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Development of oligoarginine–drug conjugates linked to new peptidic self-cleavable spacers toward effective intestinal absorption

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Abstract—We designed and synthesized new peptidic self-cleavable spacers that released a parent drug via succinimide formation and the oligoarginine-based cargo-transporter (OACT) system. The self-cleavable efficacy of these compounds was studied and the conversion time was controlled by an amino acid side-chain structure next to the succinyl moiety on the spacer. These novel self-cleavable spacers are promising for developments of the OACT system as means to potentially enhance intestinal absorption of parent drugs.

Various cell penetrating peptides (CPPs),[1–9] including human immunodeficiency virus type-1 (HIV-1) transactivator of transcription (Tat) protein derived peptide Tat(48–60),[2,6] penetratin,[4,7] and arginine-rich peptides,[8,9] are currently considered as attractive tools for intracellular delivery of substances with low membrane permeability, such as proteins,[2] oligonucleotides,[10] liposomes,[11–14] certain drug classes,[15,16] and non-covalent supra-molecular complexes.[2,17,18] High cell membrane permeability of these CPPs is mostly attributed to clusters of constitutive basic amino acids, especially the oligoarginine residues with their cationic guanidino clusters.[5,9] These highly cationic clusters can interact with negative charges on the surface of cells, thereby promoting cell permeation.[19,20] However, although a mechanism by macropinocytosis has been proposed,[21,22] the precise internalization mechanism of CPPs has not yet been clarified.

Drugs conjugated with oligoarginine have recently also been expected to increase penetration of low permeable drugs through the intestinal epithelial cell layer into blood.[23,24] Liang et al. reported stable covalent conjugation between a Tat-derived arginine-rich peptide and insulin, that effectively reduced blood glucose level when compared with insulin alone in oral administration.[24] In our preliminary human intestinal cell line (Caco-2) model experiment with a series of covalently stable fluorescein isothiocyanate (FITC)-arginine conjugates, only a slight increase in permeability was observed (data not shown). This result raised the possibility that, once the conjugates are internalized, most are retained in the intracellular compartment presumably due to the positive charges of oligoarginines. Herein, we proposed a novel self-cleavable spacer strategy.

In our previous study on water-soluble prodrugs of an HIV protease inhibitor (PI) with very low water-solubility, we developed a series of water-soluble auxiliaries possessing (1) chemical-triggered self-cleavable spacers and (2) solubilizing moieties containing an ionized amine function.[25,26] In these prodrugs, conversion to the parent drug via an intramolecular imide formation could be well controlled by the neighboring-group participation effect near the ionized basic functional group on the solubilizing moieties. We obtained water-soluble prodrugs of an HIV-PI exhibiting a wide range of

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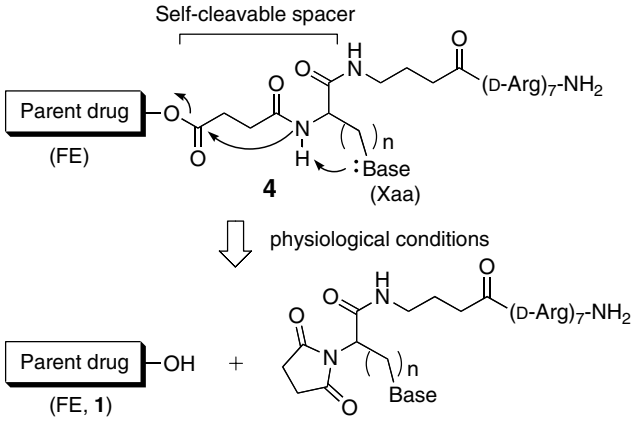


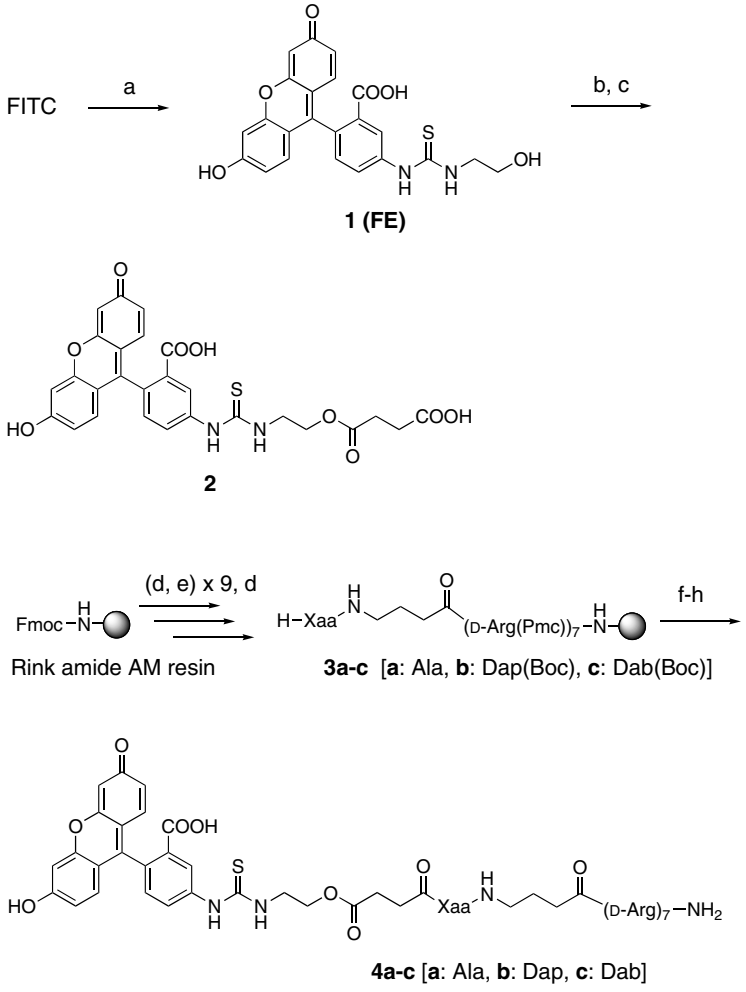
	-Base	(Xaa)
4a	none	(D-Ala)
4b	$-NH_2$	(Dap)
4c	$-CH_2NH_2$	(Dab)

Figure 1. A new peptidic self-cleavable spacer based on chemical-triggered intramolecular cyclization.

conversion times, which is crucial for increasing intestinal absorption of the parent drug.

From this background knowledge, in the present study, FITC-ethanolamine (FE, **1**) was chosen as a drug-model with low intestinal permeability, and FE–heptaarginines conjugated through new peptidic spacers were synthesized on Fmoc-based solid phase peptide synthesis (SPPS). These peptide spacers can self-catalyze under physiological conditions via an intramolecular cyclization reaction through imide formation that is triggered by the basic side chains of adjacent amino acids (Fig. 1). A CPP [D-heptaarginine] was adopted to avoid enzymatic peptide bond degradation during cell penetration. Additionally, γ-aminobutyric acid (GABA) was inserted between the self-cleavable spacer and D-heptaarginine moiety to reduce undesired effects associated with highly basic guanidine moieties.

As shown in Scheme 1, three kinds of FE–heptaarginines **4a**–**c** were designed and synthesized. Parent **1**, prepared by coupling FITC with ethanolamine in DMF, was reacted with succinic anhydride in the presence of



Scheme 1. Reagents and conditions: (a) ethanolamine, DMF; (b) succinic anhydride, DMAP, DMF, 50 °C; (c) preparative TLC; (d) 20% piperidine/DMF; (e) Fmoc-Xaa-OH (Xaa: Ala, Dap(Boc) or Dab(Boc), respectively), DIPCD, HOBt, DMF; (f) **2**, DIPCD, HOBt, DMF; (g) TFA, thioanisole, *m*-cresol; (h) preparative HPLC.

4-dimethylaminopyridine (DMAP) in DMF to afford FE-monosuccinate **2**. Protected peptide resins **3a**–**c** 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 (1,3-diisopropylcarbodiimide)–HOBt (1-hydroxy-benzotriazole) method, peptide chains were elongated by the same coupling method and the respective Fmoc groups were deprotected with 20% piperidine/DMF to obtain resins **3a**–**c**. Compound **2** was then reacted to peptide resins **3a**–**c** using the same coupling method. Finally, the peptide resins were deprotected with a TFA–thioanisole–*m*-cresol system and the resultant crude **4a**–**c** were purified by HPLC as TFA salts.[27] A control peptide **5**, FITC-GABA-(D-Arg)$_7$-NH$_2$, was also synthesized by similar SPPS.

Conversion times ($t_{1/2}$) for FE–heptaarginines **4a**–**c** to parent **1** were evaluated by HPLC under physiological conditions (Krebs–Ringer bicarbonate buffer, KRBB, pH 7.4, 37 °C).[28] As shown in Table 1 and Figure 2, peptide **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. 100 min, while the basic side chains in the spacer of FE-heptaarginines **4b** and **4c** significantly shortened 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 quick conversion with a conversion $t_{1/2}$ value of 9.4 min and almost no side reaction was observed from this conversion as exemplified in Figure 2a. Dab (L-diaminobutanoic acid) derivative **4c**, going through a six-membered ring intermediate, showed a slightly longer conversion time ($t_{1/2}$ = 18 min) than that of Dap derivative **4b**. These results clearly indicated that conversion time could be controlled by the chemical structure of Xaa, suggesting that FE–heptaarginine conjugates with more variable conversion time could be designed in controlled release FE.

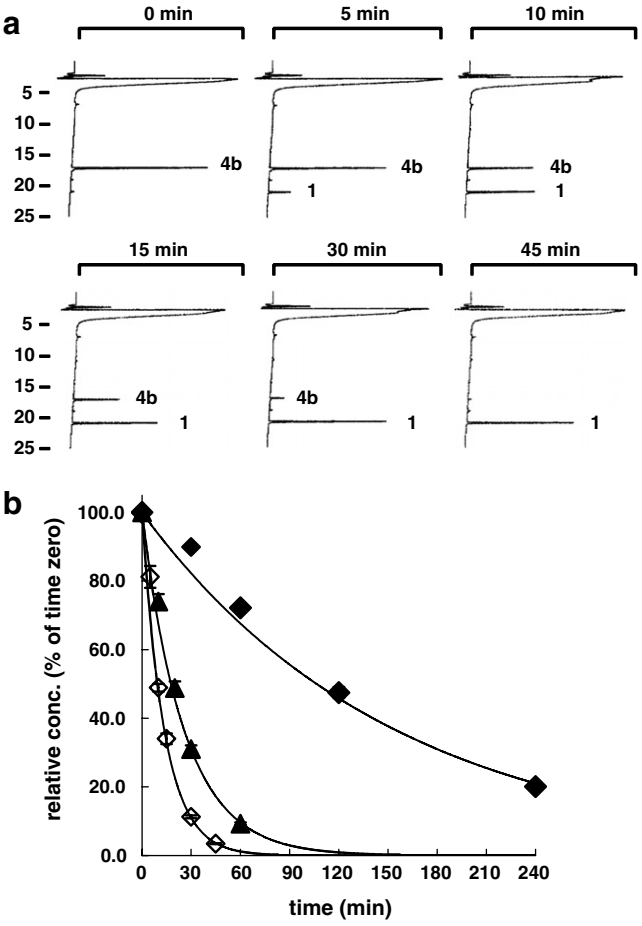


Figure 2. (a) Typical conversion profile of **4b** under physiological conditions (KRBB, pH 7.4, 37 °C). (b) Conversion of **4a**–**c**. Closed diamond, **4a**; open diamond, **4b**; closed triangle, **4c**. The conjugate concentrations at each time point are represented as relative percentages to the starting concentrations. Plots are means ± SEM of three (**4a**–**c**) experiments in KRBB (pH 7.4 at 37 °C).

As a preliminary step to investigate the efficacy of FE-heptaarginine conjugates on parent drug intestinal absorption after cellular uptake, in vitro permeation assays using Caco-2 cell monolayers were performed.[29] Conjugate **4b** having a $t_{1/2}$ value of 9 min seemed to increase transport rate of FE **1** by at least two times higher than FE **1** alone. Conjugates **4a** and **4c** with longer $t_{1/2}$ values of 106 and 18 min, respectively, exhibited only slightly higher permeation profile over FE **1** alone, while non-cleavable compound **5** had a similar permeation profile as FE **1** alone (Table 1 and Fig. 3). These results suggested that although an ideal time-dependent self-cleavage of the drug–heptaarginine conjugates seems to be an important factor in Caco-2 cell permeability, other factors may also be involved.

Table 1. Conversion times of FE–heptaarginines

Compound	Xaa	Ring size of intermediate	$t_{1/2}$ [a] (min)	Papp$_{AB}$ [b] (cm s^{-1} × 10^{-6})
4a	D-Ala	NA[c]	106 ± 2.6	0.41 ± 0.08
4b	Dap	5	9.4 ± 0.1	0.75 ± 0.10
4c	Dab	6	18 ± 0.4	0.48 ± 0.09
1	NA[c]	NA[c]	NA[c]	0.26 ± 0.11
5	NA[c]	NA[c]	NA[c]	0.38 ± 0.01

[a] Values were calculated from each degradation profile (Fig. 2).
[b] Values were calculated from the results of in vitro model permeation assays (Fig. 3), with SEM obtained from 6 (**1** and **5**) or 8 (**4a**–**c**) experiments.
[c] NA, not applicable.

Considering that (1) the conjugates are time-dependently and chemically hydrolyzed with a constant conversion time under physiological conditions, (2) compound **4b** with a self-cleavable linker exhibited improved-transport rate of parent **1** higher than non-cleavable compound **5**, and (3) FE **1** itself did not enhance cellular transport as shown in Table 1, we believe that the conjugate was delivered into the cells and the parent **1** was released intracellularly, then transferred to the basolateral side. Further biological studies are currently underway to elucidate the transfer mechanism, in order to reach our ultimate goal of enhancing intestinal absorption of parent drugs with low intestinal epithelial permeability as well as intracellular delivery of substances with low membrane permeability.

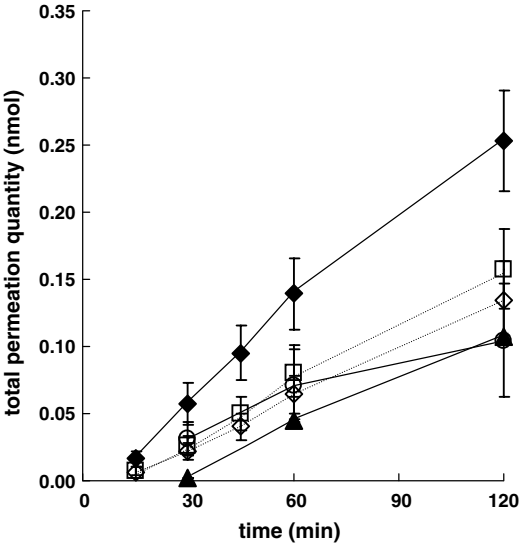


Figure 3. In vitro permeation assay of **1**, **4a**–**c**, and **5**. White diamond, **4a**; black diamond, **4b**; white square, **4c**; white circle, **1**; and black triangle, **5**. Plots are means ± SEM of 6 (**1**, **5**) or 8 (**4a**–**c**) experiments.

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References and notes

1. Futaki, S. *Biopolymers* **2006**, *84*, 241.
2. Snyder, E. L.; Dowdy, S. F. *Expert Opin. Drug Deliv.* **2005**, *2*, 43.
3. Zorko, M.; Langel, Ü. *Adv. Drug Deliv. Rev.* **2005**, *57*, 529.
4. Rothbbard, J. B.; Jessop, T. C.; Wender, P. A. *Adv. Drug Deliv. Rev.* **2005**, *57*, 495.
5. Wright, L. R.; Rothbard, J. B.; Wender, P. A. *Curr. Protein Pept. Sci.* **2003**, *4*, 105.
6. Vivès, E.; Brodin, P.; Lebleu, B. *J. Biol. Chem.* **1997**, *272*, 16010.
7. Derossi, D. A.; Joliot, H.; Chassaing, G.; Prochiantz, A. *J. Biol. Chem.* **1994**, *269*, 10444.
8. Futaki, S.; Suzuki, T.; Ohashi, W.; Yagami, T.; Tanaka, S.; Ueda, K.; Sugiura, Y. *J. Biol. Chem.* **2001**, *276*, 5836.
9. Wender, P. A.; Mitchell, D. J.; Pattabiraman, K.; Pelkey, E. T.; Steinman, L.; Rothbard, J. B. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 6043.
10. Astriab-Fisher, A.; Sergueev, D. S.; Fisher, M.; Shaw, B. R.; Juliano, R. L. *Biochem. Pharmacol.* **2000**, *60*, 1253.
11. Torchilin, V. P.; Rammohan, R.; Weissig, V.; Levchenko, T. S. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 8786.
12. Torchilin, V. P.; Levchenko, T. S.; Rammohan, R.; Volodina, N.; Papahadjopoulos-Sternberg, B.; D'Souza, G. G. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 1972.
13. Torchilin, V. P.; Levchenko, T. S.; Rammohan, R. *Curr. Protein Pept. Sci.* **2003**, *4*, 141.
14. Khalil, I. A.; Kogure, K.; Futaki, S.; Harashima, H. *J. Biol. Chem.* **2006**, *281*, 3544.
15. Rothbard, J. B.; Garlington, S.; Lin, Q.; Kirshberg, T.; Kreider, E.; McGrane, P. L.; Wender, P. A.; Khavari, P. A. *Nat. Med.* **2000**, *6*, 1253.
16. Kirshberg, T. A.; VanDeusen, C. L.; Rothbard, J. B.; Yang, M.; Wender, P. A. *Org. Lett.* **2003**, *5*, 3459.
17. Futaki, S.; Niwa, M.; Nakase, I.; Tadokoro, A.; Zhang, Y.; Nagaoka, M.; Wakako, N.; Sugiura, Y. *Bioconjug. Chem.* **2004**, *15*, 475.
18. Futaki, S.; Nakase, I.; Suzuki, T.; Nameki, D.; Kodama, E.; Matsuoka, M.; Sugiura, Y. *J. Mol. Recog.* **2005**, *18*, 169.
19. Console, S.; Marty, C.; Garcia-Echeverria, C.; Schwendener, R.; Ballmer-Hofer, K. *J. Biol. Chem.* **2003**, *278*, 35109.
20. Goncalves, E.; Kitas, E.; Seelig, J. *Biochemistry* **2005**, *44*, 2692.
21. Wadia, J. S.; Stan, R. V.; Dowdy, S. F. *Nat. Med.* **2004**, *10*, 310.
22. Nakase, I.; Niwa, M.; Takeuchi, T.; Sonomura, K.; Kawabata, N.; Koike, Y.; Takehashi, M.; Tanaka, S.; Ueda, K.; Simpson, J. C.; Jones, A. T.; Sugiura, Y.; Futaki, S. *Mol. Ther.* **2004**, *10*, 1011.
23. Morishia, M.; Kamei, N.; Ehara, J.; Isowa, K.; Takayama, K. *J. Controlled Release* **2007**, *118*, 177.
24. Liang, J. F.; Yang, V. C. *Biochem. Biophys. Res. Commun.* **2005**, *335*, 734.
25. Sohma, Y.; Hayashi, Y.; Ito, T.; Matsumoto, H.; Kimura, T.; Kiso, Y. *J. Med. Chem.* **2003**, *46*, 4124.
26. Matsumoto, H.; Sohma, Y.; Kimura, T.; Hayashi, Y.; Kiso, Y. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 605.
27. FE-succinyl-Dap-GABA-(D-Arg)$_7$-NH$_2$**4b**: FE–heptaarginine **4b** was synthesized by Fmoc-based solid phase method. 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 Fmoc-Dap(Boc)-OH, were sequentially coupled using a DIPCDI (1,3-diisopropylcarbodiimide, 0.75 mmol)–HOBt (1-hydroxybenzotriazole, 0.75 mmol) method for 2 h in DMF after removal of each Fmoc group with 20% piperidine–DMF (2× 7 mL, 2 and 20 min) to obtain H-Dap(Boc)-GABA-D-Arg(Pmc)$_7$-NH-resin **3b**. One-third of this resin **3b** 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 0.1% aqueous TFA–CH_3CN system to obtain FE–heptaarginine **4b** as a TFA salt. Yield 16%; HRMS (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).
28. In vitro conversion profiles of FE–heptaarginines **4a**–**c** to the parent **1** were determined under physiological conditions with Krebs–Ringer bicarbonate buffer (KRBB, pH 7.4). A solution of FE–heptaarginine (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.
29. (a) Fujita, T.; Majikawa, Y.; Umehisa, S.; Okada, N.; Yamamoto, A.; Ganapathy, V.; Leibach, F. H. *Biochem. Biophys. Res. Commun.* **1999**, *261*, 242; (b) Fujita, T.; Kawahara, I.; Quan, Y-S.; Hattori, K.; Takenaka, K.; Muranishi, S.; Yamamoto, A. *Pharm. Res.* **1998**, *15*, 1387.