Pharmacokinetics Research Laboratories[1]; Central Research Laboratories[2], Kissei Pharmaceutical Co., Ltd., Nagano; Chemistry Research Laboratories[3], Astellas Pharma Inc., Ibaraki; Joetsu Chemical Laboratories[4], Kissei Pharmaceutical Co., Ltd., Niigata; Department of Pharmacokinetics and Pharmacodynamics[5], Faculty of Pharmaceutical Sciences, Showa University, Tokyo, Japan

Pharmacokinetics of the amidine prodrug of a novel oral anticoagulant factor VIIa inhibitor (AS1924269-00) in rats

T. Nakabayashi[1], Y. Gotoh[2], N. Kamada[2], M. Fujioka[1], T. Ishihara[3], A. Hirabayashi[4], H. Sato[5]

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Takeshi Nakabayashi, Kissei Pharmaceutical Co.,Ltd, Pharmacokinetics, R&D, 19-48 Yoshino, 399-8710 Matsumoto-City, Nagano-Pref, Japan
takeshi_nakabayashi@pharm.kissei.co.jp

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AS1924269-00 is a promising orally applicable anticoagulant that inhibits FVIIa but has very low oral absorption. Therefore, in this study, we aimed to develop a prodrug of AS1924269-00, which possesses a carbamate-added amidine functional group, with high membrane permeability. We investigated the pharmacokinetics of the carbamate-type prodrug of AS1924269-00 in rats. The Caco-2 cell monolayer was used as an *in vitro* model and in parallel an artificial membrane permeability assay (PAMPA) was performed to examine the transport mechanisms of the prodrug. The bioavailability of the active form was determined to be only 0.3% in rats, but the oral absorption of the prodrug was markedly improved, and its bioavailability was 36%. Our *in vivo* result was consistent with the finding that compared to AS1924269-00, the prodrug showed favorable permeability in Caco-2 cells and PAMPA. We introduced carbamate into the amidine functional group of the FVIIa inhibitor, which possesses the amidine backbone, and converted it to a prodrug using carboxylic acid ethyl ester. This novel prodrug had favorable absorption and membrane permeability *in vivo* and *in vitro*. Thus, we suggest a clinical application of the carbamate-added amidine prodrug of the FVIIa inhibitor.

1. Introduction

Recently developed blood coagulation inhibitors, sibrafiban (glycoprotein IIb/IIIa receptor inhibitor) (Wittke et al. 1999), ximelagatran (antithrombin agent) (Eriksson et al. 2003), and dabigatran etexilate (antithrombin agent) (Blech et al. 2008) possess an amidine backbone, which plays a role in the inhibitory activity but reduces oral absorption; therefore, to improve oral absorption of blood coagulation inhibitors, a hydroxyl or carbamate group was further introduced at the amidine position, and the carboxylic acid was esterified. However, despite these chemical modifications, the bioavailability (BA) of the active forms was only 10–20% (Eriksson at al. 2003). Moreover, although conversion of the active forms of factor VIIa (FVIIa) and factor Xa (FXa) inhibitors to prodrugs improves pharmacokinetics in rats, the efficiency of conversion of the prodrug to the active form is low (Riggs et al. 2006; Song et al. 2003).

No previous study has shown that the conversion of blood coagulation inhibitors, which possess an amidine backbone, to prodrugs improves absorption and increases the efficiency of conversion of the prodrug to the active form *in vivo*.

We developed a novel FVIIa inhibitor with an amidine group, and we synthesized prodrugs of this inhibitor and investigated its absorption and efficiency of conversion to its active form by performing *in vitro* membrane permeability and *in vivo* pharmacokinetic (PK) studies in rats.

Abbreviations: FXa, factor Xa; FVIIa, factor VIIa; GP IIb/IIIa, glycoprotein IIb/IIIa; HPLC, high-performance liquid chromatography; LC-MS/MS, liquid chromatography-tandem mass spectrometry; PAMPA, parallel artificial membrane permeability; Papp, apparent permeability coefficient; AUC, area under the curve; BA, bioavailability; Cmax, maximum plasma concentration; Vss, volume of distribution at steady state; CLtot, total body plasma clearance; $T_{1/2}$, half-life; Fa, fraction absorbed; PK, pharmacokinetic; AP, apical; BL, basolateral; tPSA, topological polar surface area; P-gp, p-glycoprotein; MRP, multi resistance protein; AS1924269-00 (nominated as ACT), 2-{2-[({2-[(4-carbamimidoylphenyl)carbamoyl]-4-methoxyphenyl}amino)methyl]-4-(hydroxymethyl)-6-(propan-2-yloxy) phenoxy}acetic acid; AS1937090-FO (nominated as CMI), 2-[2-({[2-({4-[(amino[(ethoxycarbonyl)imino]methyl)phenyl]carbamoyl}-4-methoxy-phenyl)amino]methyl}-4-(hydroxymethyl)-6-(propan-2-yloxy)phenoxy) acetic acid; AS1932804-00 (nominated as CMP), ethyl2-[2-({[2-({4-[(amino[(ethoxycarbonyl)imino]methyl)phenyl]carbamoyl}-4- methoxy phenyl)amino]methyl}-4-(hydroxymethyl)-6-(propan-2-yloxy)phenoxy); acetate KDN-7429 (Internal standard), 2-[4-(carbamoylmethyl)-2-{[(4-chloro-2-{[4-(N'-hydroxycarbamimidoyl)phenyl]carbamoyl}phenyl)-amino]methyl}-6-ethoxyphenoxy]acetic acid.

2. Investigations and results

2.1. Transport study using Caco-2 cells

The results of the membrane permeability study using Caco-2 cells are shown in Fig. 1. The compounds tested were the prodrug, (AS1932804-00 [CMP]); the intermediate, (AS1937090-FO [CMI]); and the active form, (AS1924269-00

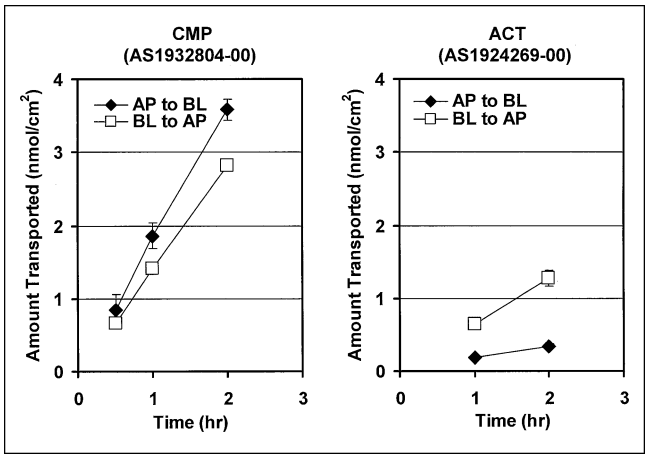


Fig. 1: Time course of transport of (A) CMP (AS1932804-00) (100 μM) and (B) ACT (AS1924269-00) (100 μM) across Caco-2 cell monolayers. Apical (AP) to basolateral (BL) transport is indicated by filled symbols and BL to AP transport is indicated by open symbols. Values are the mean of 3 measurements ± SD

[ACT]). When CMP was added, only the CMI was detected. When CMI was added, no compound was detected (ND < 0.1 μM). When the ACT was added, the ACT was detected, but the level of ACT was lower than that found after addition of the prodrug. The value of the apparent permeability coefficient (Papp) of the prodrug was 5.01×10^{-6} cm/s as CMI, which was approximately 10-fold higher than that of the ACT.

The time course of transport of CMP and ACT in both apical (AP) to basolateral (BL) and BL to AP directions across Caco-2 cell monolayers is shown in Fig. 1. The amount of CMI transported in both directions was not detected.

The amount of ACT transported in both directions increased linearly with time.

The permeability coefficient for the AP to BL transport of ACT was 0.50×10^{6} cm/s. The Papp of ACT in BL to AP direction was 3.6-fold greater than that in AP to BL direction (Table 1). These Papp are comparable with those obtained for the transport of propranolol (transcellular marker) (13.5×10^{-6} cm/s in the AP to BL direction and 12.4×10^{-6} cm/s in the BL to AP direction).

The amount of CMP transported in both directions increased linearly with time. The Papp value of CMP was 5.01×10^{-6} cm/s in the BL to AP direction, which was 10-fold greater than that of ACT (Table 1). The difference in the permeability coefficients between CMP and ACT was only 2-fold for transport in the BL to AP direction (3.56×10^{-6} cm/s for CMP and 1.80×10^{-6} cm/s for ACT). Thus, we found a marked difference in the permeability coefficients of these two compounds in terms of translocation across Caco-2 cell monolayers, which is the absorptive direction (AP to BL).

Table 1: Comparison of transport of ACT, CMI, and CMP across Caco-2 cell monolayers

		Papp (10^{-6} cm/s)		Efflux ratio
		AP to BL	BL to AP	
CMP	As CMP	ND	ND	-
	As CMI	5.01 ± 0.48	3.56 ± 0.12	0.71
	As ACT	ND	ND	-
CMI	As CMI	ND	ND	-
	As ACT	ND	ND	-
ACT	As ACT	0.50 ± 0.04	1.80 ± 0.06	3.60
Propranolol		13.5 ± 1.93	12.4 ± 0.54	0.92

Efflux ratio = Papp (BL to AP)/Papp (AP to BL)
CMP, AS1932804-00; CMI, AS1937090-FO; ACT, AS1924269-00; Each value is expressed as the mean ± SD from 3 experiments. ND, not detected

Table 2: BD-precoated parallel artificial membrane permeability assay. Apparent permeability of prodrug (CMP), intermediate (CMI), parent drug (ACT), and propranolol as a control drug in donor to acceptor direction

	Papp (10^{-6} cm/s)		
	Mean	±	SD
CMP	2.11	±	0.18
CMI		ND	
ACT		ND	
Propranolol	10.7	±	0.55

Data represent means ± SD of 3 experiments
Each value is expressed as the mean ± SD from 3 experiments. ND, not detected

Table 3: Pharmacokinetic parameters of the prodrug (carbamate type), intermediate, and active form after oral administration of CMP, CMI, and ACT to rats

Parameters		AUC (μmol·h/L)	Cmax (μmol/L)	BA (%)
		Mean ± SD	Mean ± SD	
CMP	as CMP	NC	ND	NC
	as CMI	2.43 ± 0.60	4.12 ± 0.47	1.8
	as ACT	54.3 ± 12.1	24.2 ± 8.90	36.1
	Total	56.7		
CMI	as CMI	0.59 ± 0.22	0.49 ± 0.11	0.4
	as ACT	0.25 ± 0.18	0.16 ± 0.14	0.2
	Total	0.84		
ACT	as ACT	0.40 ± 0.29	0.12 ± 0.11	0.3

Rats were orally administrated 10 mg/kg of CMP, CMI, and ACT (15.7, 16.4, and 18.6 μmol/kg). Pharmacokinetic parameters of CMP (prodrug), CMI (intermediate), and ACT (active form) were calculated as described in the Materials and Methods section. Each value is expressed as the mean ± SD from 3 rats.
ND, not detected (< 10 ng/mL) NC, not calculated

2.2. PAMPA assay

The results of the BD-precoated PAMPA membrane permeability study performed at pH 7.4 on the donor side and at pH 7.4 in the acceptor sides are shown in Table 2. The test compounds were the CMP, CMI and ACT. Compared to the ACT and CMI, CMP showed favorable membrane permeability. When CMI and ACT were added, no compounds were detected (ND < 0.1 μM). The Papp value of the prodrug was 2.11×10^{-6} cm/s as CMP.

2.3. PK Study in rats

The results of PK studies of a carbamate type prodrug (CMP), CMI and ACT in rats are shown in Fig. 2. The pharmacokinetic parameters of these compounds are shown in Table 3. The highest value of the total area under the curve (AUC) (56.73 μmol·h/L), including the prodrug, intermediate, and active form, was observed after oral administration of the prodrug, while the AUC value was as low as 0.84 μmol·h/L after oral administration of the intermediate. These findings were consistent with the Caco-2 membrane permeability-improving effect of the prodrug.

Comparison of the BA values of orally administered CMP, CMI, and ACT indicated that the BA value of CMP was the highest.

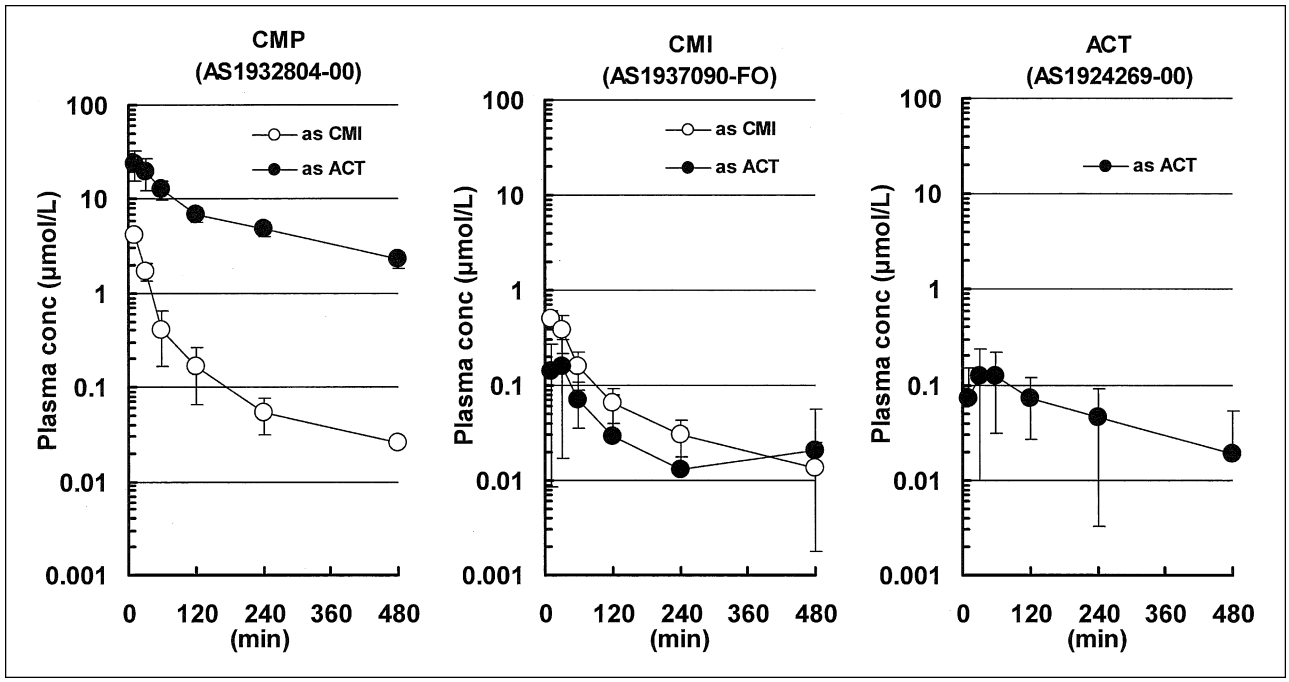


Fig. 2: Pharmacokinetic profiles of the prodrug, intermediate, and parent drug after oral administration of CMP (AS1932804-00), CMI (AS1937090-FO), and ACT (AS1924269-00) to rats. Symbols and vertical bars represent the mean ± SD from 3 rats. The vertical bars are not shown when the SD falls within the symbol

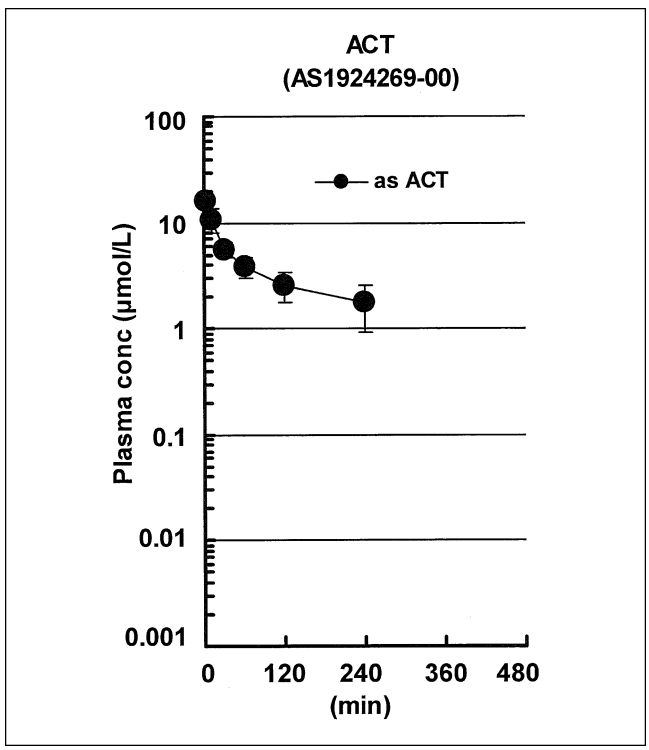


Fig. 3: Pharmacokinetic profiles after intravenous administration of ACT (AS1924269-00) to rats. Symbols and vertical bars represent the mean ± SD from 3 rats. The vertical bars are not shown when SD falls within the symbol

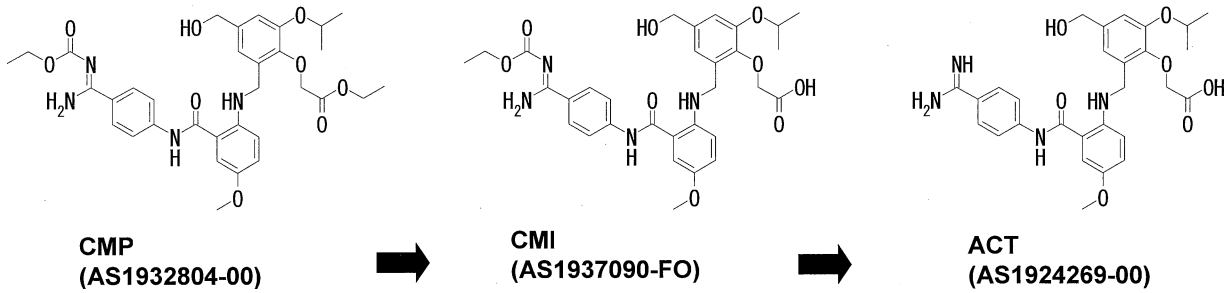
3. Discussion

Conversion of an FVIIa inhibitor (compound developed by Celera Genomics) (Riggs et al. 2006; Vijaykumar at al. 2006) and an FXa inhibitor (compound developed by Millenium Pharmaceuticals) (Song et al. 2003) to a prodrug improves the PK in rats, but the efficiency of conversion to the active form is low. Many studies have reported that conversion of active forms of drugs with poor oral absorption to prodrugs improves their membrane permeability (Hsieh et al. 2009). We investigated the improvement in absorption and efficiency of conversion to the active form to develop a novel blood coagulation FVIIa inhibitor that could be administered orally.

To our knowledge, this is the first study in which carbamate was introduced into the amidine functional group of an FVIIa inhibitor, and a prodrug-conversion strategy was combined to improve the absorption of the drug.

CMP is a prodrug of the active compound ACT, and conversion to this prodrug improved the permeability in both Caco-2 cells and PAMPA systems. Caco-2 cells assay system is a useful tool to examine the biological membrane permeability of drugs. Previous studies have shown an improvement in membrane permeability by chemical conversion to a prodrug (conversion to amidoxime) using Caco-2 cells as a strategy, which aims at improving the oral absorption of drugs (Brusewitz et al. 2006). In addition to compounds related to the blood coagulation system, an amidine compound developed as an antibacterial agent, DB75, which is also poorly absorbed after oral administration, was designed as a prodrug (methylamidoxime) for improving the membrane permeability (Zhou et al. 2002). However, long-term culture of at least 21 days is necessary to use Caco-2 cells for the evaluation of membrane permeability, which prolongs the time required to obtain experimental results. Therefore, an artificial membrane assay system, PAMPA, has recently attracted a lot of attention because of its ease of use and convenience PAMPA. PAMPA is comprised of a mixture of an organic solvent and phospholipid added to a filter. Octane, dodecane, and dodecadiene are used as organic solvents, and lecithin is used as the phospholipid (Balimane et al. 2005). Recently, artificial membrane-precoated membrane plates have been developed by Becton Dickinson (Chen et al. 2008). We used these new plates to replace Caco-2 cells for high throughput screening of drug membrane permeability at an early stage of the drug development.

However, different mechanisms are involved in drug transport across cell barriers. The important components of these are transcellular passive diffusion, paracellular diffusion, active transport with a transporter, and other types of transcytosis. In addition, the drug can be metabolized at the cell wall by cytochrome P450 3A4 (CYP3A4) and/or effluxed



Scheme 1: Metabolic pathways of CMP (AS1932804-00). CMP is converted to CMI (AS1937090-FO) and subsequently CMI is converted to ACT (AS1924269-00)

Table 4: Pharmacokinetic parameters of ACT after intravenous administration of ACT to rats

Parameters		Mean	±	SD
AUC	(μmol·h/L)	15.1	±	3.71
$T_{1/2}$	(h)	2.82	±	1.19
CLtot	(L/h/kg)	0.08	±	0.03
Vss	(L/kg)	0.28	±	0.03

Rats were intravenously administered 1 mg/kg of ACT (1.86 μmol/kg)
Pharmacokinetic parameters of ACT were calculated as described in the Materials and Methods section. Each value is expressed as the mean ± SD from 3 rats

Table 5: Physicochemical properties of test compounds

	MW	LogP	$LogD_{7.4}$	tPSA
CMP	636.71	3.86	3.86	180.03
CMI	608.65	3.36	−0.05	191.03
ACT	536.59	2.84	1.20	176.22

CMP, AS1932804-00; CMI, AS1937090-FO; ACT, AS1924269-00
The Marvin Sketch program (http://www.chemaxon.com/products/marvin) was used to evaluate the LogP, LogD, and tPSA values

by P-glycoprotein (P-gp) or multidrug resistance-related protein (MRP). Screening for Caco-2 permeability is considered more representative of human absorption *in vivo* than PAMPA. PAMPA solely provides a measure of passive diffusion whereas the Caco-2 model is a better predictor of absorption of compounds, which display active uptake or efflux or pass through the membrane via the paracellular route in humans. The results from both assays should be considered together to determine the major cause of poor drug absorption.

CMP is a carbamate prodrug, which possesses a carboxylic acid ethyl ester structure. We observed low oral absorption of the intermediate (CMI) in which the amidine functional group was present as a carboxylic acid. In contrast, blood levels of the ACT after the administration of CMP, prepared by modifying the amidine residue with carbamate in the intermediate, were markedly increased. Although several prodrug compounds are now under clinical investigation, no study has shown improvement in membrane permeability because of conversion to prodrug. The BA of the active form was only 0.3%, but that after the oral administration of a carbamate prodrug (CMP) was 36.1%, which suggested that the carbamate prodrug was rapidly hydrolyzed to its active form after oral absorption. The AUC value of CMP is about 100-fold higher than that of ACT, whereas the permeability is only 10-fold higher than that of ACT. The efflux ratio of ACT is about 5-fold higher than that of CMP.

Structural characteristics of ACT, CMI, and CMP may affect their oral absorption. The results from our *in vitro* study using the human intestinal cell model, Caco-2 cell monolayers, indicates that CMP is much more permeable than the amidine analog ACT. The transport of the drug across Caco-2 cells is significantly enhanced by masking the cationic functionalities of ACT by carbamate oxime and carboxylic acid ester moieties.

On the other hand, masking the cationic functionalities of ACT by carbamate oxime, CMI does not significantly enhance the transport across Caco-2 cells. CMI had low LogP values and high topological polar surface area (tPSA) values, and the permeability of CMI was low (Table 5).

This difference in the primary transport mechanism of these 3 compounds is consistent with the limited oral BA of ACT (Table 3).

These results provide an insight into prodrug design to improve oral absorption of a drug containing an amidine functional group. Several compounds show a good correlation between the transport through Caco-2 cell monolayers and the absorption in humans (Artursson et al. 1991). According to Artursson and Karlsson, compounds like CMP with a Papp value greater than 1×10^{-6} cm/s in Caco-2 cell monolayers may be absorbed completely from the small intestine in humans. In this study, we observed that the permeabilities of CMP in both directions of transport are not significantly different (efflux ratio, 0.7) at 100 μM, which suggest that no efflux pump was involved in the transport of CMP. The efflux ratio for ACT at 100 μM obtained in these transport studies was 3.6, which suggests that ACT may be a substrate for an efflux pump (e.g., P-gp). The potential role of P-gp in the transport of ACT and related amidino functional group is currently being investigated.

Phase I drug metabolizing enzymes (CYP3A) and some phase II enzymes that exist in the small intestine are also expressed in Caco-2 cells (Gan et al. 1997; Artursson et al. 1997). However, the activity/expression levels of some of these enzymes, e.g., CYP3A, are known to be considerably lower in Caco-2 cells in the intestinal epithelium. Therefore, the limited ability of Caco-2 cells to metabolize CMP to CMI and ACT observed in these studies does not provide sufficient information to draw conclusions about the mechanism by which the small intestine would metabolize CMP in humans.

In conclusion, we introduced carbamate into the amidine functional group of FVIIa inhibitor, which possesses an amidine backbone, and converted it to a prodrug using carboxylic acid ethyl ester. This novel prodrug showed favorable absorption and membrane permeability *in vivo* and *in vitro*. Thus, we are suggesting a clinical application of the carbamate-added amidine prodrug of the FVIIa inhibitor.

4. Experimental

4.1. Materials

AS1924269-00 (ACT), AS1937090-FO (CMI), and AS1932804-00 (CMP) (purity > 98%) were supplied by Astellas Pharma Inc (Tokyo, Japan). KDN-7429 (purity > 98%) was synthesized in Kissei Pharmaceutical Co., Ltd (Nagano, Japan). The chemical structures of these compounds are shown in Figure 1. Glucose parental solution (5%) was purchased from Otsuka Pharmaceutical Co., Ltd. (Tokushima, Japan), and heparin was purchased from Mochida Pharmaceutical Co., Ltd. (Tokyo, Japan). All

other chemicals were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), unless otherwise mentioned. All supplements and cell culture media were purchased from Invitrogen Life Technologies (Carlsbad, CA).

4.2. *In vitro experiments*

4.2.1. *Cell culture*

Caco-2 cells were obtained from the American Type Culture Collection (ATCC) (Manassas, VA) and were used between passages 40 and 50. The cells were maintained in 175-cm^2 plastic culture flasks (BD Biosciences, Franklin Lakes, NJ). Caco-2 cells were subcultured before reaching confluence; cells were treated with 0.25% trypsin and 0.25% ethylenediamine-*N,N,N′,N′*,-tetraacetic acid (EDTA) for 5–15 min at 37 °C and transferred to new flasks containing Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% non-essential amino acids, 2 mM L-glutamine, 100 U/mL penicillin/streptomycin, and 10% fetal bovine serum (FBS). The harvested cells were then seeded in 12-well Transwell cell culture chamber (3 μm, pores) (Corning Coaster, NY) at 0.5 mL/well at 0.35×10^5 cell/cm^2 for 21–25 days. They were fed basally and apically with 1.5 mL and 0.5 mL of fresh medium every other day and were incubated at 37 °C and 5% CO_2 for 21–25 days.

4.2.2. *Transport study using Caco-2 cells*

All transport experiments were performed in 12-well plates (1 cm^2 filters) on a thermostatic shaker calibrated at 37 °C with stirring of 200 rpm. Experiments were performed in a transport medium consisting of Hank's balanced salt solution (HBSS) buffered to pH 7.4 with 25 mM HEPES. Caco-2 cells were rinsed 2 times with HBSS and equilibrated at 37 °C for 1 h before transport studies. Subsequently, 0.5 mL of HBSS-containing compound (100 μM) was applied to the AP side. An aliquot of 0.1 mL was taken from the BL side (1.5 mL). At different time intervals (30, 60, and 120 min), equal volume of blank HBSS was immediately added to maintain a constant volume.

BL to AP transport was initiated by replacing the BL buffer with 1.5 mL of the drug solution after the AP medium was replaced with 0.5 mL of the transport medium. The AP solution (0.1 mL) was withdrawn and the same volume of prewarmed transport medium was added to the AP sides at 30, 60, and 120 min.

Samples were analyzed using a HPLC method. The drug concentration in the other (basal side) chamber was determined as described below. The transepithelial electrical resistance (TEER) was routinely measured using the Millicell-ERS system (Millipore Co., Bedford, MA), and the cells showed readings from 400 to 500 Ω·cm^2 before and after the transport experiments. The study was performed at pH 7.4 on the apical and basal sides.

4.2.3. *PAMPA*

The permeability assay used in this study was performed in a 96-well format using a modified PAMPA method described previously (Chen et al. 2008, Liu et al. 2003). A 96-well microtiter plate (0.3 cm^2 filters) and a 96-well filter plate (BD-precoated PAMPA plate) (Becton Dickinson, Franklin Lakes, NJ) were assembled into "sandwich" such that each composite well was separated by a 125-μm micro-filter disc (0.45-μm pores). The hydrophobic filter material of the 96-well filter plate was precoated with BD-liquid solution containing phosphatidylcholine. Subsequently, the filter plate containing 200 μL of 100 μM test compound solution (containing 1% dimethylsulfoxide) using phosphate buffer solution (pH 6.0), which constituted donor compartment (upper wells) was placed on the microtiter plate. The acceptor compartments (i.e., the lower wells) of the sandwich were hydrated using 300 μL of the phosphate buffer (pH 7.4). Then, the system was incubated at room temperature without agitation for 5 h. At the end of the incubation time, samples were removed from the lower well. A series of operations were automatically performed in Biomek FX (Beckman Coulter Inc., Brea, CA). All the permeability studies were performed in triplicate (i.e., 3 wells per compound).

4.2.4. *Preparation of Caco-2 and PAMPA samples*

To 100 μL of the samples obtained from the transport studies, 100 μL of the internal standard solution (10 μM ketoconazole) and 100 μL of acetonitrile were added. Each tube was mixed thoroughly by vortexing for 90 s. After centrifugation for 5 min at 1600 × *g*, the supernatant was transferred into clean test tubes. The supernatants were analyzed using an HPLC method.

4.2.5. *Determination of compounds by HPLC*

Samples obtained from the transport studies were determined using HPLC (CLASS-VP, Shimadzu, Kyoto, Japan) attached with a C18 reverse-phase column(ODS-3) (5 μm, 250 × 4.6 mm ID, GL Sciences, Tokyo, Japan) using a gradient system at a flow rate of 1.0 mL/min. The eluate condition was initially 10 mM ammonium acetate buffer (pH 4.5):acetonitrile (90:10) followed by a linear increase in acetonitrile to 70% acetonitrile from 0 to 14 min. The gradient was maintained at 70% acetonitrile until 19 min and then was returned to the initial running condition at 20 min. The column temperature was maintained at 50 °C. The eluate was monitored at 254 nm. Injection volume was 50 μL. Standard curves for ACT, CMI, and CMP were constructed and were found to be linear with correlation coefficients > 0.99. Precision of the assays and accuracy were assessed by adding known concentrations (0.1–30 μM). After the usual working up procedure, standard curves were constructed by linear regression analysis.

4.2.6. *Physicochemical properties*

The Marvin Sketch program (http://www.chemaxon.com/products/marvin) was used to evaluate LogP, LogD, and tPSA for ACT, CMI, and CMP. The results are summarized in Table 4.

4.2.7. *Data analysis*

Papp was estimated using the following equation: $Papp = 1/(A \cdot Co) \cdot (dQ/dt)$, where Papp is the permeability in (10^{-6} cm/s), A is the surface area, Co is the starting concentration (100 μM), and dQ/dt is the rate of change of compound quantity, in μmol/s, in the acceptor compartment with time.

4.3. *In vivo experiments*

4.3.1. *Animals*

Male Sprague–Dawley rats weighing 200–250 g and 6–7 weeks of age were purchased from Charles River (Yokohama, Japan). The animals were maintained on a 12-h light/12-h dark cycle in a room with controlled temperature (24 ± 2 °C) and acclimatized for 7 days before the study. Rats were fasted overnight with free access to water. Animal care and experiments were performed in accordance with the guidelines for the care and use of laboratory animals of the Kissei Pharmaceutical Co., Ltd.

4.3.2. *Administration of drugs*

For the oral administration of ACT, CMI, and CMP, rats were fasted for 16 h. Then each compound 10 mg/kg (18.6, 16.4, and 15.7 μmol/kg), suspended in 0.5% methyl cellulose solution was orally administered. After administration, blood samples (200 μL) were collected from the jugular vein at 15, 30, 60, 120, 240, and 480 min. For the intravenous administration of ACT, rats were fasted for 16 h and then ACT was administered via tail vein injection at a dose of 1 mg/kg in a 50% dimethylacetamide/2.5% glucose solution. After administration, blood samples (200 μL) were collected from the jugular vein at 2, 15, 30, 60, 120, 240, and 480 min and centrifuged at 1600 × *g* for 10 min to separate the plasma supernatant. To 25 μL of the plasma, 100 μL of the internal standard solution (0.1 μg/mL KDN-7429) and 875 μL of acetonitrile were added. Each tube was mixed thoroughly by vortexing for 90 s. After centrifugation for 20 min at 1600 × *g*, the supernatant was transferred into labeled clean test tubes and evaporated to dryness in an aluminum block bath at 40 °C under a stream of nitrogen. The residues were reconstituted in 100 μL of mobile phase with vortexing for 90 s. The residue solutions were analyzed using a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method.

4.3.3. *Determination of compounds by LC-MS/MS*

Chromatographic separation was performed on an Agilent HPLC (HP1100, Agilent, Santa Clara, CA) with a Cadenza CD-C18 column (3 μm, 50 × 4.6 mm, Imtakt Corporation, Kyoto, Japan) using a gradient mobile phase system at a flow rate of 0.5 mL/min. The initial chromatographic condition was 10 mM ammonium acetate buffer (pH 4.5):acetonitrile (90:10) followed by a linear increase of acetonitrile to 90% acetonitrile from 3 to 4 min. The gradient was subsequently halted at 90% acetonitrile for 7 min and was then returned to the initial condition at 7.1 min. The column temperature was maintained at 40 °C. Injection volume was 5 μL. Mass spectra were obtained using an API4000 mass spectrometer (Applied Biosystems Inc, Foster City, CA) equipped with a turbo ion-spray source. The data acquisition was ascertained by Analyst 1.4 software. Positive mode was used for the analysis. The strongest fragment of each compound was selected and was used as the Q3 ion to be monitored. The ion source parameters were set as follows: ion spray voltage = 5000 V, heated gas temperature = 600 °C. Standard curves for ACT, CMI, and CMP were constructed and were found to be linear with correlation coefficients > 0.99. Precision of the assays and

accuracy were assessed by adding known concentrations (10–50000 ng/mL). Standard curves were constructed by linear regression analysis.

4.3.4. Pharmacokinetic analysis

Pharmacokinetic parameters of ACT, CMI, and CMP, i.e., half-life ($T_{1/2}$), volume of distribution at steady state (Vss), total body plasma clearance (CLtot), and AUC from zero to end point were calculated by noncompartmental analysis using the WinNonlin ver 5.2 (Pharsight, Portland, OR).
BA was calculated as:
BA (%) = [AUC(p.o.)/Dose(p.o.)]/[AUC(i.v.)/Dose(i.v.)] × 100

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