Oligoarginine-Based Prodrugs with Self-Cleavable Spacers for Caco-2 Cell Permeation

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In the development of oligoarginine-based prodrugs with self-cleavable spacers for intestinal absorption, we previously reported a series of spacers with variable half-lives of parent compound release based on a neighboring group participation mechanism from an amino acid side-chain structure next to the succinyl moiety. In the present study, to diversify the half-life of the spacer, we first synthesized several additional fluorescein isothiocyanate ethanolamine (FE)-heptaarginine conjugates (4d—g) and evaluated their conversion time. To investigate the overall cellular uptake of FE-heptaarginine conjugates, the cellular uptakes of FE-heptaarginines 4a and 4b possessing the longest and shortest half-lives, respectively, were evaluated using HeLa cells by confocal microscopy and flow cytometry. Conjugate 4a with a longer half-life was more efficiently taken up by the cells than conjugate 4b. However, in term of the transport rate of parent FE 1 in *in vitro* Caco-2 cell permeation assay, conjugate 4b with a short half-life could function more efficiently that conjugate 4a. To understand the reason for this discrepant finding, fluorescence on the basal side medium after treatment with conjugate 4b in the permeation assay was determined. It became apparent that the fluorescence was mostly from the parent FE 1 itself, and not conjugate 4b, suggesting that the conjugate was cleaved inside the cells. Moreover, the conversion time of conjugate 4b ($t_{1/2}$=9.4 min at pH 7.4) was significantly extended in slightly acidic media. These results suggest that the conversion rate was slowed in the relatively acidic endosomal environment where the conjugate was transferred after endocytosis, and resulted in a favorable migration time across the cells. The other conjugates, including conjugate 4a, were more stable inside of the cell, resulting in very long conversion times that were ineffective in increasing the permeation rate. Therefore, spacers with shorter half lives, in order to produce a larger amount of the parent compound inside the cells are promising development for effective oligoarginine-based cargo-transporter systems to enhance intestinal absorption of parent drugs with low permeability.

Key words cell penetrating peptide; oligoarginine; self-cleavable spacer

Many kinds of cell penetrating peptides (CPPs)[1—9] including oligoarginine peptides have been widely used as attractive tools for intracellular delivery of various substances with low membrane permeability.[2,10—18] Highly cationic clusters composed of constitutive basic guanidino groups in oligoarginine peptides are known to interact with negative-charged proteoglycans on the surface of cells and internalize by macropinocytosis.[19—22] However, the precise internalization mechanism of these CPPs has yet been elaborated.

Only recently drug conjugates with oligoarginine peptides have been expected to increase the penetration of low permeable drugs through the intestinal epithelial cell layer into blood.[23—25] In our previous study, we proposed a new self-cleavable spacer in the oligoarginine-based cargo-transporter (OACT) system toward effective intestinal absorption.[25] This spacer was designed to release the parent drug from a hydrophilic oligoarginine moiety inside the cells, in order to transport the parent drug into blood through the hydrophobic basal-side cell membrane, after the conjugate had penetrated from the apical-side of cells with the assistance of oligoarginine (Fig. 1). Fluorescein isothiocyanate (FITC)-ethanolamine (FE **1**) and H-GABA-D-Arg_7-NH_2 were chosen as a drug-model with low intestinal permeability and an oligoarginine-based CPP, respectively. Using on Fmoc-based solid phase peptide synthesis (SPPS), three kinds of oligoarginine-drug model conjugates **4a**—**c**, which were connected by this chemical-triggered self-cleavable spacers, were synthesized, and their conversion times to parent FE **1** *via* an intramolecular succinimide formation was investigated.[25] The conversion times of these compounds (**4a**—**c**) were different with $t_{1/2}$ values ranging from 9 to 100 min. Moreover, the conversion time was well controlled by the neighboring-group participation of an adjacent amino acid side-chain structure to the succinyl moiety within the spacer

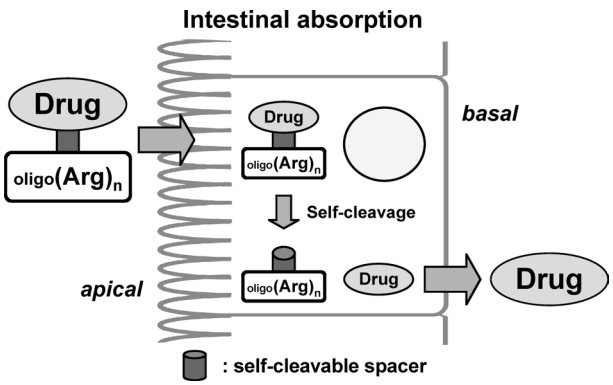

Fig. 1. A New Oligoarginine Prodrug Strategy Based on Chemically Triggered Self-Cleavage for Effective Intestinal Absorption

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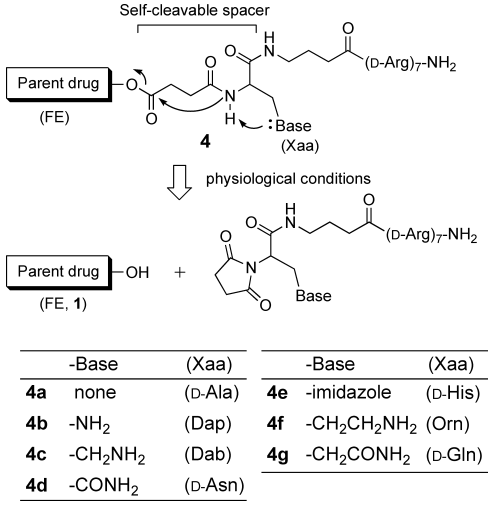


	-Base	(Xaa)		-Base	(Xaa)
4a	none	(D-Ala)	**4e**	-imidazole	(D-His)
4b	$-NH_2$	(Dap)	**4f**	$-CH_2CH_2NH_2$	(Orn)
4c	$-CH_2NH_2$	(Dab)	**4g**	$-CH_2CONH_2$	(D-Gln)
4d	$-CONH_2$	(D-Asn)			

Fig. 2. Oligoarginine-Drug Model Conjugates **4** with Self-Cleavable Spacers Based on Intramolecular Succinimide Formation

region. Furthermore, an ideal conversion time seemed to be important to increase parent compound permeability in our human intestinal cell line (Caco-2) model experiment, because only conjugate **4b** with a $t_{1/2}$ value of 9.4 min exhibited improved Caco-2 cell permeability.[25)]

In the present study, at first, to diversify the half-life of the spacer, we synthesized additional FE-heptaarginine conjugates **4d**—**g** (Fig. 2) and evaluated their conversion time. Next, we wanted to investigate the overall cellular uptake of FE-heptaarginine conjugates. The cellular uptakes of FE-heptaarginines **4a** and **4b** possessing the longest and shortest half-lives, respectively, were evaluated using HeLa cells by confocal microscopy and flow cytometry. Furthermore, the efficacy of these conjugates in *in vitro* permeation assay using Caco-2 cell monolayer was determined. The results indicated that only **4b** with a $t_{1/2}$ value of 9.4 min was the better conjugate to increase Caco-2 cells permeability. To understand the mechanism under the observed increased transport of the parent compound in **4b**, the conversion time dependency on pH, and fluorescence of FE **1** at the basal side in the *in vitro* permeation assay were determined. All synthetic details of oligoarginine conjugates **4a**—**g** are also described.

Experimental

Materials Reagents and solvents were purchased from Wako Pure Chemical Ind., Ltd. (Osaka, Japan), Nakalai Tesque (Kyoto, Japan), and Aldrich Chemical Co., Inc. (Milwaukee, WI, U.S.A.) and used without further purification. Analytical thin-layer chromatography (TLC) was performed on Merck silica gel $60F_{254}$ precoated plates. Analytical high-performance liquid chromatography (HPLC) was carried out on a C18 reverse-phase column (4.6×150 mm; YMC Pack ODS AM302) with a binary solvent system: a linear gradient of CH_3CN in 0.1% aqueous trifluoroacetic acid (TFA) at a flow rate of 0.9 ml/min, detected at UV 230 nm. Preparative TLC was performed on Merck silica gel $60F_{254}$, 2 mm precoated plates. Preparative HPLC was performed using a C18 reverse-phase column (19×100 mm; SunFire™ Prep C18 OBD™ 5 μm) with a binary solvent system: a linear gradient of CH_3CN in 0.1% aqueous TFA at a flow rate of 15 ml/min, detected at UV 228 nm and 495 nm. Solvents used for HPLC were of HPLC grade. All other chemicals were of analytical grade or better. ^{1}H nuclear magnetic resonance (NMR) spectra were obtained on a JEOL 300 MHz spectrometer with TMS as an internal standard. Fast atom bombardment mass spectrometry (FAB-MS) was performed on a JEOL JMS-SX102A spectrometer equipped with the JMA-DA7000 data system. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was performed on a Voyager-DE™ RP time-of-flight mass spectrometer equipped with the PerSeptive Biosystems.

Synthesis As shown in Chart 1, similar to a previously reported procedure for preparing FE-heptaarginines,[25)] to a solution of fluorescein isothiocyanate (FITC, 320 mg, 0.82 mmol) in DMF was added ethanolamine (75 ml, 1.23 mmol) and the reaction mixture was stirred for 2 h at room temperature. After removing DMF *in vacuo*, the residue was dissolved in EtOAc, washed with 5% citric acid and water for three-times each, dried over Na_2SO_4 to obtain title compound **1** as a yellow solid (260 mg, 70%). mp >300 °C, ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 10.10 (br s, 2H), 9.98 (s, 1H), 8.30 (s, 1H), 8.04 (br s, 1H), 7.74 (br d, J=8.4 Hz, 1H), 7.16 (d, J=8.1 Hz, 1H), 6.66 (d, J=1.8 Hz, 2H), 6.62—6.54 (m, 4H), 3.59 (m, 4H, partially overlapped with water peak). HR-MS (FAB) m/z: 451.0960 for $[M+H]^+$ (Calcd 451.0964 for $C_{23}H_{19}O_6N_2S$). *Anal.* Calcd for $C_{23}H_{18}N_2O_6S\cdot 0.5H_2O$: C, 60.12; H, 4.17; N, 6.10; Found: C, 60.38; H, 4.29; N, 6.32.

To a solution of FITC-ethanol amine **1** (200 mg, 0.444 mmol) in DMF was added succinic anhydride (49 mg, 0.490 mmol) and DMAP (18.1 mg, 0.148 mmol), and the reaction mixture was stirred for 16 h at 50 °C. After removing DMF *in vacuo*, the residue was purified by preparative TLC (20×20 cm, eluent; $CHCl_3$: MeOH : H_2O=8 : 3 : 1 upper phase) to afford title compound **2** as a yellow solid (137 mg, 56%). mp 262—264 °C, ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 9.80 (s, 1H), 8.22 (s, 1H), 7.93 (d, J=8.4 Hz, 1H), 7.75 (m, 1H), 7.58 (d, J=1 Hz, 1H), 7.09 (d, J=8.1 Hz, 1H), 6.75 (br s, 2H), 6.68 (d, J=8.4 Hz, 2H), 6.59 (d, J=8.4 Hz, 2H), 4.33 (t, J=4.8 Hz, 2H), 3.93 (dd, J=5.1, 10.1 Hz, 2H), 2.63 (m, 4H). HR-MS (FAB) m/z 551.1129 for $[M+H]^+$ (Calcd 551.1124 for $C_{27}H_{23}O_9N_2S$). *Anal.* Calcd for $C_{27}H_{22}N_2O_9S\cdot H_2O$: C, 57.04; H, 4.25; N, 4.93; Found: C, 56.82; H, 4.38; N, 4.85.

To a Rink amide AM resin (340 mg, 0.25 mmol), Fmoc-amino acids (0.75 mmol), including Fmoc-Arg(Pmc)-OH, Fmoc-GABA-OH and then Fmoc-Xaa-OH (Xaa=D-Ala, Dap, Dab, D-Asn, D-His, Orn or D-Gln) were sequentially coupled using a DIPCDI (0.75 mmol)–HOBt (0.75 mmol) method for 2 h in DMF after removal of each Fmoc group with 20% piperidine–DMF (7 ml×2 times, 2, 20 min) to obtain H-Xaa-GABA-D-Arg(Pmc)$_7$-NH-resins **3a**—**g**. One-third of this resin **3a** (Xaa=D-Ala) was reacted with compound **2** (137 mg, 0.25 mmol) by the same coupling method for 2 h and resultant protected FE-heptaarginine was deprotected with TFA–*m*-cresol–thioanisole (5.5 ml, 20 : 1 : 1) for 150 min at rt, followed by preparative HPLC purification in a 0.1% aqueous TFA–CH_3CN system to obtain FE-heptaarginine **4a** as a TFA salt. Yield: 24%; HR-MS (FAB) m/z 1798.9249 for $[M+H]^+$ (Calcd 1798.9261 for $C_{76}H_{120}O_{17}N_{33}S$), purity was >97% (HPLC analysis at 230 nm). Other FE-heptaarginines **4b**—**g** were synthesized by the same method as **4a**.

4b: Yield 16%; HR-MS (FAB) m/z: 1813.9381 for $[M+H]^+$ (Calcd 1813.9370 for $C_{76}H_{121}O_{17}N_{34}S$), purity was >96% (HPLC analysis at 230 nm).

4c: Yield: 29%; LR-MS (MADLI-TOF) m/z: 1829 for $[M+H]^+$ (Calcd 1829 for $C_{77}H_{123}O_{17}N_{34}S$), purity was >99% (HPLC analysis at 230 nm).

4d: Yield: 5%; HR-MS (FAB) m/z: 1841.9329 for $[M+H]^+$ (Calcd 1841.9319 for $C_{77}H_{121}O_{18}N_{34}S$), purity was >97% (HPLC analysis at 230 nm).

4e: Yield: 30%; HR-MS (FAB) m/z: 1864.9493 for $[M+H]^+$ (Calcd 1864.9479 for $C_{79}H_{122}O_{17}N_{35}S$), purity was >97% (HPLC analysis at 230 nm).

4f: Yield: 7%; HR-MS (FAB) m/z: 1841.9689 for $[M+H]^+$ (Calcd 1841.9683 for $C_{78}H_{125}O_{17}N_{34}S$), purity was >97% (HPLC analysis at 230 nm).

4g: Yield: 6%; HR-MS (FAB) m/z: 1855.9489 for $[M+H]^+$ (Calcd 1855.9475 for $C_{78}H_{123}O_{18}N_{34}S$), purity was >96% (HPLC analysis at 230 nm).

Conversion of FE-Heptaarginines to FE 1 *In vitro* conversion profiles of FE-heptaarginines **4a**—**g** to FE **1** were determined under physiological conditions with Krebs–Ringer bicarbonate buffer (KRBB, pH 7.4). A solution of FE-heptaarginie (0.5 mM, 10 μl) in DMSO was added to KRBB (990 μl) and the mixture was incubated at 37 °C. At the desired time point, whole samples (1 ml) were directly applied to RP-HPLC and their conversion profiles were analyzed using a C18 reverse-phase column (4.6×150 mm; YMC Pack ODS AM302) with a binary solvent system: a linear gradient of CH_3CN (0—50%, 25 min) in 0.1% aqueous TFA at a flow rate of 0.9 ml·min^{-1}, detected at UV 230 nm. Conversion time courses for compounds **4a**—**g** are shown in Fig. 3.

Cellular Uptake of FE-Heptaarginines Human cervical cancer-derived HeLa cells were maintained in α-minimum essential medium (α-MEM) with 10% heat-inactivated calf serum. These cells were grown on 100 mm dishes and incubated at 37 °C under 5% CO_2 to approximately 70%

confluence. A subculture was performed every 3—4 d.

For the flow cytometry study, HeLa cells (7.0×10^4) in fresh culture medium (1 ml) were plated into 24-well microplates (Iwaki) and cultured for 48 h in α-MEM containing 10% heat-inactivated calf serum. After complete adhesion, the cells were incubated at 37 °C for 30 min with fresh medium (200 μl) containing peptide conjugates prior to washing with PBS containing sodium heparin (0.5 mg/ml). The cells were then treated with 0.01% trypsin in PBS (200 μl) at 37 °C for 10 min prior to the addition of PBS (300 μl). The cells were centrifuged at 3000 rpm for 5 min. After the supernatant was removed, the cells were washed with 400 μl of PBS and centrifuged at 3000 rpm for 5 min. After this washing cycle was repeated, the cells were suspended in PBS (400 μl) and subjected to fluorescence analysis on a FACScalibur (BD Biosciences) flow cytometer using 488 nm laser excitation and a 515 to 545 nm emission filter.

For the confocal microscopy study, HeLa cells (2×10^5) were plated on 35 mm glass-bottomed dishes (Iwaki) and cultured in α-MEM with 10% heat-inactivated calf serum for 48 h. After complete adhesion, the culture medium was exchanged, and then the cells were incubated at 37 °C with fresh medium (200 μl) containing the peptide conjugates and washed with fresh medium (×3). Distribution of the peptide conjugates was then analyzed using a confocal scanning laser microscope FV300 (Olympus) equipped with a 40× objective without fixing the cells to avoid artifactual localization of the internalized peptides.[26]

Penetration Measurement in Caco-2 Cell Monolayer (*in Vitro* Permeation Assay) Caco-2 cells cultured on Transwell™ for 14—21 d were used for the transport assay. Transepithelial electric resistance (TEER) was measured to ensure cell monolayer integrity. Cell monolayers with TEER values greater than 500 $\Omega\cdot m^2$ were used in transport experiments. After removal of the culture medium, the cells were washed once with KRBB (pH 7.4), and preincubated for 10 min in KRBB at 37 °C. To analyze the transport of the samples from the apical-to-basal side, a stock solution of each sample (2.5 mM) in 0.01 N HCl was diluted 50 times with KRBB (pH 7.4) and this solution (500 μl) was immediately applied to the apical side (final concentration: 50 μM) after removing the preincubation media from the apical and basal sides. Fresh KRBB (1.5 ml) was also added to the basal side. The cells were incubated at 37 °C. An aliquot of each sample was taken from each basal medium at 3 or 5 time points (15, 30, 45, 60, 120 min) and the fluorescence intensity was measured using a microplate reader with an excitation wavelength at 485 nm and emission wavelength at 535 nm. FE **1** and FITC-GABA-(D-Arg)$_7$-NH$_2$ **5** were used as controls (50 μM in KRBB, pH 7.4). The results are shown in both Fig. 5A (**4b**—**d**, **1**, **5**) and Fig. 5B (**4a**, **e**—**g**, **1**, **5**).

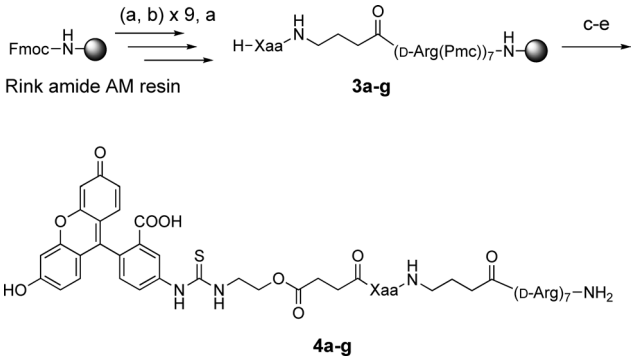
Paap Analysis From the *in vitro* permeation assay, the apparent permeability coefficient (Papp) was calculated using the equation:

$$\text{Papp (cm/s}\times10^6) = \text{Flux}/(A\times C_0\times 60)$$

where Flux (nmol/min) is the slope of the plot's best fitted line, (*i.e.* penetrated quantity per minute); A (cm^2) is the area of the membrane; and C_0 (μM) is the initial concentration of the applied sample solution (in this case, calculated as Area=1.12, and C_0=50 was a constant).

Fluorophotometric Analysis of Basolateral Medium for Compound 4b After the cells were incubated at 37 °C for 15 and 60 min in *in vitro* permeation assay, 1 ml of each basolateral medium, from the six Transwells from which the same **4b** was treated, was collected (total, 6 ml), acidified with 1 N HCl (0.5 ml) to stop the conversion, and then lyophilized. After lyophilization, each resultant powder was dissolved in 0.1 M HCl–DMSO (600 μl, 1 : 1) and the solution (50 μl) was injected to fluorophotometric HPLC to measure the fluorescence intensity using a linear gradient of CH_3CN (10—42%) in 0.1% aqueous TFA over 16 min with a flow rate of 1.0 ml/min detected at Ex. 485 nm and Em. 535 nm. Standard compounds **1** and **4b** were analyzed under the same HPLC conditions for comparison. The Fluorophotometric HPLC profiles are shown in Fig. 6.

pH-Dependent Conversion of Compound 4b *In vitro* conversion profiles of FE-heptaarginine **4b** to the FE **1** were determined under acidic conditions with KRBB (pH 6.5, 5.5). A solution of compound **4b** (1 mM, 15 μl) in DMSO was added to KRBB (985 μl) and the mixture was incubated at 37 °C. At the desired time point, a portion of the sample (200 μl) was applied to RP-HPLC and their conversion profiles were analyzed using a C18 reverse-phase column (Chromolith™ Performance RP-18 endcapped 100—4.6 mm) with a binary solvent system: a linear gradient of CH_3CN containing 0.1% TFA (5—37%, 8 min) in 0.1% aqueous TFA at a flow rate of 4 ml/min, detected at UV 215 nm.



Reagents and conditions: (a) 20% piperidine/DMF; (b) Fmoc-Arg(Pmc)-OH×7, Fmoc-GABA-OH, then Fmoc-Xaa-OH (Xaa: D-Ala, Dap(Boc), Dab(Boc), D-Asn(Trt), D-His(Trt), Orn(Boc) or D-Gln(Trt), respectively) in turn, DIPCD, HOBt, DMF; (c) FITC-ethanolamine monosuccinate **2**, DIPCD, HOBt, DMF; (d) TFA, thioanisole, *m*-cresol; (e) preparative HPLC.

Chart 1

Results and Discussion

Synthesis As shown in Chart 1, similar to a previously reported procedure,[25] seven kinds of FE-heptaarginines **4a**—**c**[25] and **4d**—**g** were designed and synthesized. Briefly, FE **1**, prepared by coupling FITC with ethanolamine in DMF, was reacted with succinic anhydride in the presence of 4-dimethylaminopyridine (DMAP) in DMF to afford FE-monosuccinate **2**. Protected peptide resins **3a**—**g** were synthesized by Fmoc-based SPPS. After loading Fmoc-D-Arg(Pmc)-OH (Pmc: 2,2,5,7,8-pentamethylchroman-6-sulfonyl), to a Rink amide AM resin by DIPCD-HOBt method (DIPCD: 1,3-diisopropylcarbodiimide; HOBt: 1-hydroxybenzotriazole), peptide chains were elongated by the same coupling method and the respective Fmoc groups were deprotected with 20% piperidine/DMF to obtain H-Xaa-GABA-D-Arg(Pmc)$_7$-NH-resins **3a**—**g**. Compound **2** was then reacted to peptide-resins **3a**—**g** using the same coupling method. Finally, the peptide resins were deprotected with a TFA–thioanisole–*m*-cresol system and the resultant crudes **4a**—**g** were purified by HPLC as TFA salts. A control peptide, FITC-GABA-(D-Arg)$_7$-NH$_2$ (**5**), was also synthesized by similar SPPS.

Conversion of Compounds 4a—g to FE 1 In addition to the previously reported compounds **4a**—**c**,[25] the conversion time ($t_{1/2}$) for synthesized FE-heptaarginines **4d**—**g** to FE **1** was evaluated by HPLC under physiological conditions (Krebs–Ringer bicarbonate buffer, KRBB, pH 7.4, 37 °C). As shown in Table 1 and Fig. 3, in combination with the previously reported data,[25] conjugate **4a** having D-Ala with no basic side chain functionality next to the succinyl moiety (Xaa site), exhibited the longest $t_{1/2}$ of *ca.* 106 min, while the basic side chains of FE-heptaarginines **4b**—**g** in the spacer significantly shortened the conversion time. Especially, Dap (L-diaminopropanoic acid) derivative **4b**, which is expected to undergo a nucleophilic neighboring-group participation through a five-membered ring intermediate, exhibited a quick conversion with a $t_{1/2}$ value of 9.4 min and almost no side reaction was observed from this conversion as previously reported.[25] Dab (L-diaminobutanoic acid) and D-Asn derivatives **4c** and **4d**, respectively, going through a six-membered ring intermediate, showed a slightly longer conversion time ($t_{1/2}$=18, 28 min, respectively). The difference in conversion times between **4c** and **4d** indicates that the more nucleophilic

Table 1. Biological Evaluations of FE-Heptaarginines

Compd	Xaa	Ring size of intermediate	$t_{1/2}$ [a] (min)	$Papp_{AB}$ [b] ($cm/s \times 10^{-6}$)
4a [c]	D-Ala	—	106±2.6	0.41±0.08
4b [c]	Dap	5	9.4±0.10	0.75±0.10
4c [c]	Dab	6	18±0.37	0.48±0.09
4d	D-Asn	6	28±0.69	0.50±0.06
4e	D-His	6	65±1.2	0.44±0.08
4f	Orn	7	85±2.1	0.41±0.07
4g	D-Gln	7	88±3.2	0.33±0.04
FE **1** [c]	—	—	—	0.26±0.11
5 [c,d]	—	—	—	0.38±0.01
LY [e]	—	—	—	0.18±0.02

a) Values were calculated from each degradation profile (Fig. 3). *b*) Values were calculated from the results of *in vitro* model permeation assays (Fig. 4), with S.E.M. obtained from 8 (**4a**—**g**), 6 (FE **1** and **5**) or 4 (**6**) experiments. *c*) See ref. 25. *d*) **5**: FITC-GABA-$(D\text{-}Arg)_7$-NH_2. *e*) LY: lucifer yellow. —: not applicable.

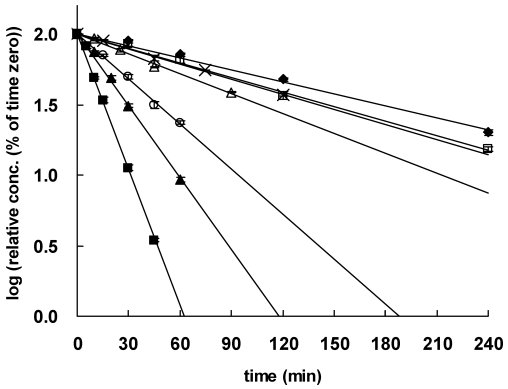


Fig. 3. Conversion of FE-Heptaarginines **4a**—**g**

Black diamond: **4a**; black square: **4b**; black triangle: **4c**; white circle: **4d**; white triangle: **4e**; ×-mark: **4f** and white square: **4g**. The conjugate concentrations at each time point are represented as relative percentages to the starting concentrations. Plots are means±S.E.M. of three experiments in KRBB (pH 7.4 at 37 °C). Data for **4a**—**c** were obtained from ref. 25.

amine in Dab increased the conversion rate, although this effect is not as significant as the steric stability of the intermediate participating neighboring-groups (*i.e.*, ring size). FE-heptaarginines **4f** and **4g** with respective Orn and D-Gln, undergoing a seven-membered ring intermediate, exhibited much longer conversion times ($t_{1/2}$=85, 88 min, respectively). These results suggest a correlation between the chemical structure of Xaa and conversion time. Interestingly, a conversion time of 65 min was observed for D-His derivative **4e**. Thus, by introducing amino acids of different basicity to the Xaa position in the novel spacers, we successfully developed FE-heptaarginines conjugates with variable conversion times ranging from 9 to 106 min, resulting in controllable release of FE **1**.

Cellular Uptake of FE-Heptaarginines We investigated the overall cellular uptake of FE-heptaarginines using HeLa cells. The cellular uptakes of FE-heptaarginines **4a** and **4b** possessing the longest and shortest half-lives, respectively, were evaluated by confocal microscopy and flow cytometry. In confocal microscopy, the cells were incubated at 37 °C with fresh medium (200 μl) containing the peptide conjugates and observed after washing with serum-containing medium (×3). In flow cytometry, FE-heptaarginines **4a**, **4b** and FE **1** (50 μM each) were added to HeLa cells, incubated at 37 °C in α-MEM containing 10% serum, washed, and trypsinized. The amount of the respective peptide taken up by these cells was then analyzed by FACS. The majority of the cell-surface adsorbed peptides were removed by heparin-containing (0.5 mg/ml) PBS wash and trypsin treatment, and thus the data reflected the total cellular uptake of the peptides (see Experimental).[27] As shown in Fig. 4A, a brighter intracellular fluorescence in the 120 min treatment of FE-heptaarginines **4a** and **4b** than that of FE **1** was observed by microscopy. Corresponding results that both FE-heptaarginines **4a** and **4b** could increase intracellular fluorescence were obtained by flow cytometric analysis (Fig. 4B). Although FE-heptaarginine **4a** or **4b** in the 120 min incubation increased or slightly increased the total amount of fluorescence delivered into cells than that in the 30 min incuba-

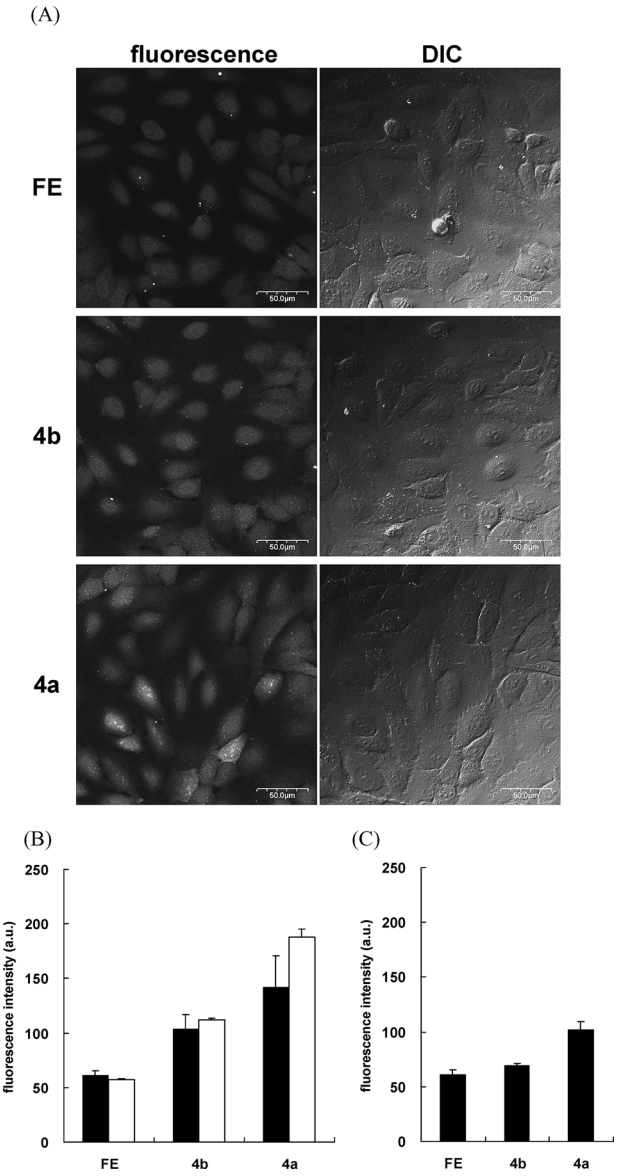


Fig. 4. Cellular Uptake of FE-Heptaarginines

(A) Confocal microscopic images; cell line: HeLa; incubation: 50 μM, 120 min, 37 °C, α-MEM(+); cells were washed three times with α-MEM(+) after incubation and then observed by confocal microscopy. (B, C) Flow cytometric analysis; cell line: HeLa; incubation: 50 μM, 30 min (B, black), 120 min (B, white) 30 min after cell-free pre-incubation for 30 min (C), 37 °C, α-MEM(+); means±S.D. of three experiments are shown in flow cytometric analysis.

tion, respectively (Fig. 4B), FE-heptaarginine **4a** more effectively increased intracellular fluorescence than conjugate **4b**. Furthermore, under the conditions of cell-free pre-incubation for 30 min before adding the conjugates to the cells (Fig. 4C), only FE-heptaarginine **4a** enhanced intracellular fluorescence relative to FE **1** alone. Consequently, we concluded that FE-heptaarginine **4a** possessing a longer conversion time was continuously taken up into the cells for 120 min, while the cellular uptake of FE-heptaarginine **4b** having a $t_{1/2}$ value of 9.4 min was mostly completed within 30 min, probably due to the self-cleavage of the heptaarginine moiety outside the cells.

***In Vitro* Permeation Assay** To investigate the efficacy of FE-heptaarginine conjugates on parent drug intestinal cell permeation after cellular uptake, *in vitro* permeation assays using Caco-2 cell monolayer were performed.[28,29] The quantity of compounds permeated to the basal side was evaluated as fluorescence intensity, derived from both FE-heptaarginine and produced FE **1**, in the basal medium. Paap values were calculated based on this basal fluorescence. Among all FE-heptaarginine including previously reported **4a**—**c**,[25] conjugate **4b** having a $t_{1/2}$ value of 9.4 min increased the transport rate of FE **1** by at least two times higher than FE **1** alone. Other FE-heptaarginine with longer conversion times exhibited similar or slight increase in permeation over FE **1** alone (Table 1, Fig. 5). To investigate the mechanism that increased permeation in conjugate **4b**, compounds with fluorescence in the basal medium were analyzed by fluorophotometric HPLC. A tiny amount of conjugate **4b** was detected only in the 15 min incubation. The major fluorescence detected was mostly from FE **1** in both 15 and 120 min incubations (Fig. 6). The Papp value for lucifer yellow (LY), a paracellular transport marker, was only 0.18 ± 0.02, suggesting that paracellular transport is not involved in increasing the transport rate in **4b**. These results suggest that compound **4b** is transported intracellularly, mostly converted to its FE **1**, and then migrates to the basal medium.

In the cellular uptake of fluorescence in HeLa cells and *in vitro* permeation of parent FE **1** in Caco-2 cells experiments, the observed discrepancy between FE-heptaarginines **4a** and **4b** with different FE **1** formation rates might be explained as follows; FE-heptaarginine **4a** with a longer half-life could be taken up intracellularly better than conjugate **4b** with a shorter half-life. However, the intracellular free FE **1** concentration would be increased for conjugate **4b** more quickly than that of conjugate **4a**. The reason is that self-cleavable elimination of the highly hydrophilic heptaarginine moiety and the formation of relatively hydrophobic and small molecular weight FE **1**, would lead to enhanced transport of FE **1** to the basal medium.

pH-Dependent Conversion of Compound 4b We evaluated the conversion of the conjugates to FE **1** under different pH conditions such as 7.4, 6.5 and 5.5. In FE-heptaarginine **4b** with enhanced transport of FE **1**, in spite of being at $t_{1/2}=9.4$ min at pH 7.4, the conversion time extended to 15 and 59 min at pH 6.5 and 5.5, respectively. A similar pH-dependent succinimide formation has been described in water-soluble prodrug studies based on a chemical mechanism.[30] In cellular assay, the prolonged conversion to FE **1** under slightly acidic conditions in endosomes (pH >5) is presumably associated with the observed gradual transport of FE **1**

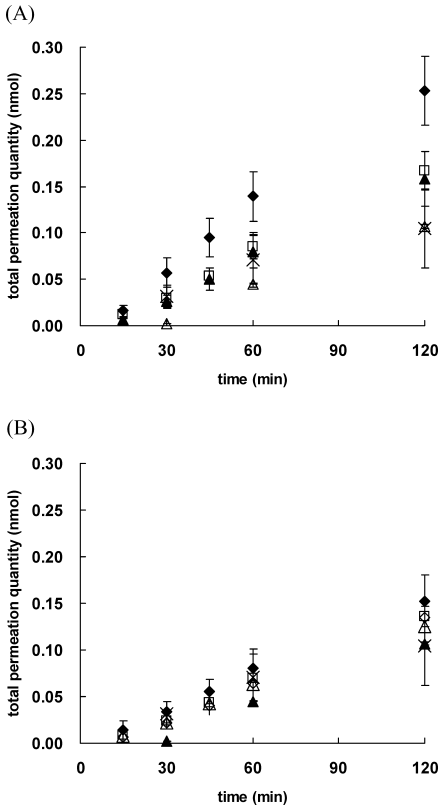


Fig. 5. *In Vitro* Permeation Assay

(A) Compounds **4b**—**d** (possessing shorter half life), FE **1** and **5**. Black diamond: **4b**; black triangle: **4c**; white square: **4d**; ×-mark: FE **1** and white triangle: **5**. Plots are means±S.E.M. of 8 (**4b**—**d**) or 6 (FE **1**, **5**) experiments. (B) Compounds **4a**, **e**—**g** (possessing longer half-lives), FE **1** and **5**. White diamond: **4a**; black diamond: **4e**; white square: **4f**; black triangle: **4g**; ×-mark: FE **1** and black triangle: **5**. Plots are means±S.E.M. of 6 (FE **1**, **5**) or 8 (**4a**, **e**—**g**) experiments.

in FE-heptaarginine **4b**, up to 120 min despite its very short $t_{1/2}$ value at pH 7.4, that resulted in at least two-times higher transport than FE **1** alone. The other oligoarginine conjugates with longer conversion times are expected to be more stable under endosomal conditions and might result in slower transportation rates in the Caco-2 permeation assay. Additionally, the quantum efficiency of fluorescein is known to be reduced with environmental acidification.[31] Endosomal fluorescence of conjugates and/or FE **1** that was taken up by the cells *via* endocytosis might fade, and the amount of internalized fluorescence might be underestimated in our flow cytometric analysis. Taking into account all of these considerations, a shorter conversion time at pH 7.4 is effective for improving permeability, and thereby suggesting the importance of conversion time controlled by the self-cleavable spacer.

Because 1) the conversion time from the conjugate to FE **1** is dependent on the chemical structure of the spacer and the pH of the environment, 2) FE **1** itself, which was added to the apical medium, did not enhance cellular transport as shown in Table 1, 3) the detected major fluorescence in the basal medium was mostly derived from FE **1** and not from conjugate **4b** that was added to the apical medium in Caco-2 permeation assay (Fig. 6), and 4) a conjugate can internalize into the cell using the oligoarginine moiety, it is reasonable to consider that the conjugate was delivered into the cells and most FE **1** was released from FE-heptaarginine **4b** intracellularly, then transferred to the basal medium.

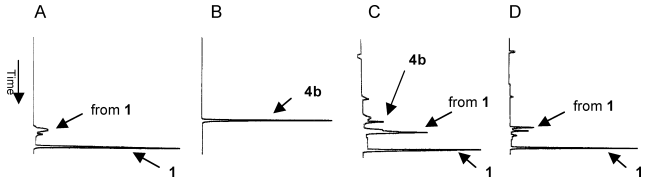


Fig. 6. Fluorophotometric HPLC Profiles

(A) **1** alone, (B) **4b** alone, (C) a sample taken from the basal medium at 15 min after **4b** was added to the apical medium, and (D) a sample taken from the basal medium at 120 min after **4b** was added to the apical medium.

Although the sterically unhindered ester in the present self-cleavable model drug-oligoarginine conjugates might be hydrolyzed by esterases in *in vivo* intestinal fluids, a more sterically hindered ester, to be developed in future studies, could be effective in avoiding such enzymatic cleavage, when taking into consideration that our previously developed chemically cleavable water-soluble paclitaxel prodrug (isotaxel), which has a bulky secondary ester bond, was stable against esterases.[30] This design could realize into a practical OACT system based on chemical cleavage for effective intestinal absorption of a parent drug. Consequently, our system might be applicable to orally non-bioavailable or relatively low-bioavailable drugs such as taxoids and aspartic protease inhibitors[30,32,33] that contain bulky secondary hydroxyl moieties. Furthermore, we believe that this system is useful for the design of prodrugs, because the parent drugs are quantitatively reproduced.

In conclusion, oligoarginine-model drug conjugates possessing a series of peptidic self-cleavable spacer, converting to their parent drugs with different half-lives *via* succinimide formation, were developed. The conversion time is controlled by a basic amino acid side-chain structure next to the succinyl moiety on the spacer. Caco-2 permeation assays indicated that an ideal time-dependent self-cleavage of the peptide spacer seems to be important for improving permeability of drugs. These novel peptidic self-cleavable spacers with shorter conversion times are promising for the development of safe and effective oligoarginine-based cargo-transporter (OACT) systems, to enhance Caco-2 cell permeability of parent drugs with low permeability, as well as intracellular delivery of substances with low membrane permeability.

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