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

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Received November 23, 2007; accepted August 26, 2008; published online September 2, 2008

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 (t1/2=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 negatively 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-Arg7-NH2 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 t1/2 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.

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

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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 \textit{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 \textit{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\(_3\)CN 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\textsuperscript{TM} Prep C18 OBD\textsuperscript{TM} 5 μm) with a binary solvent system: a linear gradient of CH\(_3\)CN 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.\(^{1}\)

\(^1\)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\textsuperscript{TM} 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 \textit{vacuo}, the residue was dissolved in EtOAc, washed with 5% citric acid and water for three-times each, dried over Na\(_2\)SO\(_4\), to obtain title compound 1 as a yellow solid (260 mg, 70%), mp >300 °C.\(^{1}\)H-NMR (300 MHz, DMSO-d\(_6\)) \(\delta\): 10.10 (br s, 2H), 9.98 (s, 1H), 8.30 (s, 1H), 8.04 (brs, 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) \(m/z\): 545.0960 for [M+H]\(^+\) (Calcd 545.0964 for C\(_{25}\)H\(_{22}\)N\(_2\)O\(_9\)S·0.5H\(_2\)O). Anal. Calcd for C\(_{25}\)H\(_{22}\)N\(_2\)O\(_9\)S·0.5H\(_2\)O: C, 56.82; H, 4.38; N, 4.93.

To a solution of FITC-ethanolamine \(1\) (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 at 16 °C for 50 h. After removing \textit{in vacuo}, the residue was purified by preparative TLC (20×20 cm, eluent; CHCl\(_3\) : MeOH : H\(_2\)O 15:15:1). The results indicated that only conjugate 4b with a \(t_{1/2}\) value of 9.4 min exhibited improved Caco-2 cell permeability.\(^{(25)}\)
confluence. A subculture was performed every 3—4 d.

For the flow cytometry study, HeLa cells (7.0 × 10⁴) in fresh culture medium (1 ml) were plated into 24-well microplates (Corning) 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 0.4% NaCl to remove excess peptide. The cells were then washed with PBS for 10 min to remove any unbound peptide. 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 × 10⁴) were plated on 35 mm glass-bottomed dishes (MatTek) 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 (Olympus) equipped with a 40× objective without fixing the cells to avoid artificial localization of the internalized peptides.29

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 for the transport assay. Transepithelial electric resistance (TEER) was measured to ensure cell monolayer integrity. Cell monolayers with TEER values above 1000 Ω·cm² 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 (50 μl) was immediately applied to the apical side (final concentration: 50 μM) containing the peptide conjugates and washed with fresh medium (×3). Distribution of the peptide conjugates was then analyzed using a confocal scanning laser microscope (Olympus) equipped with a 40× objective without fixing the cells to avoid artificial localization of the internalized peptides.29

**Results and Discussion**

Synthesis As shown in Chart 1, similar to a previously reported procedure,25 seven kinds of FE-heptaarginines 4a—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-di-methylaminopyridine (DMAP) in DMF to afford FE mono-succinate 2. Protected peptide resins 3a—g were synthesized by Fmoc-based SPPS. After loading Fmoc-Arg(Pmc)-OH (Pmc: 2,2,5,7,8-pentamethylchroman-6-sulfonil), to a Rink Amide resin by DIPC-DHBt method (DIPC: 1,3-di-isopropylcarbodiimide; DHBt: 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)NH₂ 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 crude 4a—g were purified by HPLC as TFA salts. A control peptide, FITC-GABA-d-Arg(Pmc)NH₂ (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 (t₁/₂) for synthesized FE-heptaarginines 4d—g to FE 1 was evaluated by HPLC under physiological conditions (Kreb’s–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 t₁/₂ 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-diaminopropionic 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 t₁/₂ 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 4e and 4d, respectively, going through a six-membered ring intermediate, showed a slightly longer conversion time (t₁/₂ = 18, 28 min, respectively). The difference in conversion times between 4c and 4d indicates that the more nucleophilic

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**Diagram**

![Diagram](image)

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**Table**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 5.5, 5.5</td>
<td>5.0—5.5</td>
</tr>
<tr>
<td>pH 6.5, 5.5</td>
<td>5.0—5.5</td>
</tr>
<tr>
<td>pH 7.4, 7.4</td>
<td>5.0—5.5</td>
</tr>
</tbody>
</table>

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**Figure**

![Figure](image)
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 (ranging from 9 to 106 min, resulting in controlable release 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).

Table 1. Biological Evaluations of FE-Heptaarginines

<table>
<thead>
<tr>
<th>Compd</th>
<th>Xaa</th>
<th>Ring size of intermediate</th>
<th>$t_{1/2}^{a,b}$ (min)</th>
<th>$P_{app}^{a,b}$ (cm/s × 10$^{-6}$)</th>
</tr>
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<tbody>
<tr>
<td>4a$^{c}$</td>
<td>D-Ala</td>
<td>—</td>
<td>106±2.6</td>
<td>0.41±0.08</td>
</tr>
<tr>
<td>4b$^{d}$</td>
<td>Dap</td>
<td>5</td>
<td>94±0.10</td>
<td>0.75±0.10</td>
</tr>
<tr>
<td>4c$^{e}$</td>
<td>Dab</td>
<td>6</td>
<td>18±0.37</td>
<td>0.48±0.09</td>
</tr>
<tr>
<td>4d</td>
<td>d-Asn</td>
<td>6</td>
<td>28±0.69</td>
<td>0.50±0.06</td>
</tr>
<tr>
<td>4e</td>
<td>d-His</td>
<td>6</td>
<td>65±1.2</td>
<td>0.44±0.08</td>
</tr>
<tr>
<td>4f</td>
<td>Orn</td>
<td>7</td>
<td>85±2.1</td>
<td>0.41±0.07</td>
</tr>
<tr>
<td>4g</td>
<td>d-Gln</td>
<td>7</td>
<td>88±3.2</td>
<td>0.33±0.04</td>
</tr>
<tr>
<td>FE 1$^{i}$</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.26±0.11</td>
</tr>
<tr>
<td>5$^{e}$</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.38±0.01</td>
</tr>
<tr>
<td>LY$^{i}$</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.18±0.02</td>
</tr>
</tbody>
</table>

$^{a}$) 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-Arg)-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.

**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.\textsuperscript{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,\textsuperscript{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 Paap 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 produg studies based on a chemical mechanism.\textsuperscript{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 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 transport rates in the Caco-2 permeation assay. Additionally, the quantum efficiency of fluorescein is known to be reduced under endosomal conditions and might result in slower transport rates in the Caco-2 permeation assay. Additionally, the quantum efficiency of fluorescein is known to be reduced with environmental acidification.\textsuperscript{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.
Although the sterically unhindered ester in the present self-cleavable model drug-oligoarginine conjugates might be hydrolyzed by esterases in 
\textit{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 (iso-taxel), which has a bulky secondary ester bond, was stable against esterases. 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 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 succinimide 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 drugs. These novel peptidic self-cleavable spacers with shorter conversion times are promising for the development of drugs with low permeability, as well as intracellular delivery of substances with low membrane permeability.

Acknowledgements This research was supported by various grants from MEXT (Ministry of Education, Culture, Sports, Science and Technology-Japan, including the 21st Century COE Program and Japan Society for the Promotion of Science’s Post-Doctoral Fellowship for Foreign Researchers. K. T. is grateful for JSPS Research Fellowship for Young Scientists. We are grateful to Ms. K. Oda and Mr. T. Hamada for mass spectra measurements.

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