration shows the fraction dose absorption (Fa):

\[ Fa = 1 - \frac{C_m}{C_0} \]  

Equation (8) can be converted to the following:

\[ Fa = 1 - e^{-4GzP_{\text{eff}}^*} \]  

Gz of the human intestine is estimated to be approximately 0.5, the intestinal length (L) is 500 cm, the flow rate (Q) is 0.5 mL/min, and the water phase diffusive constant (D) of 3 × 10^{-4} cm^2/min. Consequently, Eq. (9) becomes

\[ Fa = 1 - e^{-2P_{\text{eff}}^*} \]  

If the assumption is made that P*aq is not limiting, Eq. (10) becomes

\[ Fa = 1 - e^{-2P_w^*} \]

Intravenous and portal vein administration to rats: Rats were anesthetized with sodium pentobarbital (30
mg/kg) by intraperitoneal injection. Following midabdominal incision, DX-9065a solution in saline (0.1 mg/0.2 mL/head) was administered by injection into the left jugular vein or the portal vein. Blood samples were withdrawn at 1, 5, 15, 30, 60, 120 and 240 min after administration from the right jugular vein under ether anesthesia.

**Stability in the intestinal fluid:** Intestinal fluid was collected from the small intestine of rats using the method of Takada et al.,11 with some modifications. Rat small intestine was removed under ether anesthesia, and 20 cm of the jejunum was washed out with saline. For stability experiments, 0.5 mL of a DX-9065a saline solution (0.5 or 5 mg/mL) was added to 4.5 mL of the intestinal fluid or 50 mM phosphate buffer (pH 6.8) and incubated at 37°C for up to 180 min. Two hundred microliter samples were then withdrawn from the incubated mixture, and stored at 4°C until HPLC analysis.

**Everted and non-everted sac experiment:** Everted sac experiment was performed according to the method of Niibuchi et al.,12 with some modifications. Briefly, the cannulated small intestine was then perfused with modified Ringer’s solution at a rate of 2.0 mL/min for 60 min. Intestinal mucus was removed by perfusion with Ringer’s solution containing 0.02% sodium deoxycholate (w/v). Following surgical excision of a 2 cm portion of small intestine from 10 cm below the pylorus, the everted or non-everted intestine was applied to Wiseman’s perfusion apparatus, which was maintained at 37°C or 4°C, and 60 mL of DX-9065a Ringer’s solution (0.42 mg/mL) was added to the mucus side and 20 mL of Ringer’s solution to the serosal side, respectively. In the experiments examining the effect of sodium deoxycholate perfusion on DX-9065a transport, a 0.042 mg/mL of DX-9065a solution was used. Both sides of the intestinal membrane were gassed with 5% CO₂ in oxygen. After perfusion, a 200 μL portion of perfusate was withdrawn from the serosal side, and stored at 4°C until HPLC analysis.

**Rat oral bioavailability study:** Rat bile duct ligation was prepared using the following method. Rats were anesthetized with ether, and midabdominal incision was performed. The bile duct was ligated with surgical thread, and the abdomen was then closed. After recovering from anesthesia, rats were allowed a recovery period of approximately 1 h. DX-9065a (0.5 mg/0.5 mL/head in saline solution) was orally administered to normal rats and rats ligated bile duct by gastric tube. Blood samples were taken at 0.5, 1, 2, 4 and 8 h after administration from the jugular vein under ether anesthesia. Bioavailability of DX-9065a after oral administration was calculated from the AUC ratio after oral and intravenous administration of DX-9065a.

**Equilibrium dialysis:** DX-9065a was dissolved in isotonic phosphate buffer (pH 6.8) at a concentration of 0.2–20 mg/mL, and the bile components were dissolved in the same buffer at a concentration of 20 mg/mL. Then 0.4 mL of DX-9065a buffer solution was added to 0.4 mL of each bile component solution and dialyzed against the same buffer solution at 37°C for 20 h.

**HPLC assay:** DX-9065a concentration in plasma, perfusate, and samples from equilibrium dialysis was assayed by HPLC. HPLC (Waters 600E system, Milford, MO, USA) was equipped with ultraviolet (UV) detector (Model 484, Waters) set at 240 nm and a reversed-phase column (L-column ODS, 4.6 mm i.d. × 150 mm length, Chemicals Inspection and Testing Institute, Tokyo, Japan) was used. The mobile phase consisted of pH 6.8 phosphate buffer and acetonitrile (100:14) containing 5 mM octylamine. Samples were eluted at a flow rate of 1.0 mL/min. The DX-9065a concentration was determined from the peak area using a calibration curve.

Solid-phase extraction method was used to clean plasma samples suitable for HPLC analysis. A Sep-Pak Plus C18 Cartridge (Waters) was conditioned with 10 mL of methanol and 10 mL of distilled water. A mixed solution of 0.2 mL of the rat plasma, 0.1 mL of the internal standard solution and 5.7 mL distilled water was applied to the column. After washing with 10 mL distilled water, DX-9065a and IS were eluted with 6 mL of methanol containing 1% acetic acid. The elution medium was evaporated with a centrifuge evaporator. The dried residue was redissolved with 100 μL of the mobile phase, and 20 μL of the solution was injected into the HPLC.

**Data analysis:** Pharmacokinetic parameters were calculated using the SAG-CP statistical analysis and graphics program (ASMedica, Tokyo, Japan) for data obtained following oral administration.

**Results**

**Estimation of human oral absorption:** The intrinsic wall permeability (Pₑ) obtained from rat intestinal single-perfusion experiments is shown in Table 1. The Pₑ values were within a range of 0.07 to 0.21 in the jejunum and ileum, and the predicted fraction absorbed (Fa) of DX-9065a calculated from Eq. (11) was approximately 15–35% in humans.

**Influence of hepatic first-pass effect on DX-9065a absorption:** To examine the low bioavailability of DX-9065a, we focused on the hepatic first-pass effect for oral absorption of DX-9065a. After intravenous injection, the plasma concentration-time profile of DX-9065a was compared with that of portal vein administration in rats (Fig. 2). Plasma DX-9065a concentration-time profiles for intravenous and portal vein administration were similar at all sampling times, suggesting that the bioavailability of DX-9065a is not
Characteristics of Gastrointestinal Absorption of DX-9065a

<table>
<thead>
<tr>
<th>DX-9065a concentration</th>
<th>Jejunum pH 6.5</th>
<th>Jejunum pH 7.5</th>
<th>Ileum pH 7.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 mM</td>
<td>0.206 ± 0.119</td>
<td>0.102 ± 0.066</td>
<td>0.104 ± 0.061</td>
</tr>
<tr>
<td>1 mM</td>
<td>0.067 ± 0.053</td>
<td>0.122 ± 0.088</td>
<td>0.080 ± 0.029</td>
</tr>
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</table>

Data are shown as the mean ± S.E., n = 4.

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Intrinsic wall permeability (P* w) of DX-9065a in rat intestine

Intestinal metabolism was also evaluated by incubation of DX-9065a with the intestinal homogenate. As the result, no metabolism was observed in 30 min (Data not shown).

Stability of DX-9065a in intestinal fluid: The stability of DX-9065a against digestive enzymes was examined by incubating DX-9065a in intestinal fluid at 37°C. As shown in Fig. 3, no degradation of DX-9065a was found with incubation in intestinal fluid for up to 180 min.

In vitro intestinal perfusion: The permeability of DX-9065a in isolated intestine with and without washing off intestinal mucus by deoxycholate perfusion was determined, to examine the interaction between DX-9065a and mucus components. The intestinal permeability of DX-9065a did not change, irrespective of washing off mucus or not (Fig. 4).

In an attempt to clarify the mechanism of transport of DX-9065a, permeability across everted and non-everted intestine was determined at 37°C. As shown in Fig. 5, there were no differences in time course of DX-9065a transport across everted and non-everted intestine. No changes in DX-9065a transport across the intestine were observed under the condition of 4°C, either.

In vivo absorption in rats with bile duct ligation:

In an attempt to clarify the mechanism of transport of DX-9065a, permeability across everted intestine perfused with (●) or without (○) 0.02% deoxycholate. Each value represents the mean ± S.E. (n = 3).

Plasma concentration-time profiles of DX-9065a after oral administration to rats with or without bile duct ligation are shown in Fig. 6, and the pharmacokinetic parameters are summarized in Table 2. AUC after intravenous administration of DX-9065a (0.1 mg/head) was 1713 ng·h/mL in rats (average, n = 5). The absolute bioavailability of DX-9065a after oral administration was approximately 3% in normal rats. Absorption was significantly higher in rats with bile duct ligation than in normal rats, and Cmax, and AUC were approximately 2.5-fold and 6-fold greater, respectively, in the former than the latter. Approximately 17% bioavailability was obtained in rats with bile duct ligation.

Fig. 2. Plasma concentration profiles after intravenous (●) or portal vein (○) injection of DX-9065a (0.1 mg/head) in rats. Each value represents the mean ± S.E. (n = 5).

Fig. 3. Stability of DX-9065a in rat intestinal fluid at 37°C at 50 (●) and 500 (○) μg/mL of DX-9065a concentration. The data is expressed as a percentage of the initial value. Each value represents the mean ± S.E. (n = 3).

Fig. 4. The permeability of DX-9065a across everted intestine perfused with (●) or without (○) 0.02% deoxycholate. Each value represents the mean ± S.E. (n = 3).

Fig. 5. Stability of DX-9065a in rat intestinal fluid at 37°C at 50 (●) and 500 (○) μg/mL of DX-9065a concentration. The data is expressed as a percentage of the initial value. Each value represents the mean ± S.E. (n = 3).

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Interaction between DX-9065a and bile components:

The interaction between DX-9065a and bile components was examined in the equilibrium dialysis experiment. In a preliminary experiment, it was confirmed that the time to attain equilibrium of DX-9065a concentration was
over 16 h at 37°C, and that no degradation during incubation, or adsorption to the apparatus, occurred under the conditions used in the present experiment (data not shown). As shown in Fig. 5, the equilibrium concentration of DX-9065a was decreased in the presence of rat bile and almost all of the bile acids. An especially large decrease in DX-9065a concentration was observed with the addition of deoxycholate derivatives, such as deoxycholate, chenodeoxycholate and taurodeoxycholate. Moreover, the magnitude of decrease was increased with increasing DX-9065a concentration of DX-9065a was decreased in the presence of rat bile and almost all of the bile acids. An especially large decrease in DX-9065a concentration was observed with the addition of deoxycholate derivatives, such as deoxycholate, chenodeoxycholate and taurodeoxycholate. 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concentration. With deoxycholate, chenodeoxycholate and taurodeoxycholate, precipitation occurred in the presence of 10 mg/mL DX-9065a.

Discussion

DX-9065a is a zwitterion compound that has a carboxyl group and two amino groups; pKa: 3.3, 11.2 and 13.3. Therefore, DX-9065a exists in ionized form at intestinal pH, indicating that DX-9065a does not have a high degree of membrane permeability. The low lipophilicity of DX-9065a makes it far from suitable for intestinal absorption.\(^\text{13}\) The molecular weight, lipophilicity and bioavailability of DX-9065a are similar to those of certain peptide drugs such as thyrotropin-releasing hormone (TRH) analogues.\(^\text{14}\) The low oral bioavailability of azetirelin, a TRH analogue, was due to its high degree of hydrophilicity rather than to degradation in the gastrointestinal tract.\(^\text{15}\) It thus appears that the physicochemical properties of DX-9065a are in part responsible for its poor intestinal permeability.

The boundary layer theory is very useful for prediction of the human oral bioavailability of compounds that are absorbed not only by passive diffusion, but also by active transport.\(^\text{9}\) In this study, we attempted to predict the oral bioavailability of DX-9065a using the method of rat intestinal perfusion. The results of this experiment suggested that the predicted Fa of DX-9065a after oral administration was approximately 15–35% in humans, however, the actual bioavailability (2–3% in clinical trials) was significantly lower than the predicted value. These findings suggest that gastrointestinal absorption of DX-9065a is inhibited by a certain factor. The following factors were considered: 1) rate-limited dissolution, 2) the first-pass effect, 3) degradation in the gastrointestinal tract, 4) binding with the intestinal mucus, 5) influence of the efflux system, P-gp etc., 6) interaction with components of intestinal fluid. Factor 1) was negligible, because of the high solubility of DX-9065a. Consequently, the other inhibiting factors of DX-9065a absorption were evaluated in detail.

The plasma concentration profiles of DX-9065a following intravenous and portal vein injection were compared to estimate the hepatic first-pass effect. The mean plasma concentration profiles obtained following different administration routes showed no differences. This finding demonstrated that the low bioavailability of DX-9065a was not due to the hepatic and intestinal first-pass effect. DX-9065a was not degraded in rat intestinal fluids within 3 h. Furthermore, DX-9065a did not interact with the intestinal mucus. Levine and Pelikan reported that quaternary ammonium compounds interacted with intestinal mucus and formed a nonabsorbable complex.\(^\text{10}\) In addition, Niibuchi \textit{et al.} found that the intestinal absorption of gentamicin and cephaloridin was significantly increased in intestine treated with surfactant to wash off the intestinal mucin.\(^\text{11}\) In the present study, intestinal absorption of DX-9065a was not increased following washing off the mucin by surfactant treatment. These findings indicated that degradation in the gastrointestinal tract and interaction with the intestinal mucin did not affect oral absorption of DX-9065a. Consequently, factors 2), 3) and 4) did not attribute to the low bioavailability of DX-9065a.

To clarify the mechanism of membrane permeability of DX-9065a, \textit{in-vitro} perfusion experiments using rat jejunum were performed. No significant differences were observed in the permeability of DX-9065a between everted and non-everted intestine. The permeability of DX-9065a in everted intestine was not altered, even at a low temperature (4\(^\circ\)C), either. It was confirmed that DX-9065a transport across the small intestine was not saturated at the tested concentration range. These findings suggest that the mechanism of intestinal absorption of DX-9065a was passive diffusion, and that it was not affected by the efflux systems, P-gp etc. Consequently, factor 5) was not considered to be a factor of low bioavailability of DX-9065a.

Bile salts are known to increase the intestinal absorption of some poorly water-soluble drugs.\(^\text{17}\) In contrast, the bioavailability of DX-9065a after oral administration was enhanced in rats with bile duct ligation. This finding suggests that oral absorption of DX-9065a is inhibited by bile. A few studies have reported a reduction in drug absorption due to bile. Neomycin and kanamycin were reported to form precipitates with bile.\(^\text{18}\) Furthermore, it has been reported that the absorption of \(\beta\)-adrenergic blocking agents such as nadolol\(^\text{19}\) and atenolol\(^\text{20}\) is inhibited by bile salts. In an attempt to elucidate the details of the interaction between DX-9065a and bile salts, equilibrium dialysis experiments were performed. A reduction in the equilibrium concentration of DX-9065a was observed in the presence of bile acids. It is possible that macromolecular complexes were formed by the intermolecular interaction between DX-9065a and bile acid, since they could not across the cellulose membrane used in these experiments.

In conclusion, the actual human bioavailability was lower than the predicted human Fa. Our findings indicate that the low oral bioavailability of DX-9065a is due to interaction with bile components in the intestinal tract. These findings suggest that human oral bioavailability of DX-9065a may be enhanced by inhibition of the interaction of DX-9065a and bile acids in the intestinal tract.

References

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