Table 1
CXCR4-binding activity, anti-HIV activity and cytotoxicity of compounds **12–47**

Compd no.	R^1 [a]	R^2 [b]	R^3 [c]	Inhibition[d] (%)	EC_{50}[e] (μM)	CC_{50}[f] (μM)	Compd no.	R^1 [a]	R^2 [b]	R^3 [c]	Inhibition[d] (%)	EC_{50}[e] (μM)	CC_{50}[f] (μM)
12	i	(*R*)	A	0	>20	35	**30**	i	(*S*)	A	30 ± 1.1	>4	11
13	i	(*R*)	B	4 ± 1.7	>4	23	**31**	i	(*S*)	B	25 ± 3.3	>20	24
14	i	(*R*)	C	6 ± 0.7	>20	37	**32**	i	(*S*)	C	27 ± 1.7	>20	41
15	i	(*R*)	D	24 ± 1.7	n.d.	n.d.	**33**	i	(*S*)	D	60 ± 1.5	13	65
16	i	(*R*)	E	12 ± 3.0	>20	39	**34**	i	(*S*)	E	16 ± 1.2	8	44
17	i	(*R*)	F	16 ± 2.2	n.d.	n.d.	**35**	i	(*S*)	F	20 ± 1.3	10	44
18	ii	(*R*)	A	3 ± 0.9	>20	38	**36**	ii	(*S*)	A	36 ± 1.8	>20	37
19	ii	(*R*)	B	6 ± 3.9	>20	41	**37**	ii	(*S*)	B	0	>20	43
20	ii	(*R*)	C	11 ± 0.8	>20	45	**38**	ii	(*S*)	C	14 ± 1.4	>20	57
21	ii	(*R*)	D	22 ± 4.1	n.d.	n.d.	**39**	ii	(*S*)	D	32 ± 8.4	n.d.	n.d.
22	ii	(*R*)	E	6 ± 2.7	>20	45	**40**	ii	(*S*)	E	13 ± 15	>20	51
23	ii	(*R*)	F	12 ± 1.9	n.d.	n.d.	**41**	ii	(*S*)	F	25 ± 13	>20	47
24	iii	(*R*)	A	15 ± 2.1	n.d.	n.d.	**42**	iii	(*S*)	A	16 ± 5.1	>4	9.9
25	iii	(*R*)	B	13 ± 0.6	>20	27	**43**	iii	(*S*)	B	23 ± 14	>4	13
26	iii	(*R*)	C	79 ± 14	11	47	**44**	iii	(*S*)	C	36 ± 13	n.d.	n.d.
27	iii	(*R*)	D	69 ± 5.0	>11	11	**45**	iii	(*S*)	D	35 ± 5.2	n.d.	n.d.
28	iii	(*R*)	E	44 ± 5.4	n.d.	n.d.	**46**	iii	(*S*)	E	26 ± 23	n.d.	n.d.
29	iii	(*R*)	F	0	n.d.	n.d.	**47**	iii	(*S*)	F	51 ± 6.6	n.d.	n.d.
KRH-1636				100	0.33	80	FC131				100	0.16	>10
AMD3100				n.d.	0.062	55	1 (ST34)				n.d.	7.4	66
AZT				n.d.	0.058	100							

[a,c] The structures of R^1 and R^3 are shown in Fig. 2 as i–iii and **A–F**, respectively.
[b] The absolute configuration in stereochemistry of R^2 shown in Fig. 2 is described.
[d] CXCR4-binding activity was assessed based on the inhibition of the [^{125}I]-CXCL12 binding to Jurkat cells. Inhibition percentages of all the compounds at 10 μM were calculated relative to the inhibition percentage by T140 (100%).
[e] EC_{50} values are the concentrations for 50% protection from X4-HIV-1 (NL4-3 strain)-induced cytopathogenicity in MT-4 cells.
[f] CC_{50} values are the concentrations for 50% reduction of the viability of MT-4 cells. All data are the mean values from at least three independent experiments.

significant anti-HIV activity at concentrations below 11 μM because of high cytotoxicity (CC_{50} = 11 μM). With the exception of **27**, **30**, **42** and **43**, the tested compounds showed no significant cytotoxicity (CC_{50} >20 μM, Table 1). On the other hand, compounds **26**, **27**, **33**, **34** and **35** at concentrations below 100 μM failed to show significant protective activity against macrophage-tropic (R5-) HIV-1 (NL(AD8) strain)-induced cytopathogenicity in PM-1/CCR5, whereas the EC_{50} of the CCR5 antagonist SCH-D[17] in this assay was 0.055 μM (data not shown). Since instead of CXCR4, R5-HIV-1 strains use the chemokine receptor CCR5, a member of the GPCR family, as the major co-receptor for their entry, this suggests that these compounds do not bind to CCR5. Thus, compounds **26**, **27**, **33**, **34** or **35** have highly selective affinity for CXCR4. Compounds **34** and **35**, which have significant anti-HIV activity, have a pyridyl group with a nitrogen atom at the γ-position, in contrast to compounds **26**, **27** and **33** which also show CXCR4-binding activity, but have a pyridyl group with a nitrogen atom at the β-position. Furthermore, compounds **34** and **35** have R^1 = 4-fluorobenzyl and R^2 = (*S*)-1-naphthylethylamine. A possible explanation of these observations is that compounds **34** and **35** compete with HIV-1 in binding to CXCR4 while compounds **26** and **33** compete with HIV-1 and CXCL12. Compound **27** does not compete with HIV-1 because of its high cytotoxicity. This suggests that the CXCR4 binding site used by compounds **34** and **35** differs slightly from that used by compounds **26**, **27** and **33**.

Low molecular weight CXCR4 ligands with two types of recognition modes for CXCR4 have been obtained in this study: one causes competition with HIV-1 on CXCR4 whereas the other causes competition with HIV-1 and CXCL12. These compounds have selective affinity for CXCR4 because they do not significantly bind to CCR5. Further structural modification studies of these CXCR4 ligands are the subject of an ongoing project.

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15. *For example, the synthesis of compound* ***30***: To a stirred solution of **8Ai** (176 mg, 0.415 mmol, HCl salt) in DMF (4 mL) were added EDCI·HCl (104 mg, 0.454 mmol), $HOBt·H_2O$ (58.4 mg, 0.381 mmol), Et_3N (301 μL, 2.16 mmol) and ***(S)*-11** (320 mg, 0.657 mmol, HCl salt) at 0 °C. The mixture was stirred at room temperature for 43 h. The reaction mixture was diluted with $CHCl_3$ and washed with saturated citric acid, saturated $NaHCO_3$ and brine, and dried over $MgSO_4$. Concentration under reduced pressure followed by flash column chromatography over silica gel with $CHCl_3$/MeOH (20/1) gave the condensation product (175 mg, 0.208 mmol, 50% yield) as white powder. To this compound were added *m*-cresol (75.0 μL, 0.714 mmol), 1,2-ethanedithiol (225 μL, 2.68 mmol), thioanisole (225 μL, 1.91 mmol), TFA (3 mL) and bromotrimethylsilane (495 μL, 3.82 mmol) with stirring at 0 °C, and the stirring was continued at room temperature for 3.5 h under N_2. The reaction mixture was concentrated under reduced pressure, followed by addition of Et_2O to precipitate the product. After washing with Et_2O, the crude product was purified by preparative HPLC and lyophilized to give the compound **30** (15.6 mg, 0.0236 mmol, 13%) as white powder. ^1H NMR δ_H (400 MHz; DMSO-d_6) 1.49 (m, 2H), 1.51 (d, J = 7.2 Hz, 3H), 1.80–1.62 (m, 2H), 3.07 (dd, J = 6.4, 12.8 Hz, 2H), 3.85 (s, 2H), 3.91 (s, 4H), 4.54 (m, 1H), 5.72 (m, 1H), 7.13 (t, J = 8.8 Hz, 2H), 7.40 (m, 1H), 7.60–7.45 (m, 10H), 7.75–7.95 (m, 5H), 8.10 (m, 1H), 8.40 (d, J = 8.0 Hz, 1H), 8.58 (m, 1H), 8.65 (d, J = 7.6 Hz, 1H); LRMS (ESI), *m/z* calcd for $C_{39}H_{42}FN_7O_2$ $(MH)^+$ 660.34, found 660.31.
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Evaluation of a synthetic C34 trimer of HIV-1 gp41 as AIDS vaccines

Chie Hashimoto [a], Wataru Nomura [a,*], Aki Ohya [a], Emiko Urano [b], Kosuke Miyauchi [b], Tetsuo Narumi [a], Haruo Aikawa [a], Jun A. Komano [b], Naoki Yamamoto [c], Hirokazu Tamamura [a,*]

[a] Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, Chiyoda-ku, Tokyo 101-0062, Japan
[b] AIDS Research Center, National Institute of Infectious Diseases, Shinjuku-ku, Tokyo 162-8640, Japan
[c] Yong Loo Lin School of Medicine, National University of Singapore, Singapore 117597, Singapore

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ABSTRACT

An artificial antigen forming the C34 trimeric structure targeting membrane-fusion mechanism of HIV-1 has been evaluated as an HIV vaccine. The C34 trimeric molecule was previously designed and synthesized using a novel template with C3-symmetric linkers by us. The antiserum produced by immunization of the C34 trimeric form antigen showed 23-fold higher binding affinity for the C34 trimer than for the C34 monomer and showed significant neutralizing activity. The present results suggest effective strategies of the design of HIV vaccines and anti-HIV agents based on the native structure mimic of proteins targeting dynamic supramolecular mechanisms in HIV fusion.

1. Introduction

Highly active anti-retroviral therapy (HAART) involving new anti-HIV drugs such as protease inhibitors and integrase inhibitors has been brought a great success to us. Antibody-based therapy is also promising, and several AIDS antibodies have been developed by normal immunization[1] and by de novo techniques of monoclonal antibodies (mAb) using molecular evolution methods such as phage display.[2] Antibodies including anti-gp41 and anti-gp120 have been identified as human mAbs capable of highly and broadly neutralizing HIV. A transmembrane envelope glycoprotein, gp41 is hidden beneath an outer envelope glycoprotein gp120 and its ectodomain contains helical amino-terminal and carboxy-terminal leucine/isoleucine heptad repeat (HR) domains HR1 and HR2. These HR1 and HR2 regions are designated as the N-terminal helix (N36) and C-terminal helix (C34), respectively. In the membrane fusion of HIV-1, these helices join to form a six-helical bundle consisting of a central parallel trimer of N36 surrounded by C34 in an antiparallel hairpin fashion. A useful strategy to produce broadly neutralizing antibodies is therefore to synthesize molecules that mimic the natural trimer as it appears on viral surface proteins. Walker et al. reported that antibody recognition for the trimer form is important in HIV vaccine strategies, because antibodies that specifically recognize the trimer formation might have broad and potent neutralizing activity.[3] To date, several gp41 mimetics, especially for N36 regions, which assemble these helical peptides with branched peptide-linkers have been synthesized as antigens.[4–7] Previously, we synthesized a three-helix bundle mimetic, which corresponds to the trimeric form of N36, with a novel template with C3-symmetric linkers of equal lengths.[8] Immunization with the equivalent trimeric form of N36 mimetic produced antibodies with stronger binding affinity for N36 trimer than for N36 monomer. The structure-specific antibodies produced in this way showed significant neutralization activity against HIV-1 infection. Several potent anti-HIV-1 peptides based on the gp41 C-terminal HR2 region have been discovered[9,10] and an HR2-peptide, T20, has subsequently been developed into a clinical anti-HIV-1 drug, enfuvirtide (Roche/Trimeris).[11–14] The C-terminal helix C34 is also an HR2-derived peptide containing the amino acid residues required for docking into the hydrophobic pocket of the trimer of the N-terminal HR1 region, and potently inhibits HIV-1 fusion in vitro.[15] Recently, we also synthesized a three-helix bundle mimetic, which corresponds to the trimeric form of C34, with a novel different template with C3-symmetric linkers of equal lengths.[16] The C-terminal ends of three peptide strands are assembled in the C34 trimer, while the N-terminal ends of three peptide strands are assembled in N36 trimer. As an anti-HIV agent, the C34 trimer peptide showed two orders of magnitude higher inhibitory potency than the C34 monomer peptide. This study demonstrates a useful strategy for the design of effective inhibitors against viral infections that proceed by membrane fusion with host cells. In the present study, we have investigated the activity of the equivalent trimeric

* Corresponding authors. Tel.: +81 3 5280 8036; fax: +81 3 5280 8039.
E-mail addresses: nomura.mr@tmd.ac.jp (W. Nomura), tamamura.mr@tmd.ac.jp (H. Tamamura).

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form of C34 as an antigen peptide producing structure-specific antibodies. We have performed comparative studies of antisera isolated from mice immunized with the C34 trimer in binding affinity for the C34 trimer and for the C34 monomer.

2. Materials and methods

2.1. Immunization and sample collection

Six-week-old male BALB/c mice, purchased from Sankyo Laboratory Service Corp. (Tokyo, Japan), were maintained in an animal facility under specific pathogen-free conditions. The experimental protocol used was approved by the ethical review committee of Tokyo Medical and Dental University. Freund incomplete adjuvant and PBS were purchased from Wako Pure Chemical Industries (Osaka, Japan). DMSO (endotoxin free) was purchased from Sigma–Aldrich (St. Louis, MO).

All mice were bled one week before immunization. One hundred micrograms of antigen (C34 monomer C34REG) was dissolved in PBS (50 μL) and DMSO (1 μL). The antigen C34 trimer triC34e (100 μg) was dissolved in PBS (50 μL). This solution was mixed with Freund incomplete adjuvant (50 μL) and the mixture was injected subcutaneously under anesthesia on days 0, 7, 14, 21 and 28. Mice were bled on days 5, 12, 19, 26 and 33. Serum was separated by centrifugation (1500 rpm) at 4 °C for 10 min, and inactivated at 56 °C for 30 min. Sera were stored at −80 °C before use.

2.2. Serum titer ELISA

Tween-20 (polyoxyethylene (20) sorbitan monolaurate) and hydrogen peroxide (30%) were purchased from Wako Pure Chemical Industries (Osaka, Japan). 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS) was purchased from Sigma-Aldrich. Anti-mouse IgG (H+L)(goat)-HRP was purchased from EMD Chemicals (San Diego, CA). Ninety-six well microplates were coated with 25 μL of a synthetic peptide in a 10 μg/mL solution in PBS at 4 °C overnight. The coated plates were washed 10 times with deionized water and blocked with 150 μL of blocking buffer (0.02% PBST, PBS with 0.02% Tween 20, containing 5% skim milk) at 37 °C for 1 h. The plates were washed with deionized water 10 times. Mice sera were diluted in 0.02% PBST with 1% skim milk, and 50 μL of twofold serial dilutions of sera from 1/200 to 1/409600 were added to the wells and allowed to incubate at 37 °C for 2 h. The plates were again washed 10 times with deionized water. HRP-conjugated anti-mouse IgG, diluted 1:2000 in 0.02% PBST (25 μL), was added to each well. After incubation for 45 min, the plates were washed 10 times and 25 μL of HRP substrate, prepared by dissolving ABTS (10 mg) in 200 μL of HRP staining buffer—a mixture of 0.5 M citrate buffer (pH 4.0, 1 mL), H_2O_2 (3 μL), and H_2O (8.8 mL)—was added. After 30 min incubation, the reaction was stopped by addition of 25 μL/well 0.5 M H_2SO_4, and optical densities at 405 nm were recorded.

2.3. Virus preparation

For virus preparation, 293FT cells in a 60 mm dish were transfected with 10 μg of the pNL4-3 construct by the calcium phosphate method. The supernatant was collected 48 h after transfection, passed through a 0.45 μm filter, and stored at −80 °C as the virus stock.

2.4. Neutralizing assay (P24 assay)

For viral neutralizing assay, the NL4-3 virus (5 ng of p24) was bound to MT-4 cells (5×10^4 cells/200 μL) by spinoculation at 2100 g for 20 min at 4 °C. After removal by washing out of unbound virus, cells were resuspended with 200 μL of medium containing 10 μL sera from immunized or pre-immunized mice and were cultured. Half of the culture medium was changed every 2 or 3 days. At 7 days after infection, the level of p24 in the culture supernatant was determined by the p24 ELISA kit (PerkinElmer, MA).[17]

3. Results and discussion

In the C34 trimer, triC34e, which was previously synthesized,[16] the triplet repeat of arginine and glutamic acid (RERERE) was added to the C-terminal end of the C34 sequence to increase solubility in buffer solution, and glycine was fused to the C-terminus (Fig. 1A and B). The C3-symmetric template with three hydrophilic branches of equal length was adopted to assemble three peptide strands. As a control peptide, which corresponds to the monomeric form of C34, C34REG having RERERE and Gly in the C-terminus was used (Fig. 1C).[16]

To investigate whether antibodies are efficiently produced, mice were immunized with C34REG and triC34e and the increase in the titer in 5 weeks' immunization was observed (Fig. 2). Titers and specificity of antisera isolated from mice immunized with C34REG or triC34e were evaluated by serum titer ELISA against coated synthetic antigens. In each case, the increase in antibody production was observed as time passed. The most active antiserum for each antigen was utilized for the evaluation of binding activity by ELISA (Fig. 3). The C34REG-induced antibody showed approximately 1.2 times higher antibody titer against the coated C34REG than against the coated triC34e; the serum dilutions at 50% bound are 1.06×10^{-3} and 1.30×10^{-3}, respectively (Fig. 3A and B). The triC34e-induced antibody showed approximately 23 times higher titer against the coated triC34e than against the coated C34REG; the 50% bound serum dilutions are 3.15×10^{-4} and 7.30×10^{-3}, respectively (Fig. 3A and B). C34REG-induced or triC34e-induced antibody did not show any significant binding titer against an unrelated control peptide (Fig. 3C and D). Although purified monoclonal antibodies were not used for this evaluation, the antibodies produced exploited specific affinity for each antigen of the monomer or the trimer. These results suggest the synthesis of structure-involving antigens leads to the production of antibodies with structural specificity.

It is important to know if the antisera produced have inhibitory activity against HIV-1 infection. Accordingly, the inhibitory activity of the antisera was assessed by p24 assays utilizing the antisera bled from three mice that showed antibody production for each antigen (Fig. 4). The experiments using HIV-1 was performed in the biosafety level 3 laboratory #5 in the National Institute of Infectious Diseases. Sera from mice immunized with the monomer C34REG and the trimer triC34e antigens contained antiviral activities compared to control sera. Any significant difference of inhibitory effects was not observed between the sera isolated from C34REG-immunized mice and those from triC34e-immunized mice. The synthetic C34 trimeric antigen induces antibodies with a structural preference, but the levels of neutralization activity of sera from mice immunized with the C34 trimer were similar with those of sera from the C34 monomer-immunized mice. This suggests that antibodies with structural specificity against the gp41-C34-derived region do not always have more potent neutralization activity. The difference of recognition mechanism of two types of antibodies might cause different neutralizing mechanism although their levels of neutralization activity are almost the same. This result is not consistent with the data of the synthetic antigen molecules derived from N36, in which the N36 trimer-specific antibodies showed higher neutralization activity against HIV-1 infection than the N36 monomer-specific antibodies.[8] In any case,

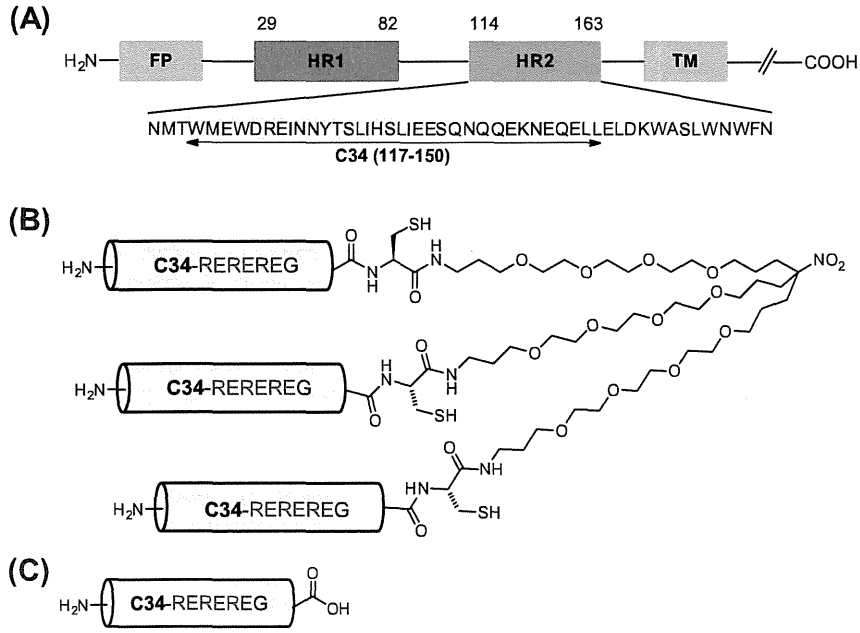

Figure 1. The sequence of C34 in gp41 (NL4-3) (A). FP and TM represent hydrophobic fusion peptide and transmembrane domain, respectively. Structures of C34-derived peptides, the C34 trimer with a C3-symmetric linker, triC34e (B), and the C34 monomer, C34REG (C).

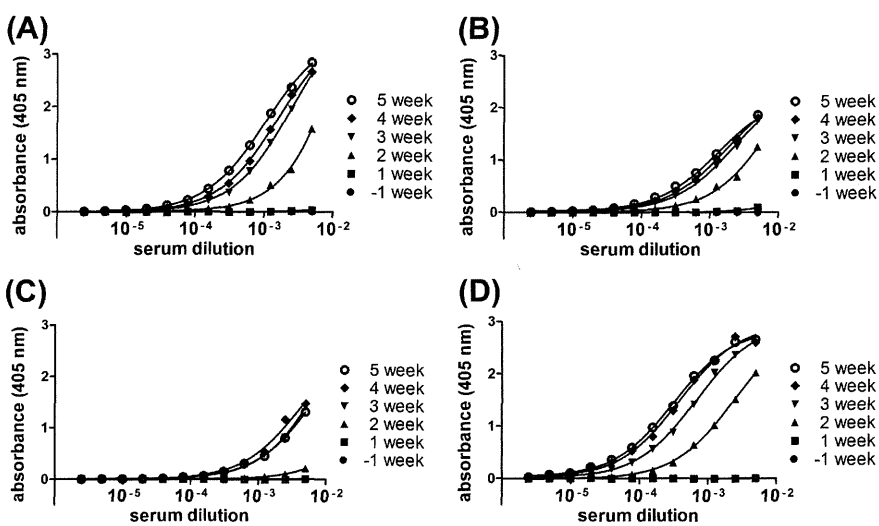

Figure 2. Results of serum titer ELISA of antisera collected during immunization (from one week before start to five weeks after immunization start) to determine the immunogenicity of designed antigens. The titers were evaluated as followings; antiserum against C34REG binding to C34REG (A); antiserum against C34REG binding to triC34e (B); antiserum against triC34e binding to C34REG (C); antiserum against triC34e binding to triC34e (D).

the synthetic C34 trimeric antigen induces antibodies with a structural preference and potent neutralization activity. In case antibodies bind to the gp41 C-terminal HR2 region and suppress membrane fusion, they may recognize the primary amino acid sequence of the C34 region or its structural conformation, because the C34 region is located outside in the formation of a six-helical bundle. It is suggested that suppressant potencies of these types of antibodies are almost similar. In addition, the action of these antibodies might be orthogonal and supplementally effective.

Recently, broadly active and potent neutralization antibodies, PG-9 and PG-16, were isolated from sera of HIV-1 infected individuals.[3] The antibodies can neutralize ~80% of HIV-1 isolates across all clades with approximately one order of magnitude higher potency than those of broad neutralizing mAbs reported previously. It is interesting that the epitopes for these mAbs are quaternary, and preferentially displayed on Env trimers, as expressed on the surface of virions and transfected cells. These results suggest that there may be production mechanisms for antibodies recognizing epitope structures.[18–20] The sera obtained from immunization of the C34 trimer antigen have structural specificity and neutralization activity. Thus, our trimer antigens, including the N36 trimer,[8] could work efficiently as a new class of HIV-1 vaccines.

Concerning inhibitory activity of these C-region peptides against HIV-1 entry, the potency of triC34e is one hundred times higher than that of C34REG.[16] It indicates that a trimeric form is critical as the active structure of the inhibitor, although as vaccines

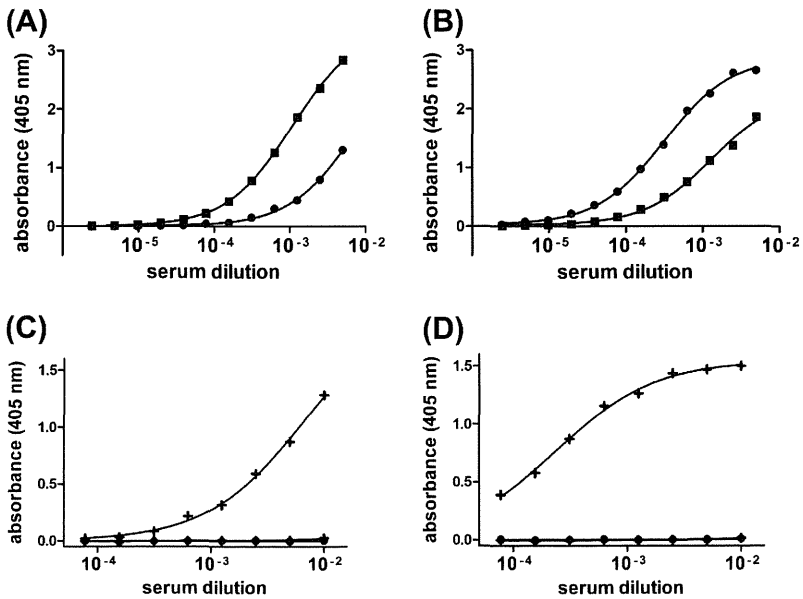

Figure 3. Serum titers of the antibodies produced by the fifth immunization of the C34REG antigen and the fourth immunization of the triC34e antigen. These titers were evaluated against ELISA templates of C34REG (monomer) (A) and triC34e (trimer) (B), using sera obtained from a C34REG-immunized mouse (■) and a triC34e-immunized mouse (●) as each representative. Titers of C34REG-induced antibodies were evaluated against ELISA templates of C34REG (+) and an unrelated control peptide (●) (C), and titers of triC34e-induced antibodies were evaluated against ELISA templates of triC34e (+) and an unrelated control peptide (●) (D). Unrelated control peptide: CH_3CO-GELDKWEKIRLRPGGGC(CH_2CONH_2)-NH_2.

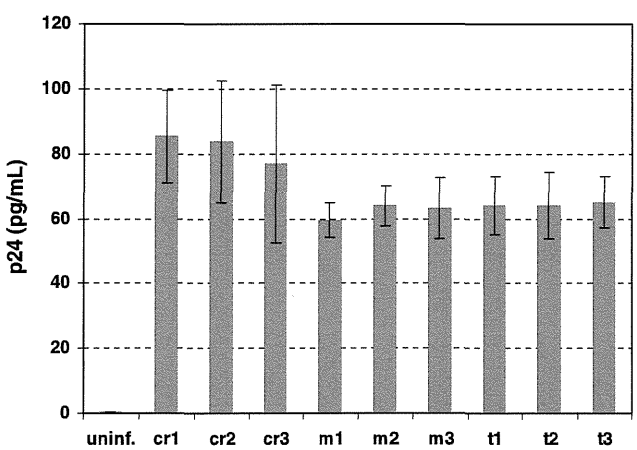

Figure 4. Determination of neutralization activity of the antibodies produced by immunization of C34REG and triC34e antigens. Inhibition of HIV-1 (NL4-3 strain) infection by produced antibodies was evaluated by the p24 assay in MT-4 cells. Y-axis shows the amount of p24 production. Uninf means uninfected cells. Pre-immunization sera (−1 week) were used as controls (cr1-3). C34REG- and triC34e-immunization sera (5 weeks) were used (m1-3 and t1-3, respectively). Experiments were conducted in triplicate. Error bars show standard error of mean.

there is no significant difference in neutralization activity of induced antibodies between the monomer and the trimer.

The exposed timing of epitopes of the helical region trimers is limited in the fusion step,[21] and carbohydrates are not included in the amino acid residues of the regions. The effectiveness of the vaccine design based on the gp41 helical regions is supported by the critical advantages cited above. Our developed N36 and C34 trimer-form specific antibodies might have the above properties. The designs of antigens and inhibitors targeting the dynamic supramolecular mechanism of HIV-1 fusion will be useful for future studies on AIDS vaccines and inhibitors.

Acknowledgments

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A Synthetic C34 Trimer of HIV-1 gp41 Shows Significant Increase in Inhibition Potency

Wataru Nomura,[a] Chie Hashimoto,[a] Aki Ohya,[a] Kosuke Miyauchi,[b] Emiko Urano,[b] Tomohiro Tanaka,[a] Tetsuo Narumi,[a] Toru Nakahara,[a] Jun A. Komano,[b] Naoki Yamamoto,[c] and Hirokazu Tamamura*[a]

The development of new anti-HIV-1 drugs such as inhibitors of protease and integrase has been contributed to highly active anti-retroviral therapy (HAART) for the treatment of AIDS.[1] The entry of human immunodeficiency virus type 1 (HIV-1) into target cells is mediated by its envelope glycoprotein (Env), a type I transmembrane protein that consists of surface subunit gp120 and noncovalently associated transmembrane subunit gp41.[2] Sequential binding of HIV-1 gp120 to its cell receptor CD4 and a co-receptor (CCR5 or CXCR4) can trigger a series of conformational rearrangements in gp41 to mediate fusion between viral and cellular membranes.[3–5] The protein gp41 is hidden beneath gp120, and its ectodomain contains helical N- and C-terminal leucine/isoleucine heptad repeat domains, N-HR and C-HR. Particular regions of N-HR and C-HR are involved in membrane fusion, and 36-mer and 34-mer peptides, which are derived from N-HR and C-HR, have been designated as the N-terminal helix (N36) and C-terminal helix (C34), respectively. In the membrane fusion of HIV-1, these helices assemble to form a six-helical bundle (6-HB) consisting of a central parallel trimer of N36 surrounded by C34 in an antiparallel hairpin fashion. Synthetic peptides derived from these helices have potent antiviral activity against both laboratory-adapted strains and primary isolates of HIV-1.[6–9] They inhibit the membrane fusion stage of HIV-1 infection in a dominant-negative manner by binding to the counterpart regions of gp41 (N-HR or C-HR), blocking formation of the viral gp41 core.

Several potent anti-HIV-1 peptides based on the C-HR region have been discovered,[7,8] and T20 was subsequently developed as the clinical anti-HIV-1 drug enfuvirtide (Roche/Trimeris).[8,10–13] It is a 36-mer peptide derived from the gp41 C-HR sequence and can bind to the N-HR to prevent formation of the 6-HB in a dominant-negative fashion.[10] T20 therapy has brought safety, potent antiretroviral activity, and immunological benefit to patients, but its clinical application is limited by the development of resistance. The C-terminal helix C34 is also a C-HR-derived peptide, and contains the amino acid residues required for docking into the hydrophobic pocket, termed the "deep pocket", of the trimer of the N-HR region. This peptide potently inhibits HIV-1 fusion in vitro.[14] To date, several gp41 mimetics, especially those of N36 regions, which assemble these helical peptides with branched peptide linkers, have been synthesized as antigens.[15–19]

Recently, by using a novel template with C3-symmetric linkers of equal length, we synthesized a three-helix bundle mimetic that corresponds to the trimeric form of N36.[20] The antisera obtained from mice immunized by the peptide antigen showed strong recognition against the N36 trimer peptide with structural preference. At the same time, the trimer peptide was also investigated as a fusion inhibitor. However, the trimer N36 showed only a threefold increase in inhibition of HIV-1 fusion relative to the N36 monomer.[20] In terms of N36 content, the trimer and monomer have nearly the same inhibitory potency. This phenomenon is consistent with the results from other studies.[21–23] The multimerization of the functional unit, such as synthetic ligands against receptors, show synergistic binding and strong binding activity. Thus, we hypothesized that our strategy using C3-symmetric linkers in the design of trimer mimics of gp41 could be applied to the C34 peptide, which shows significant inhibition potency in the monomeric form. In the present study, we designed and synthesized a novel three-helical bundle structure of the trimeric form of C34. This equivalent mimic of the trimeric form of C34 was evaluated as a novel form of fusion inhibitor.

The C-terminal region of gp41 is known to be an assembly site involving a trimeric coiled-coil conformation. In the design of the C34-derived peptides C34REG-thioester (Figure 1 A) and C34REG (Figure 1 B), the triplet repeat of arginine and glutamic acid (RERERE) was added to the C-terminal end of the C34 sequence (residues 628–661) to increase aqueous solubility, and for C34REG-thioester, a glycine thioester was fused to the C terminus. To form a triple helix corresponding precisely to the gp41 pre-fusion form, we designed the novel C3-symmetric template depicted in Figure 1 C. This designed template linker has three branches of equal length, a hydrophilic structure, and a ligation site for coupling with C34REG-thioester. The template was synthesized as shown in Scheme 1. This approach uses native chemical ligation for chemoselective coupling of unprotected C34REG-thioester with a three-armed cysteine scaffold to produce triC34e (Figure 2).[24,25]

Circular dichroism (CD) spectra of C34REG and triC34e are shown in Figure 3 A. The peptides were dissolved in 50 mM sodium phosphate buffer with 150 mM NaCl, pH 7.2. Both spectra display minima at ~200 nm, indicating that these peptides form random structures. We previously reported that the

[a] Dr. W. Nomura, C. Hashimoto, A. Ohya, Dr. T. Tanaka, Dr. T. Narumi, T. Nakahara, Prof. Dr. H. Tamamura
Institute of Biomaterials and Bioengineering
Tokyo Medical and Dental University
2-3-10 Kandasurugadai, Chiyoda-ku, Tokyo 101-0062 (Japan)
E-mail: tamamura.mr@tmd.ac.jp

[b] Dr. K. Miyauchi, Dr. E. Urano, Dr. J. A. Komano
AIDS Research Center, National Institute of Infectious Diseases
1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640 (Japan)

[c] Prof. Dr. N. Yamamoto
Department of Microbiology, Yong Loo Lin School of Medicine
National University of Singapore
5 Science Drive 2, Singapore 117597 (Singapore)

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cmdc.201100542.

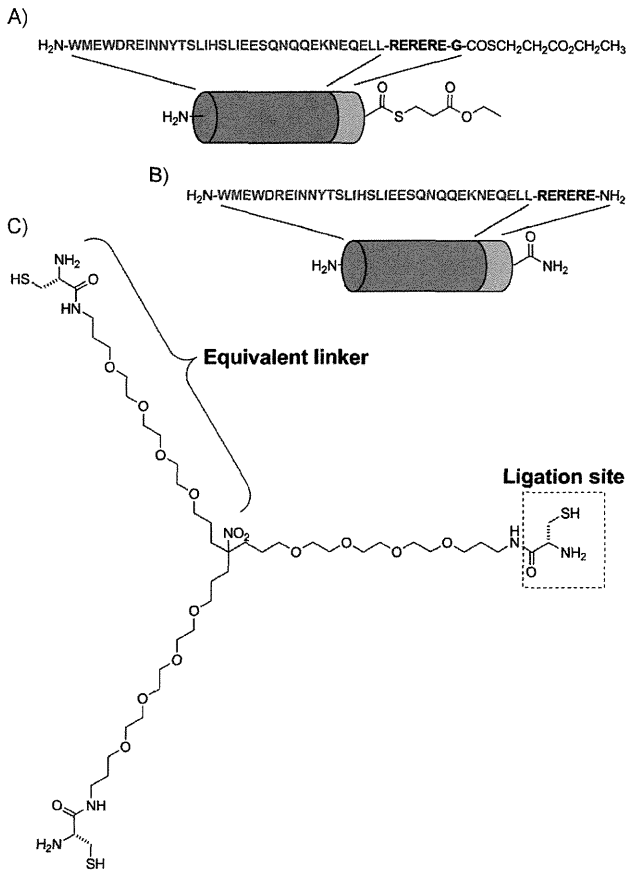


Figure 1. C34-derived peptides: A) C34REG-thioester and B) C34REG. C) The design of a C3-symmetric template.

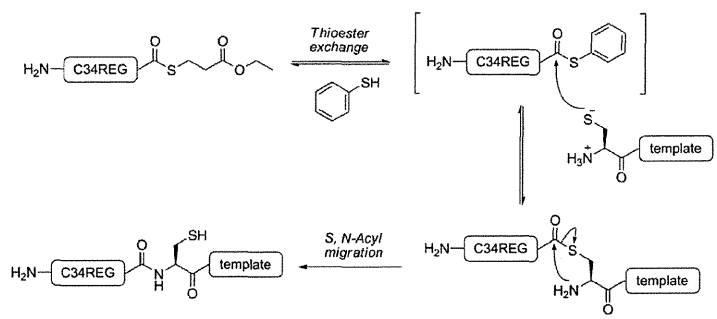
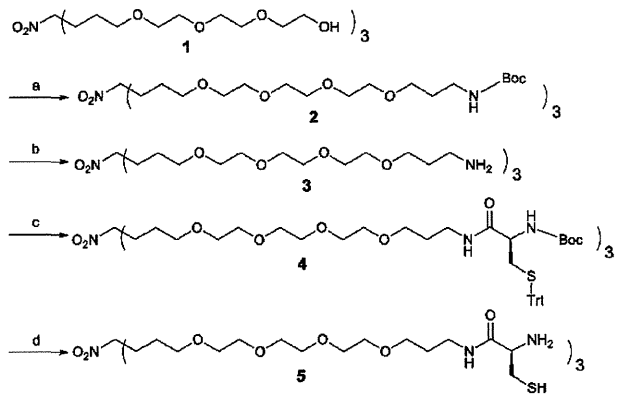


Figure 2. The native chemical ligation used for assembly of the C34REG-thioester on the template.



Scheme 1. Synthesis of the equivalently branched template **5**. *Reagents and conditions*: a) (3-bromopropyl)carbamic acid *tert*-butyl ester, NaH, THF; b) 4 M HCl/dioxane; c) Boc-Cys(Trt)-OH, EDCI·HCl, HOBt·H_2O, Et_3N, DMF; d) 90% aq. TFA.

helical content of the trimer triC34e and N36RE mixture is lower than that of the monomer C34REG and N36RE mixture. This is evidence that relative to the monomer C34REG, the trimer triC34e interacts with N36 only with difficulty, due to the assembly of three peptide strands by covalent bonds.

As the trimeric C34 was proven to interact with N36 helices, the potential HIV-1 inhibitory activities of the C-terminal peptides, C34REG and triC34e, were evaluated. The C34 peptide without the solubility-increasing sequence (3×[Arg-Glu], obtained from NIAID) was used as the monomeric control.[27] All peptides showed potent inhibitory activity in the viral fusion assay (Table 1), with the potency of triC34e being 100- and 40-fold higher than that of C34REG and C34 peptides, respectively. Notably, the triC34e trimer peptide is remarkably more potent in anti-HIV-1 activity than the monomer, indicating that a trimeric form is critical for inhibitory activity. Cytotoxicity from the peptides was not observed at concentrations of 15 μM for C34REG and C34, and 5 μM for triC34e.

We next carried out an assay for the inhibition of viral replication. As shown in Table 2, triC34e showed 30- and 20-fold higher inhibitory activity than peptides C34 and C34REG, respectively. In the two anti-HIV-1 assays, triC34e showed a great enhancement of activity over the C34 monomers. The IC_{50} values obtained in the assays are different, and this can be

N36 monomer N36RE and the N36 trimer triN36e form a highly structured α helix, and that the helical content of triN36e was greater than that of N36RE.[20,26] These results suggest that in contrast to N36-derived peptides, C34-derived peptides tend to form random structures both in the monomeric and trimeric forms. To assess the interaction of triC34e with N36, CD spectra of a mixture of triC34e with an N36-derived peptide, N36RE, were measured (Figure 3B). The spectrum of the C34REG and N36RE mixture and that of the triC34e and N36RE mixture showed double minima at λ 208 and 222 nm, indicating that the peptide mixture forms an α-helical structure and that the

Table 1. IC_{50} and CC_{50} values determined by viral fusion inhibition and cell viability assays.

	C34 peptide[a]	C34REG	triC34e
IC_{50} [μM][b]	0.044	0.12	0.0013
CC_{50} [μM][c]	>15	>15	>5

[a] HIV-1 IIIB C34 peptide. [b] IC_{50} values are based on luciferase signals in TZM-bl cells infected with HIV-1 (NL4-3 strain). [c] CC_{50} values are based on the decrease in viability of TZM-bl cells. All data are the mean values from at least three experiments.

Table 2. IC_{50} values determined by inhibition assay based on p24 ELISA.			
	C34 peptide	C34REG	triC34e
IC_{50} [μM][a]	1.59	1.06	0.0547

[a] IC_{50} values are based on the production of p24 in MT-4 cells infected with HIV-1 (NL4-3 strain). All data are the mean values from at least three experiments.

explained through differences in experimental procedures. In the fusion inhibition assay, cells were treated with peptides before viral infection. In contrast, in the viral replication inhibition assay, peptides were treated after viral adsorption to cells. Therefore, in the latter case, the infection by HIV-1 might precede peptide binding to gp41.

It has been shown that T-1249, an analogue of enfuvirtide, and its hydrophobic C-terminal region inhibit HIV-1 fusion by interacting with lipid bilayers.[28] The tryptophan-rich domain of T-1249 was shown to play important roles in HIV-1 fusion.[29–31] As enfuvirtide shows weak interaction with the gp41 core structure, and the C34 sequence lacks the C-terminal lipid binding domain, it has been suggested that C34 has a mechanism of action distinct from that of enfuvirtide.[32] Thus, it is of interest to discern the mechanism of the enhanced inhibition observed with triC34e relative to the monomer. Two explanations can be envisaged: 1) the α helicity of the C34 trimer is higher than that of the monomer, as shown in Figure 3A, and as a result, the C34 trimer binds more strongly to the N36 trimer; and 2) in the mixture with the N36 monomer, the C34 trimer shows less α helicity than its monomer (Figure 3B). As shown in Figure 3A, the molar ellipticity at 222 nm is similar for both the C34 trimer and the monomer. Thus, the decrease at 222 nm in the mixture with N36 might be due to a decrease in the α helicity of N36. These results suggest that the C34 trimer might destabilize helix formation in N36 and thus exert potent inhibitory activity. It has been shown that a dimeric C37 (residues 625–661) variant does not show a significant difference in IC_{50} value against HIV-1 from wild-type C37, although the dimeric peptide shows tighter binding to the gp41 N-HR coiled-coil than the C37 monomer.[33] Thus, the mechanism of action of the C34 trimer could be different from that of the dimeric C-peptide. The detailed action mechanism of the trimer as a fusion inhibitor and the reasons behind its remarkable increased anti-HIV-1 activity will be the subjects of future studies in our research group.

A C-terminal helical peptide of HIV-1 gp41 has been designed as a new HIV fusion inhibitor and was synthesized with a novel template and three branched linkers of equal length. The native chemical ligation proceeded by chemoselective coupling in an aqueous medium of an unprotected C34 derivative containing a C-terminal thioester with a three-cysteine-armed scaffold. This process led to the production of triC34e. As a fusion inhibitor, triC34e has potent anti-HIV-1 activity, 100-fold greater than that of the C34REG monomer, although the anti-HIV-1 activity of the N36 trimer is threefold higher than that of the N36 monomer, and the N36 content is the same in both cases.[20] A trimeric form of C34 is evidently critical as the

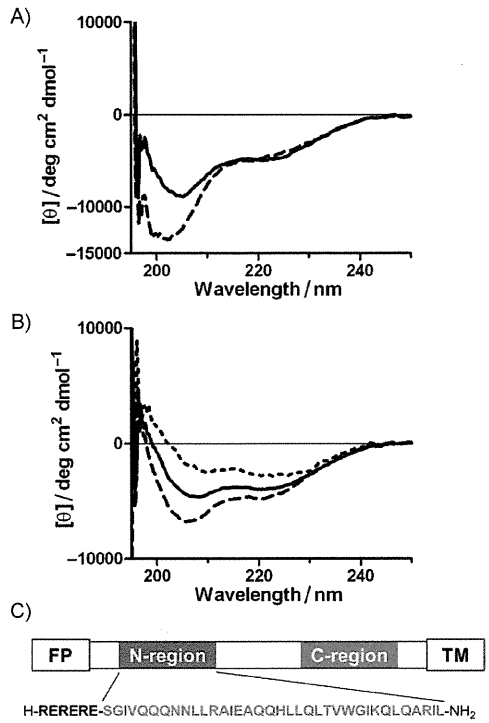


Figure 3. A) CD spectra of C34REG (monomer, -----, 6 μM) and triC34e (trimer, ——, 2 μM). B) CD spectra in the presence or absence of the N36 monomer N36RE:[20] -----, [C34REG (6 μM) + N36RE (6 μM)]; ——, [triC34e (2 μM) + N36RE (6 μM)]; ••••, N36RE (6 μM). In the amino acid sequence of N36RE, the triplet repeat of arginine and glutamic acid is located at the N-terminus of the original N36 sequence.[20] C) Amino acid sequence of N36RE: FP and TM represent the hydrophobic fusion peptide and transmembrane domains, respectively.

active structure of the fusion inhibitor. The soluble C34 derivative, SC34, retains potent inhibitory effects against enfuvirtide-resistant viruses,[34] and this suggests that the present highly potent trimeric inhibitor could be effective for enfuvirtide-resistant HIV-1 strains. The design of inhibitors that target the dynamic supramolecular mechanism of HIV-1 fusion will be useful for future studies of anti-HIV-1 agents.

Experimental Section

Conjugation of C34REG-thioester and the template to produce triC34e

TCEP·HCl (773 μg, 2.67 μmol) and thiophenol (9 μL, 89 μmol) were dissolved in 0.1 M sodium phosphate buffer (60 μL) containing 6 M urea and EDTA (pH 8.5, 2 mM) under a nitrogen atmosphere. Compound **5** (100 μg, 0.0899 μmol), C34REG-thioester (1.77 mg, 0.297 μmol), and CH_3CN (20 μL) were added. The reaction was stirred for 5 h at 37 °C and monitored by HPLC. The ligation product (triC34e) was separated as an HPLC peak and characterized by ESI-ToF-MS (*m/z* calcd for $C_{703}H_{1108}N_{205}O_{245}S_6$ $[M+H]^+$: 16533.9, found: 16543.8). Purification was performed by reversed-phase HPLC (Cosmosil $5C_{18}$-AR II column, 10×250 mm, Nacalai Tesque, Inc.) with elution using a 33–43% linear gradient of CH_3CN (0.1% TFA) over 40 min. Purified triC34e, obtained in 17% yield, was identified by ESI-ToF-MS. Details of the synthesis of these peptides are described in the Supporting Information.

CD spectra

Circular dichroism measurements were performed with a J-720 CD spectropolarimeter equipped with a thermoregulator (Jasco). The wavelength dependence of molar ellipticity [θ] was monitored at 25 °C from λ 195 to 250 nm. The peptides were dissolved in PBS (50 mM sodium phosphate, 150 mM NaCl, pH 7.2).

Virus preparation

For virus preparation, 293FT cells in a 60 mm dish were transfected with the pNL4-3 construct (10 μg) by the calcium phosphate method. The supernatant was collected 48 h after transfection, passed through a 0.45 μm filter, and stored at −80 °C as the virus stock.

Anti-HIV-1 assay

For the viral fusion inhibition assay, TZM-bl cells (2×10^4 cells per 100 μL) were cultured with the NL4-3 virus (5 ng of p24) and serially diluted peptides. After culture for 48 h, cells were lysed, and the luciferase activity was determined with the Steady-Glo luciferase assay system (Promega, Fitchburg, WI, USA).[35] For the viral replication inhibition assay, MT-4 cells (5×10^4 cells) were exposed to HIV-1 NL4-3 (1 ng of p24) at 4 °C for 30 min. After centrifugation, cells were resuspended with 150 μL medium containing indicated concentrations of serially diluted peptides. Cells were cultured at 37 °C for 3 days, and the concentration of p24 in the culture supernatant was determined by HIV-1 p24 antigen ELISA kit (ZeptoMetrix, Buffalo, NY, USA).

Cytotoxicity assay

The cytotoxic effects of peptides were determined by the CellTiter 96 AQueous One Solution Cell Proliferation assay system (Promega) under the same conditions, but in the absence of viral infection.

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Keywords: antiviral agents · C34 trimers · fusion inhibitors · gp41 · HIV-1

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Conjugation of cell-penetrating peptides leads to identification of anti-HIV peptides from matrix proteins

Tetsuo Narumi [a], Mao Komoriya [a], Chie Hashimoto [a], Honggui Wu [b,c], Wataru Nomura [a], Shintaro Suzuki [a], Tomohiro Tanaka [a], Joe Chiba [c], Naoki Yamamoto [d], Tsutomu Murakami [b,*], Hirokazu Tamamura [a,*]

[a] *Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, Chiyoda-ku, Tokyo 101-0062, Japan*
[b] *AIDS Research Center, National Institute of Infectious Diseases, Shinjuku-ku, Tokyo 162-8640, Japan*
[c] *Department of Biological Science Technology, Tokyo University of Science, Noda, Chiba 278-8510, Japan*
[d] *Yong Loo Lin School of Medicine, National University of Singapore, Singapore 117597, Singapore*

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ABSTRACT

Compounds which inhibit the HIV-1 replication cycle have been found amongst fragment peptides derived from an HIV-1 matrix (MA) protein. Overlapping peptide libraries covering the whole sequence of MA were designed and constructed with the addition of an octa-arginyl group to increase their cell membrane permeability. Imaging experiments with fluorescent-labeled peptides demonstrated these peptides with an octa-arginyl group can penetrate cell membranes. The fusion of an octa-arginyl group was proven to be an efficient way to find active peptides in cells such as HIV-inhibitory peptides.

1. Introduction

Several anti-retroviral drugs beyond reverse transcriptase inhibitors, including effective protease inhibitors[1] and integrase inhibitors[2,3] are currently available to treat human immunodeficiency virus type 1 (HIV-1) infected individuals. We have also developed several anti-HIV agents such as coreceptor CXCR4 antagonists,[4–7] CD4 mimics,[8–10] fusion inhibitors[11] and integrase inhibitors.[12,13] However, the emergence of viral strains with multi-drug resistance (MDR), which accompanies the development of any antiviral drug, has encouraged a search for new types of anti-HIV-1 drugs with different inhibitory mechanisms.

Matrix (MA) proteins are essential for assembly of the virion shell. MA is a component of the Gag precursor protein, Pr55Gag, and is located within the viral membrane.[14,15] It has been reported that MA-derived peptides such as MA(47–59) inhibit infection by HIV,[16] and that MA-derived peptides such as MA(31–45) and MA(41–55) show anti-HIV activity.[17] In addition, Morikawa et al. report that MA(61–75) and MA(71–85) inhibit MA dimerization, a necessary step in the formation of the virion shell.[18] However, the question of whether the above MA peptides can penetrate cell membranes was not addressed in these reports. We speculate that to achieve antiviral activity it is essential that the MA-derived peptides penetrate the cell membrane and function intracellularly. In this paper, we report our design and construction of an overlapping library of fragment peptides derived from the MA protein with a cell membrane permeable signal. Our aim is the discovery of potent lead compounds, which demonstrate HIV inhibitory activity inside the host cells.

2. Materials and methods

2.1. Peptide synthesis

MA-derived fragments and an octa-arginyl (R_8) peptide were synthesized by stepwise elongation techniques of Fmoc-protected amino acids on a Rink amide resin. Coupling reactions were performed using 5.0 equiv of Fmoc-protected amino acid, 5.0 equiv of diisopropylcarbodiimide and 5.0 equiv of 1-hydroxybenzotriazole monohydrate. Ac_2O–pyridine (1/1, v/v) for 20 min was used to acetylate the N-terminus of MA-derived fragments, with the exception of fragment 1. Chloroactylation of the N-terminus of the R_8 peptide, was achieved with 40 equiv of chloroacetic acid, 40 equiv of diisopropylcarbodiimide and 40 equiv of 1-hydroxybenzotriazole monohydrate, treated for 1 h. Cleavage of peptides from resin and side chain deprotection were carried out by stirring for 1.5 h with a mixture of TFA, thioanisole, ethanedithiol, *m*-cresol

* Corresponding authors. Tel.: +81 3 5285 1111; fax: +81 3 5285 5037 (T.M.); tel.: +81 3 5280 8036; fax: +81 3 5280 8039 (H.M.).
E-mail addresses: tmura@nih.go.jp (T. Murakami), tamamura.mr@tmd.ac.jp (H. Tamamura).

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and triisopropylsilane (8.15/0.75/0.75/0.25/0.25/0.1, v/v). After removal of the resins by filtration, the filtrate was concentrated under reduced pressure, and crude peptides were precipitated in cooled diethyl ether. All crude peptides were purified by RP-HPLC and identified by ESI-TOFMS. In the conjugation of the R_8 peptide (or iodoacetamide), the peptide (or iodoacetamide) solution in 0.1 M phosphate buffer, pH 7.8 was added to MA fragments which were synthesized as described above. The reaction mixture was stirred at room temperature under nitrogen. After 24 h (or 1 h for the conjugation of iodoacetamide), purification was performed by RP-HPLC. The purified peptides were identified by ESI-TOF MS and lyophilized. Purities of all final compounds were confirmed to be >95% by analytical HPLC. Detailed data are provided in Supplementary data.

2.2. Anti-HIV-1 assay

Anti-HIV-1 (NL4-3 or NL(AD8)) activity was determined by measurement of the protection against HIV-1-induced cytopathogenicity in MT-4 cells or PM1/CCR5 cells. Various concentrations of test peptide solutions were added to HIV-1 infected MT-4 or PM1/CCR5 cells at multiplicity of infection (MOI) of 0.001 and placed in wells of a 96-well microplate. After 5 day incubation at 37 °C in a CO_2 incubator, the number of viable cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. The anti-HIV-1 (JR-CSF) activity was also determined by measuring capsid p24 antigen concentrations of the culture supernatant in the infected cultures by a commercially available ELISA assay (ZeptoMetrix Corp., Buffalo, NY).

2.3. CD spectroscopy

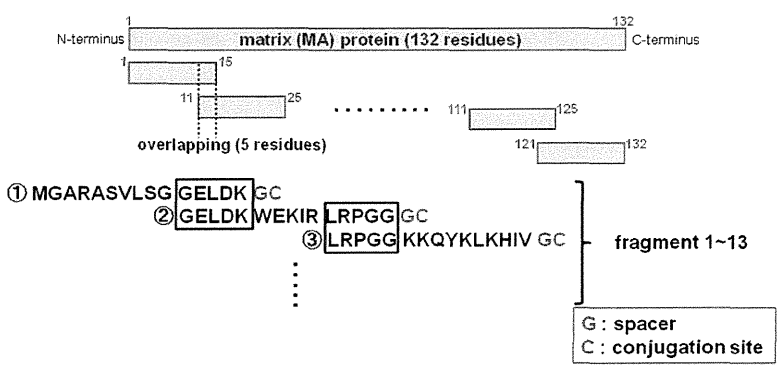
CD spectra were recorded on a JASCO J-720 spectropolarimeter at 25 °C. The measurements were performed using a 0.1 cm path length cuvette at a 0.1 nm spectral resolution. Each spectrum represents the average of 10 scans, and the scan rate was 50 nm/min. The concentrations of samples 8L and 9L were 28.2 and 64.7 μM, respectively, in PBS buffer (pH 7.4).

2.4. Fluorescent imaging of cell-penetrating MA peptides

Cells were seeded on 35 mm glass-bottom dish (2×10^5 cells/dish for HeLa and A549, 1×10^5 cells/dish for CHO-K1) one day before the experiments. The cells were cultured in DMEM/10% FBS/Penicillin–Streptomycin for HeLa and A549, or Ham's F12/10% FBS/Penicillin–Streptomycin for CHO-K1 at 37 °C/5% CO_2. Before the addition of MA peptides, cells were washed with Hanks' balanced salt solutions (HBSS) once. Peptides were added at 5 μM and further cultured for 30 min at 37 °C/5% CO_2. After incubation, cells were washed three times with HBSS and observed under a confocal laser-scanning microscopy (Zeiss LSM510).

3. Results and discussion

An overlapping peptide library spanning the whole sequence of the MA domain, p17, of NL4-3, the Gag precursor Pr55 of HIV-1 was designed. The full sequence of MA consists of 132 amino acid residues. In the peptide library, the MA sequence was divided from the N-terminus in 15-residue segments with an overlap of 5



fragment number	sequence
1	H-MGARASVLSGGELDKGC-NH_2
2	CH_3CO-GELDKWEKIRLRPGGGC-NH_2
3	CH_3CO-LRPGGKKQYKLKHIVGC-NH_2
4	CH_3CO-LKHIVWASRELERFAGC-NH_2
5	CH_3CO-LERFAVNPGLLETSEGC-NH_2
6	CH_3CO-LETSEGSRQILGQLQGC-NH_2
7	CH_3CO-LGQLQPSLQTGSEELGC-NH_2
8	CH_3CO-GSEELRSLYNTIAVLGC-NH_2
9	CH_3CO-TIAVLYSVHQRIDVKGC-NH_2
10	CH_3CO-RIDVKDTKEALDKIEGC-NH_2
11	CH_3CO-LDKIEEEQNKSKKKAGC-NH_2
12	CH_3CO-SKKKAQQAAADTGNNGC-NH_2
13	CH_3CO-DTGNNSQVSQNYGC-NH_2

Figure 1. The construction of MA-based overlapping peptide library.

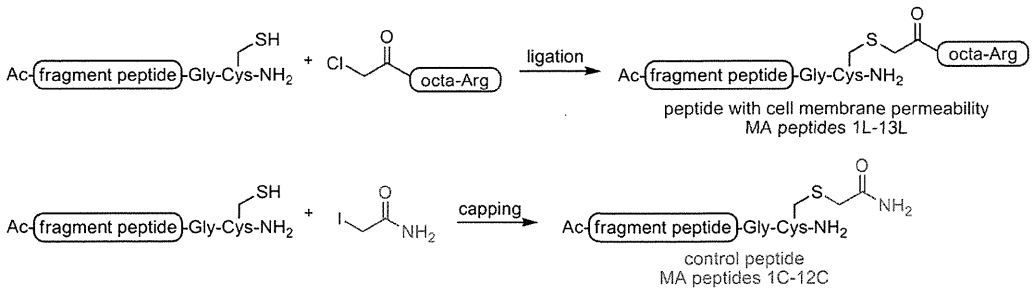


Figure 2. The design of MA peptides with cell membrane permeability (upper) and their control peptides (lower).

residues to preserve secondary structures (Fig. 1). Cys residues of the original MA sequence were changed into Ser residues because of the facility of peptide synthesis. Thirteen MA fragment peptides (1–13) were designed with the addition of Gly as a spacer and Cys as a conjugation site at the C-terminus. To impart cell membrane permeability to these peptides, the N-terminal chloroacetyl group of an octa-arginyl (R_8) peptide[19] was conjugated to the side-chain thiol group of the Cys residue of the above peptides. This resulted in the MA peptides 1L–13L (Fig. 2). R_8 is a cell membrane permeable motif and its fusion with parent peptides is known to produce bioactive peptides with no significant adverse properties.[12,13,20–24] In addition, the R_8-fusion can increase the solubility of MA

Table 1
Anti-HIV activity and cytotoxicity of control MA peptides

MA peptide	MT-4 cell	PM1/CCR5 cell		MT-4 cell
	NL4-3 (MTT assay) EC_{50}[a] (μM)	NL(AD8) (MTT assay) EC_{50}[a] (μM)	JR-CSF (p24 ELISA) EC_{50}[a] (μM)	(MTT assay) CC_{50}[b] (μM)
1C	>50	ND	ND	>50
2C	17 ± 1.4	1.0	ND	>50
3C	>50	ND	ND	>50
4C	No inhibition at 12.5 μM	ND	ND	14
5C	>50	ND	ND	>50
6C	37 ± 12	24% inhibition at 6.25 μM	25% inhibition at 50 μM	>50
7C	>50	ND	ND	>50
8C	>50	ND	ND	>50
9C	29 ± 1.4	13	8.1	>50
10C	No inhibition at 12.5 μM	ND	ND	17
11C	>50	ND	ND	>50
12C	>50	ND	ND	>50
14C	>50	ND	ND	>50
AZT	0.020	0.459	0.17	>100
SCH-D	ND	0.026	0.0014	ND

X4-HIV-1 (NL4-3 strain)-induced cytopathogenicity in MT-4 cells and R5-HIV-1 (NL(AD8) strain)-induced cytopathogenicity in PM1/CCR5 cells evaluated by the MTT assay, and inhibitory activity against R5-HIV-1 (JR-CSF strain)-induced cytopathogenicity in PM1/CCR5 cells evaluated by the p24 ELISA assay.

[a] EC_{50} values are the concentrations for 50% protection from HIV-1-induced cytopathogenicity in MT-4 cells.

[b] CC_{50} values are the concentrations for 50% reduction of the viability of MT-4 cells. All data are the mean values from at least three independent experiments. ND: not determined.

Table 2
Anti-HIV activity and cytotoxicity of MA peptides with cell membrane permeability

MA peptide	MT-4 cell	PM1/CCR5 cell		MT-4 cell
	NL4-3(MTT assay) EC_{50} (μM)	NL(AD8)(MTT assay) EC_{50} (μM)	JR-CSF(p24 ELISA) EC_{50} (μM)	(MTT assay) CC_{50} (μM)
1L	30	30	40	>50
2L	21 ± 4.2	>31	ND	32 ± 4.2
3L	no inhibition at 25 μM	ND	ND	36
4L	no inhibition at 3.13 μM	ND	ND	3.7
5L	40	42% inhibition at 50 μM	42	>50
6L	40 ± 8.9	49% inhibition at 50 μM	31	>50
7L	35 ± 1.5	37% inhibition at 50 μM	35% inhibition at 50 μM	>50
8L	2.3 ± 0.3	5.8	7.8	9.0 ± 2.4
9L	2.1 ± 0.5	0.43	0.58	5.7 ± 2.1
10L	43 ± 8.5	42% inhibitionat 50 μM	27	>50
11L	18 ± 3.0	17% inhibition at 25 μM	23	>50
12L	41 ± 5.5	30% inhibition at 25 μM	27	>50
13L	20 ± 2.1	0.43	11	>50
14L	no inhibition at 25 μM	ND	ND	36
AZT	0.020	0.459	0.17	>100
SCH-D	ND	0.026	0.0014	ND

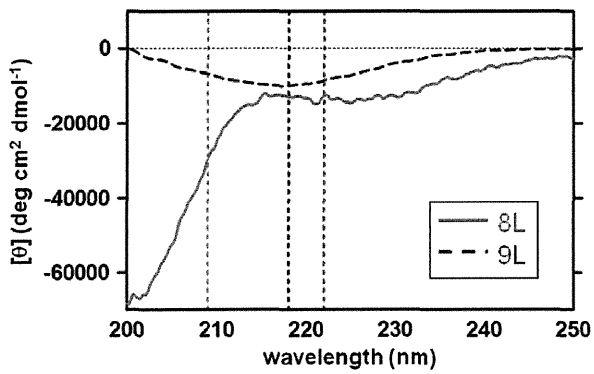


Figure 3. CD spectra of MA peptides 8L (28 μM) and 9L (65 μM) in PBS buffer, pH 7.4 at 25 °C.

peptides whose hydrophobicity is relatively limited. On the other hand, to develop control peptides lacking cell membrane permeability, iodoacetamide was conjugated to the thiol group of the Cys residue to prepare MA peptides 1C–12C (Fig. 2). MA peptide 13C was not synthesized because MA fragment 13 is insoluble in PBS buffer.

The anti-HIV activity of MA peptides 1L–13L and MA peptides 1C–12C, was evaluated. Inhibitory activity against T-cell line-tropic (X4-) HIV-1 (NL4-3 strain)-induced cytopathogenicity in MT-4 cells and against macrophage-tropic (R5-) HIV-1 (NL(AD8) strain)-induced cytopathogenicity in PM1/CCR5 cells was assessed by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay, and inhibitory activity against R5-HIV-1 (JR-CSF strain) replication in PM1/CCR5 cells was determined by the p24 ELISA assay. The results are shown in Tables 1 and 2. The control MA peptides 6C and 9C showed slight anti-HIV activity against NL4-3, NL(AD8) and JR-CSF strains, and 2C showed high anti-HIV activity against NL4-3 and NL(AD8) strains, but the other control MA peptides showed no significant anti-HIV activity. 2C showed significant anti-HIV activity against both X4-HIV-1 and R5-HIV-1 strains, suggesting that this region of the MA domain is relevant with Gag localization to the plasma membrane (PM)[25] and that 2C might inhibit competitively the interaction between MA and PM. On the other hand, the MA peptides with the exception of 3L and 4L, showed moderate to potent anti-HIV activity against all three strains. These peptides expressed almost the same level of anti-HIV activity against both X4-HIV-1 and R5-HIV-1 strains. The MA peptides 8L and 9L in particular, showed significant anti-HIV activity. These results suggest that MA peptides achieve entry into target cells as a result of the addition of R_8, and inhibit viral replication within the cells. The adjacent peptides 8L and 9L possess an overlapping sequence TIAVL. Such peptides exhibited relatively high cytotoxicity and the MA peptide 4L showed the highest cytotoxicity although it did not show any significant anti-HIV activity. The control MA peptides 1C–12C were relatively weakly cytotoxic. The MA peptides 8C and 9C exhibited no significant cytotoxicity, although the addition of R_8, giving 8L and 9L, caused a remarkable increase in cytotoxicity. This suggests that the octa-arginyl (R_8) sequence is correlated with the

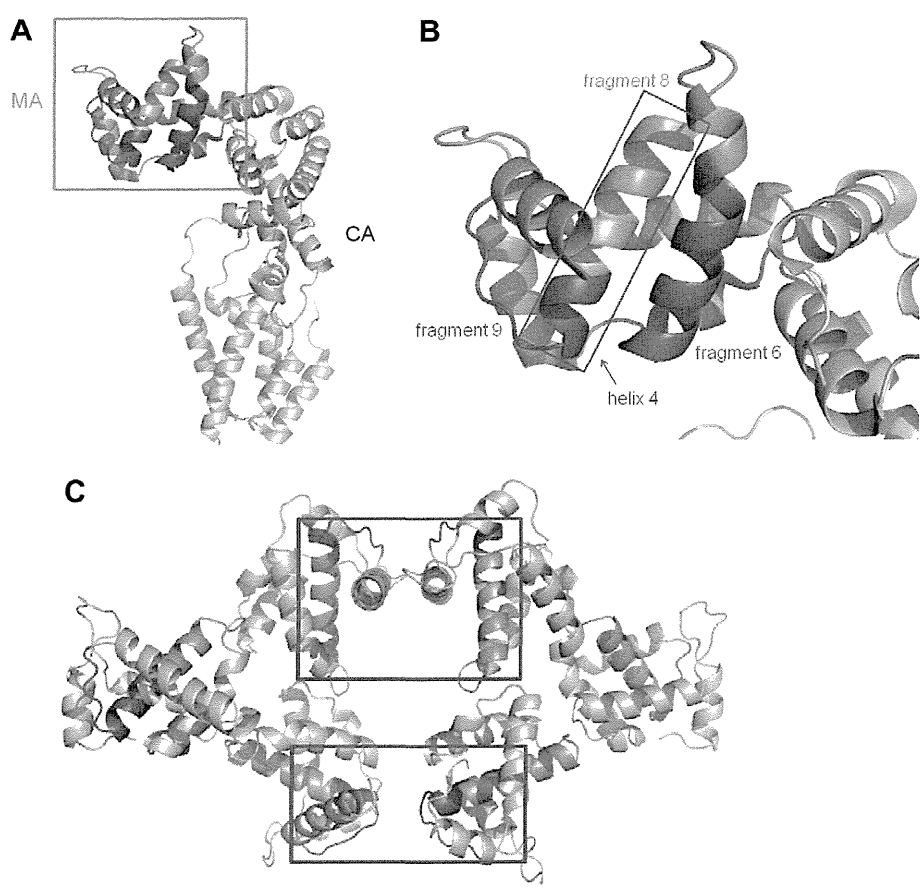


Figure 4. (A) The complete structure of MA and CA proteins (PDB ID: 2gol). (B) The enlarged structure of the highlighted region of (A). (C) The structure of an MA hexamer. Red-colored squares show interfaces between two MA trimers (PDB ID: 1hiw). Orange- and pink-colored helical ribbons represent fragments 8 and 9, respectively.

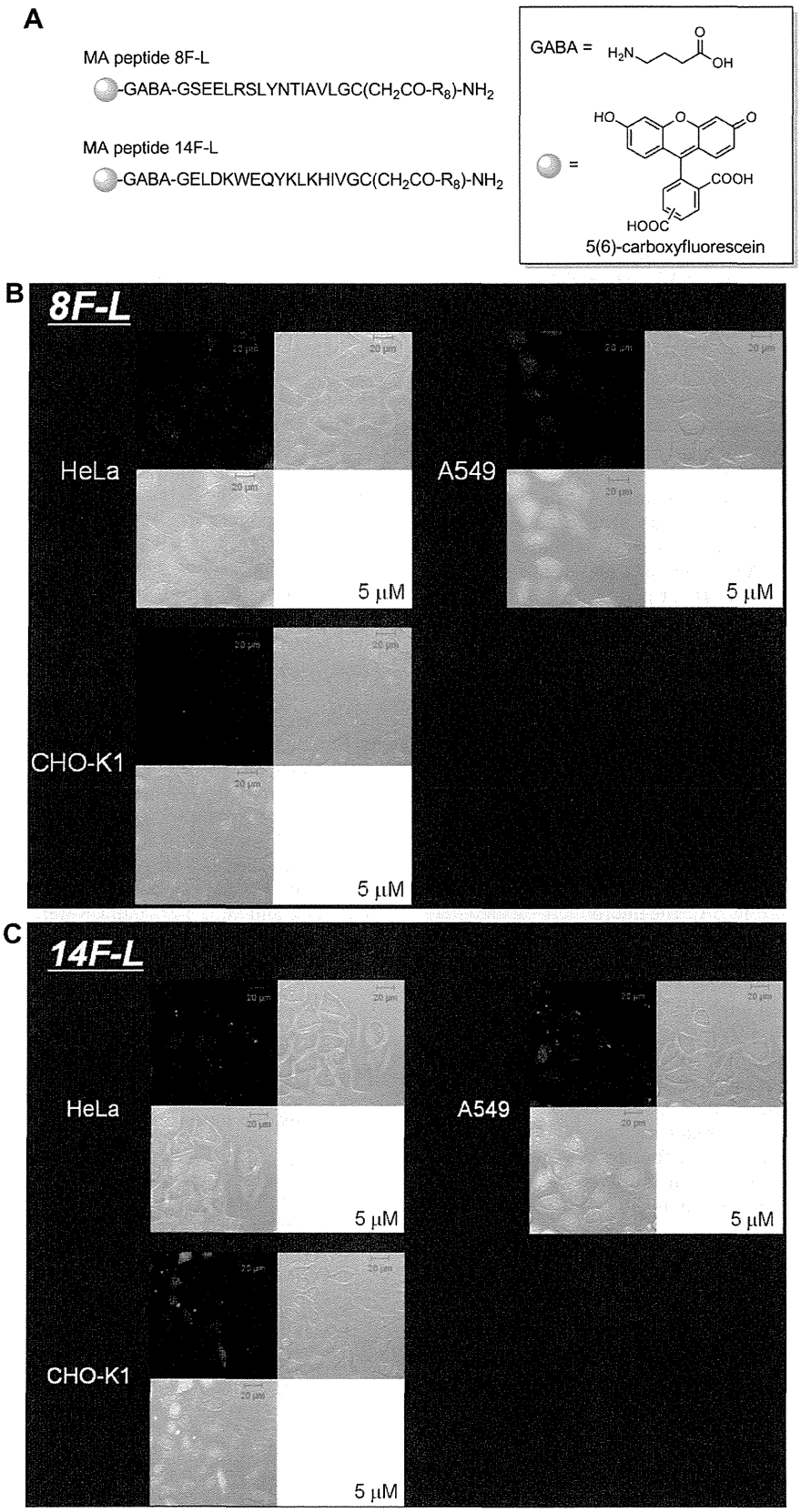


Figure 5. (A) The structures of fluorophore-labeled MA peptides 8F–L and 14F–L. (B) The fluorescent imaging of live cells HeLa, A549 and CHO-K1 by 8F–L. (C) The fluorescent imaging of live cells HeLa, A549 and CHO-K1 by 14F–L.

expression of cytotoxicity and in future, a different effective strategy for cell penetration may be advisable.

In the present assay, the control MA peptides 6C and 9C, which cover MA(51–65) and MA(81–95), respectively, showed significant anti-HIV activity. This is consistent with the previous studies, in which MA(41–55), MA(47–59) and MA(71–85) showed anti-HIV or dimerization inhibitory activity as discussed above.[16–18] These peptides have no R_8 sequence and thus cannot penetrate cell membranes. They exhibit inhibitory activity on the surface of cells, not intracellularly.

The structures of MA peptides 8L and 9L, dissolved in PBS buffer (2.7 mM KCl, 137 mM NaCl, 1.47 mM KH_2PO_4, 9.59 mM Na_2HPO_4) at pH 7.4, were determined by CD spectroscopy (Fig. 3). When peptides form α-helical structures, minima can be observed at approximately 207 and 222 nm in their CD spectra. The amino acid residues covering fragments 8 and 9 corresponding to 8L and 9L are located in an α-helical region (helix 4) of the parent MA protein (Fig. 4), and peptides 8L and 9L were presumed to have an α-helical conformation.[26–28] However, the CD spectra shown in Figure 3, suggest that these peptides lack any characteristic secondary structure. This is because the 15-mer peptide derived from MA is not sufficiently long to form a secondary structure even though Gly, Cys and octa-Arg are attached to their C-terminus. Analysis of the CD spectra suggests MA fragment peptides need a longer sequence in order to form a secondary structure. The CD spectra of the control MA peptides 8C and 9C were not determined because the aqueous solubility of these peptides is inadequate.

Fluorescent imaging of live cells was used to evaluate the cell membrane permeability of the MA peptides 8L and 14L, which showed high and zero significant anti-HIV activity, respectively. The MA fragment 14 is a hybrid of the fragments 2 and 3, and the MA peptides 14L and 14C, which are based on the conjugation of the N-terminal chloroacetyl group of an R_8 peptide and iodoacetamide to the thiol group of the Cys residue, respectively (Supplementary data), are control peptides lacking significant anti-HIV activity (Tables 1 and 2). These peptides were labeled with 5(6)-carboxyfluorescein via a GABA linker at the N-terminus to produce 8F–L and 14F–L (Fig. 5A). The fluorophore-labeled peptides 8F–L and 14F–L were incubated with live cells of HeLa, A549 and CHO-K1, and the imaging was analyzed by a fluorescence microscope (Fig. 5B and C). A549 cells are human lung adenocarcinomic human alveolar basal epithelial cells.[29] Similar penetration of both peptides 8F–L and 14F–L into these cells was observed. Even peptides without significant anti-HIV activity can penetrate cell membranes. The penetration efficiency of both peptides into A549 was relatively high and into HeLa was low. In CHO-K1 the penetration efficiency of 8F–L is relatively low, but that of 14F–L is high. These imaging data confirm that the MA peptides with the R_8 sequence can penetrate cell membranes and suggest that MA peptides such as 8L and 9L should be able to inhibit HIV replication inside cells.

4. Conclusions

Several HIV-1 inhibitory fragment peptides were identified through the screening of an overlapping peptide library derived from the MA protein. Judging by the imaging experiments, peptides possessing the R_8 group can penetrate cell membranes and might exhibit their function intracellularly thus inhibiting HIV replication.

Two possible explanations for the inhibitory activity of these MA fragment peptides can be envisaged: (1) The fragment peptides might attack an MA protein and inhibit the assembly of MA proteins. (2) These peptides might attack a cellular protein and inhibit its interaction with MA. Further studies to elucidate detailed action mechanisms and identify the targets of these peptides will be performed in future. The technique of addition of the R_8 group to peptides enabled us to screen library peptides that function within cells. Thus, the design of an overlapping peptide library of fragment peptides derived from a parent protein with a cell membrane permeable signal is a useful and efficient strategy for finding potent cell-penetrating lead compounds.

In the present study, the MA peptides 8L and 9L were shown to inhibit HIV-1 replication with submicromolar to micromolar EC_{50} values in cells using the MT-4 assay (NL4-3 and NL(AD8) strains) and the p24 ELISA assay (JR-CSF strain). Our findings suggest that these peptides could serve as lead compounds for the discovery of novel anti-HIV agents. Amino acid residues covering fragments 8 and 9 corresponding to 8L and 9L are located in the exterior surface of MA, and in particular in the interface between two MA trimers (Fig. 4C).[26–28] The interaction of two MA trimers leads to the formation of an MA hexamer, which is the MA assembly with physiological significance. Thus, the region covering fragments 8 and 9 is critical to oligomerization of MA proteins. This suggests that MA peptides 8L and 9L might inhibit the MA oligomerization through competitive binding to the parent MA, and that more potent peptides or peptidomimetic HIV inhibitors could result from studies on the mechanism of action of these MA peptides and identification of the interaction sites. Taken together, some seeds for anti-HIV agents are inherent in MA proteins, including inhibitors of the interaction with PM such as the MA peptide 2C.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.12.055.

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CD4 mimics as HIV entry inhibitors: Lead optimization studies of the aromatic substituents

Tetsuo Narumi [a], Hiroshi Arai [a], Kazuhisa Yoshimura [b,c], Shigeyoshi Harada [b,c], Yuki Hirota [a], Nami Ohashi [a], Chie Hashimoto [a], Wataru Nomura [a], Shuzo Matsushita [b], Hirokazu Tamamura [a,*]

[a] Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, Chiyoda-ku, Tokyo 101-0062, Japan
[b] Center for AIDS Research, Kumamoto University, Kumamoto 860-0811, Japan
[c] AIDS Research Center, National Institute of Infectious Diseases, Shinjuku-ku, Tokyo 162-8640, Japan

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ABSTRACT

Several CD4 mimics have been reported as HIV-1 entry inhibitors that can intervene in the interaction between a viral envelope glycoprotein gp120 and a cell surface protein CD4. Our previous SAR studies led to a finding of a highly potent analogue **3** with bulky hydrophobic groups on a piperidine moiety. In the present study, the aromatic ring of **3** was modified systematically in an attempt to improve its antiviral activity and CD4 mimicry which induces the conformational changes in gp120 that can render the envelope more sensitive to neutralizing antibodies. Biological assays of the synthetic compounds revealed that the introduction of a fluorine group as a *meta*-substituent of the aromatic ring caused an increase of anti-HIV activity and an enhancement of a CD4 mimicry, and led to a novel compound **13a** that showed twice as potent anti-HIV activity compared to **3** and a substantial increase in a CD4 mimicry even at lower concentrations.

1. Introduction

The first step of HIV entry into host cells is the interaction of a viral envelope glycoprotein gp120 with the cell surface protein CD4.[1] Such a viral attachment process is an attractive target for the development of the drugs to prevent the HIV-1 infection of its target cells.[2] Several small molecules including BMS-806,[3] IC-9564[4] and NBDs[5] have been identified that inhibit the viral attachment process by binding to gp120. Recently, we and others have been exploring the potentials of NBDs-derived CD4 mimics as a novel class of HIV entry inhibitors (Fig. 1).[6–8]

Small molecular CD4 mimics identified by an HIV syncytium formation assay showed potent cell fusion and virus cell fusion inhibitory activity against several HIV-1 laboratory and primary isolates.[5] Furthermore, the interaction of CD4 mimics with a highly conserved and functionally important pocket on gp120, known as the 'Phe43 cavity', induces conformational changes in gp120,[9] a process which occurs with unfavorable binding entropy, leading to a favorable enthalpy change similar to those caused by binding of the soluble CD4 binding to gp120. These unique properties render CD4 mimics valuable not only for the development of entry inhibitors, but which also, when combined with neutralizing antibodies function as envelope protein openers-putatively, stimulants.[10]

The structure of the complex formed by NBD-556 (**1**) bound to the gp120 core from an HIV-1 clade C strain (C1086) was recently determined by X-ray analysis (PDB: 3TGS).[11] As expected with molecular modeling by us[8a] and others,[6a] NBD-556 binds with Phe43 cavity with its *p*-chlorophenyl ring inserted into the cavity, and in addition multiple contacts were observed, with Trp112, Val255, Phe382, Ile424, Asn425, Trp427, Gly473, and Val430 of gp120 were observed (Fig. 2). However, no obvious interaction with Arg59 of CD4 was observed, although the salt bridge formation between Arg59 of CD4 and Asp368 of gp120 is a critical interaction of the viral attachment.[12] Based on this binding model, several potent compounds were recently identified.[6c,7]

Prior to those studies, we performed structure–activity relationship (SAR) studies based on the modification of the piperidine moiety of CD4 mimics to interact with Val430 and/or Asp368. These resulted in the discovery of a potent compound **3** which has bulky hydrophobic groups on its piperidine ring, and shows significant anti-HIV activity and lower cytotoxicity than other known CD4 mimics.[8c] Our study of the docking of **3** into the Phe43 cavity of gp120 suggests that the cyclohexyl group of **3** can interact hydrophobically with the isopropyl group of Val430.

We hypothesized that the optimization of the aromatic ring of **3** would lead to an increase of antiviral activity and CD4 mimicry, the latter inducing the conformational changes in gp120. Here, we de-

* Corresponding author. Tel.: +81 3 5280 8036; fax: +81 3 5280 8039.
E-mail address: tamamura.mr@tmd.ac.jp (H. Tamamura).

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scribe the systematic modification of the aromatic ring of **3** for further optimization to evaluate substituent effects on anti-HIV activity, cytotoxicity and CD4 mimicry.

2. Results and discussion

The co-crystal structure of **1** with the gp120 core revealed that the aromatic group of **1** binds to gp120 by several aromatic–aromatic and hydrophobic interactions (Fig. 2). In particular, hydrophobic space surrounded by the hydrophobic amino acid residues Trp112, Val255, Phe382, and Ile424 is likely to be affected by substituents at the *meta*- and *para*-positions of the aromatic ring, and consequently we decided to investigate substituents at these positions (Fig. 3).

Initially, we selected a chlorine or a methyl group to serve as the *para*-substituent of the aromatic group because CD4 mimic compounds such as **1** (NBD-556) with a *p*-chloro substituent, and because **3** showed significant anti-HIV activity compared to other substituents. Further, CD4 mimic structures such as **2** with a *p*-methyl substituent also showed potent anti-HIV activity and exhibits lower cytotoxicity than those with the *p*-chlorophenyl derivatives.[8a] Next, we chose several halogens including F, Cl and Br, to be the *meta*-substituent on the aromatic group since previous SAR studies revealed that the introduction of an appropriate group with an electron-withdrawing ability at the *meta*-position leads to an increase of binding affinity and antiviral activity.[6a] Furthermore, to investigate whether electron withdrawal and hydrophobicity of the *meta*-position are appropriate, the CD4 mimics with a *meta*-methyl substituent, which has electron-donating properties and is similar in size to bromine, were also synthesized. Finally, two piperidine scaffolds (the 2,2,6,6-tetramethylpiperidine **A** and the dicyclohexylpiperidine **B**) were combined with these aromatics via the oxalamide linker.

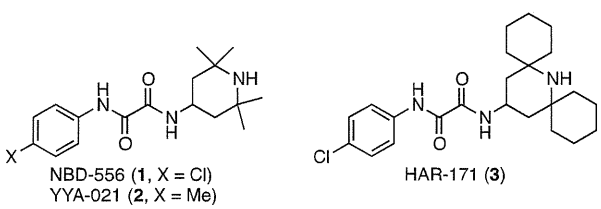

Figure 1. Structures of NBD-556 (**1**), YYA-021 (**2**) and HAR-171 (**3**).

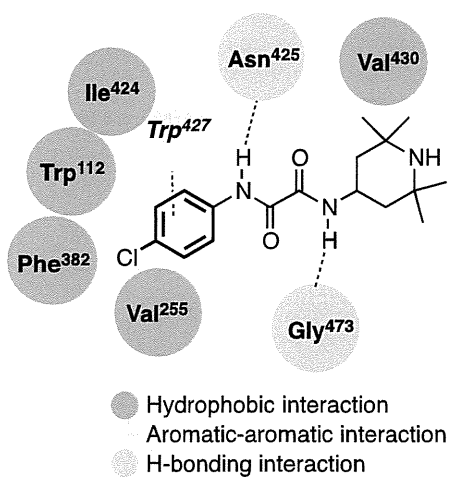

Figure 2. Major interactions between NBD-556 and Phe43 cavity of gp120.

2.1. Chemistry

The syntheses of novel compounds are depicted in Schemes 1 and 2. Starting from the appropriate aniline with *m*- and *p*-substituents, coupling with ethyl chloroglyoxylate in the presence of Et_3N gave the corresponding amidoesters **6a–c** and **7a–c**. Subsequently, microwave-assisted aminolysis[13] of **6a–c** and **7a–c** with commercially available 4-amino-2,2,6,6-tetramethylpiperidines afforded the desired compounds **8a–c** and **9a–c** (Scheme 1). A series of CD4 mimics with two cyclohexyl groups **13a–c** and **14a–c** were prepared from 2,2,6,6-tetramethylpiperidin-4-one **10** by the method previously reported,[8c] with slight modification (Scheme 2). Briefly, treatment of **10** with cyclohexanone in the presence of ammonium chloride gave a 2,6-substituted piperidin-4-one **11** via Grob fragmentation followed by intramolecular cyclization.[14] Reductive amination with *p*-methoxybenzyl amine, acidic treatment with TMSBr/TFA, and oxidative cleavage of *p*-methoxybenzyl group with cerium(IV) ammonium nitrates (CAN) furnished the corresponding 4-aminopiperidines (**12**) with higher yields and less burdensome purifications than the previous method. Finally, coupling of **12** with the corresponding esters **6a–c** and **7a–c** under microwave irradiation provided the desired compounds **13a–c** and **14a–c**.

2.2. Biological evaluation

The anti-HIV activity of the synthetic compounds was evaluated against an R5 primary isolate YTA strain. IC_{50} values were determined by the WST-8 method as the concentrations of the compounds that conferred 50% protection against HIV-1-induced cytopathogenicity in PM1/CCR5 cells. Cytotoxicity of the compounds based on the viability of mock-infected PM1/CCR5 cells was also evaluated using the WST-8 method. The assay results for compounds **8a–c** and **13a–c** with a *p*-chlorophenyl group are shown in Table 1. The parent compound **1** and compound **8a**,[6a] known as JRC-II-191, showed significant anti-HIV activities (IC_{50}

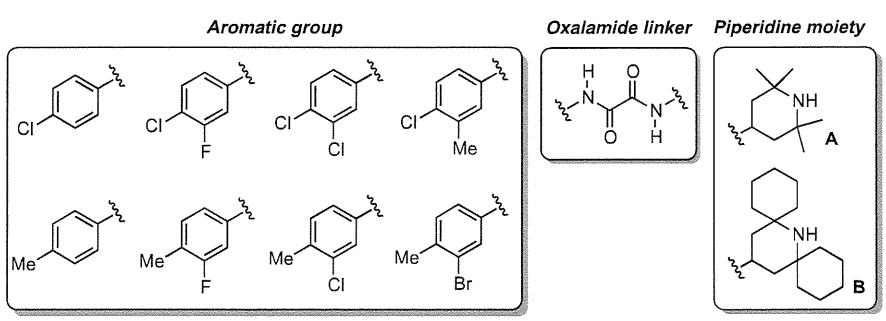

Figure 3. The structures of scaffolds in the design of novel CD4 mimics.