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 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.

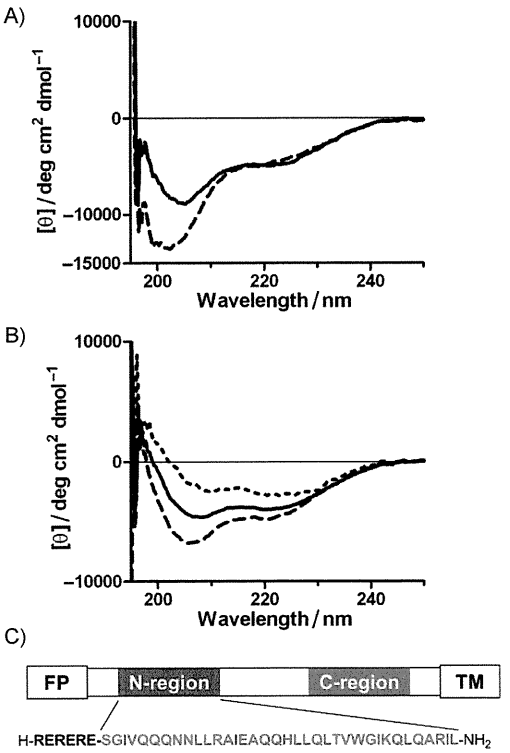

Figure 3. A) CD spectra of C34REG (monomer, -----, 6 μM) and triC34e (trimer, ——, 2 μM). B) CD spectra in the presence or absence of the N36 monomer N36RE:[20] -----, [C34REG (6 μM) + N36RE (6 μM)]; ——, [triC34e (2 μM) + N36RE (6 μM)]; ••••, N36RE (6 μM). In the amino acid sequence of N36RE, the triplet 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.

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

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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

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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

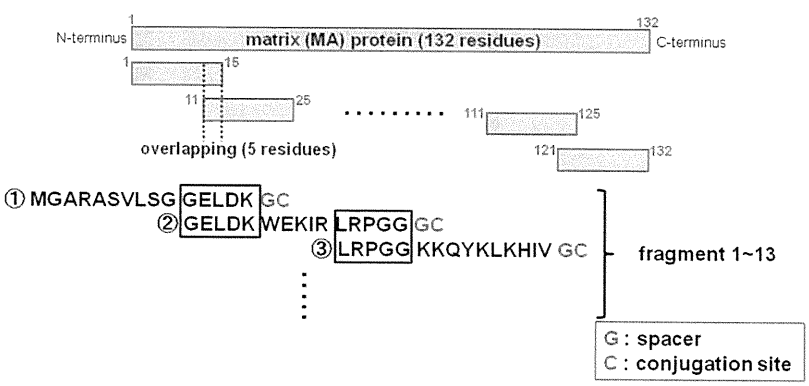
CD spectra were recorded on a JASCO J-720 spectropolarimeter at 25 °C. The measurements were performed using a 0.1 cm path length cuvette at a 0.1 nm spectral resolution. Each spectrum represents the average of 10 scans, and the scan rate was 50 nm/min. The concentrations of samples 8L and 9L were 28.2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

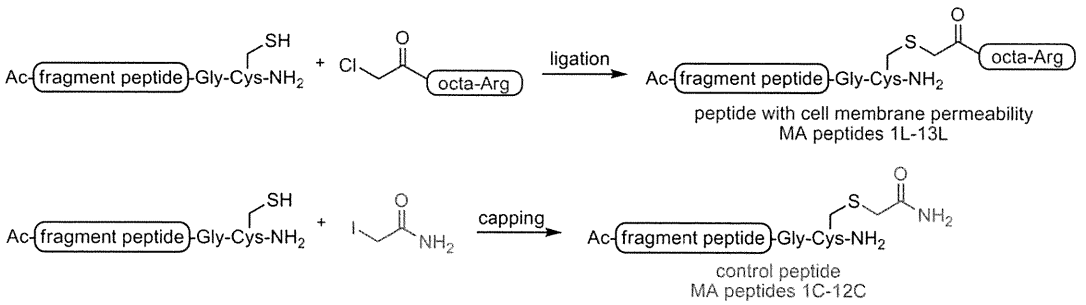


Figure 2. The design of MA peptides with cell membrane permeability (upper) and their control peptides (lower).

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

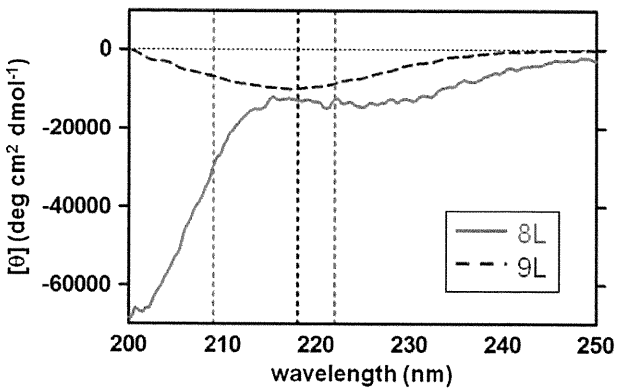


Figure 3. CD spectra of MA peptides 8L (28 μM) and 9L (65 μM) in PBS buffer, pH 7.4 at 25 °C.

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

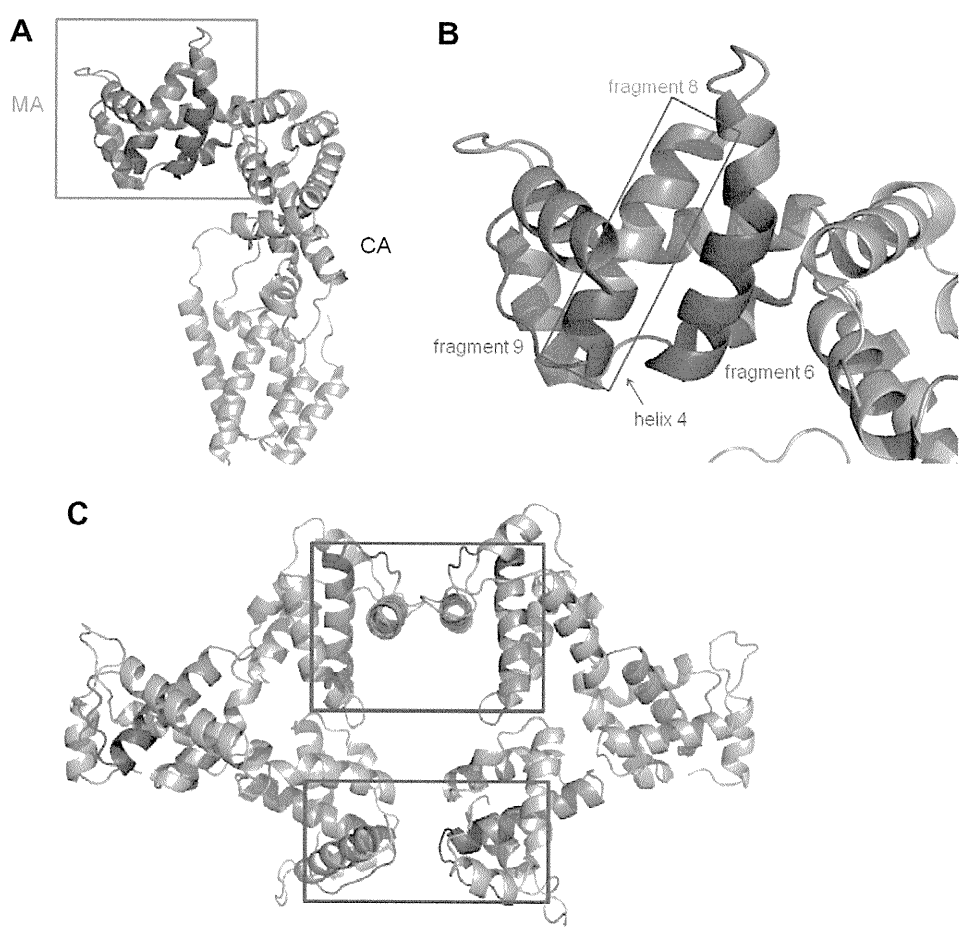


Figure 4. (A) The complete structure of MA and CA proteins (PDB ID: 2gol). (B) The enlarged structure of the highlighted region of (A). (C) The structure of an MA hexamer. Red-colored squares show interfaces between two MA trimers (PDB ID: 1hiw). Orange- and pink-colored helical ribbons represent fragments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

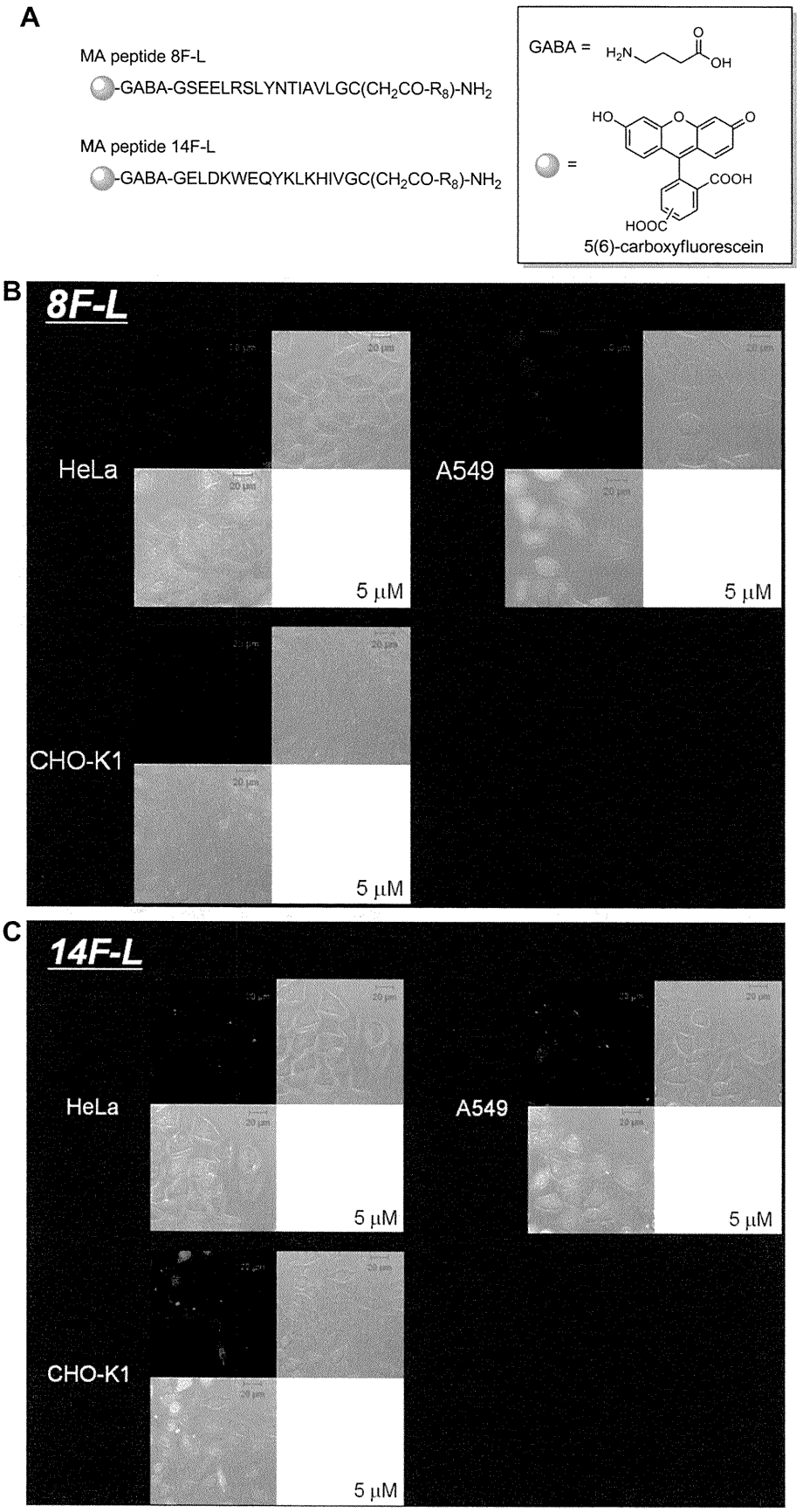


Figure 5. (A) The structures of fluorophore-labeled MA peptides 8F–L and 14F–L. (B) The fluorescent imaging of live cells HeLa, A549 and CHO-K1 by 8F–L. (C) The fluorescent imaging of live cells HeLa, A549 and CHO-K1 by 14F–L.

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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Evaluation of a synthetic C34 trimer of HIV-1 gp41 as AIDS vaccines

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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

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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,

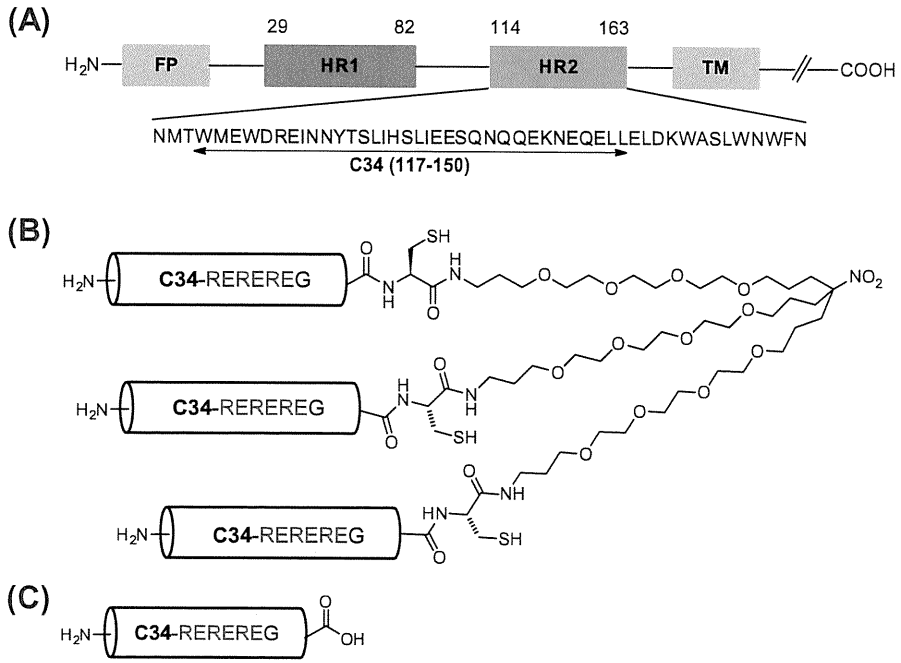

Figure 1. The sequence of C34 in gp41 (NL4-3) (A). FP and TM represent hydrophobic fusion peptide and transmembrane domain, respectively. Structures of C34-derived peptides, the C34 trimer with a C3-symmetric linker, triC34e (B), and the C34 monomer, C34REG (C).

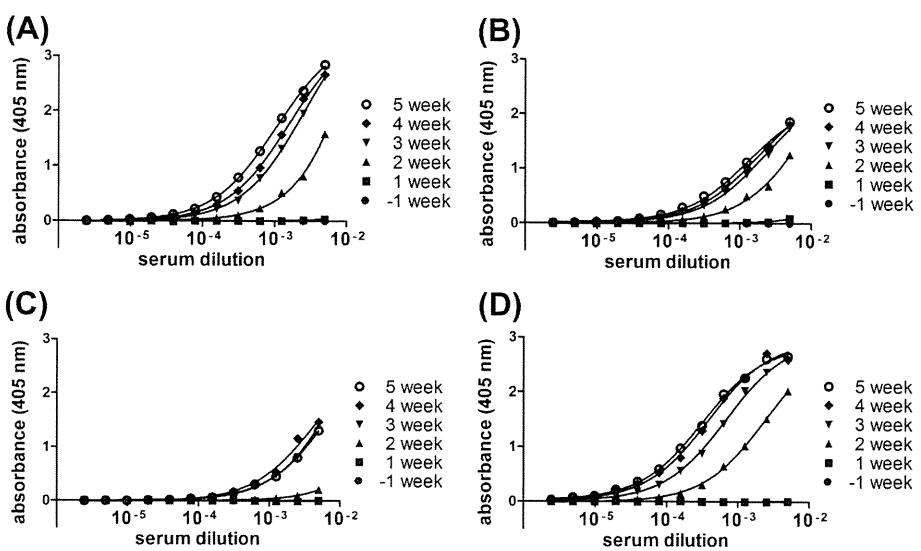

Figure 2. Results of serum titer ELISA of antisera collected during immunization (from one week before start to five weeks after immunization start) to determine the immunogenicity of designed antigens. The titers were evaluated as followings; antiserum against C34REG binding to C34REG (A); antiserum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

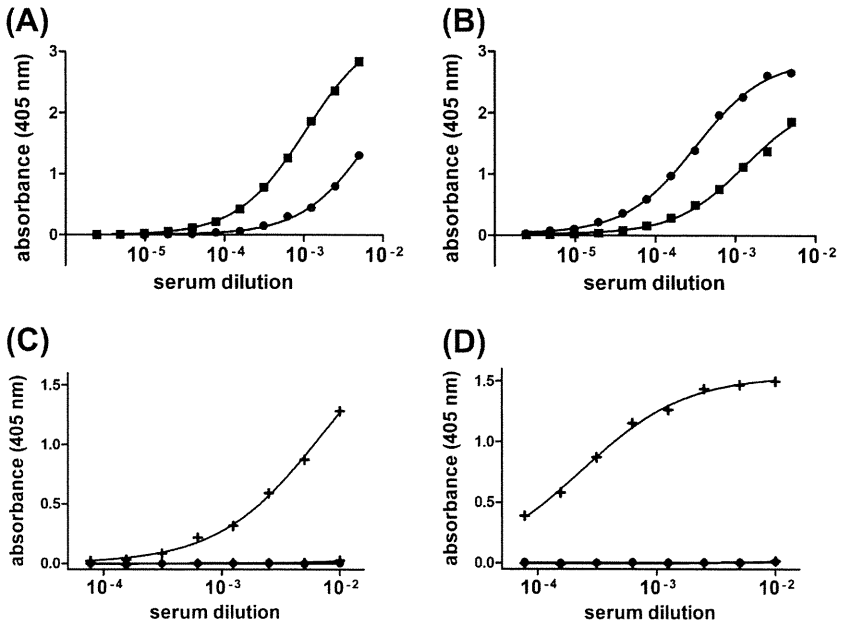

Figure 3. Serum titers of the antibodies produced by the fifth immunization of the C34REG antigen and the fourth immunization of the triC34e antigen. These titers were evaluated against ELISA templates of C34REG (monomer) (A) and triC34e (trimer) (B), using sera obtained from a C34REG-immunized mouse (■) 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.

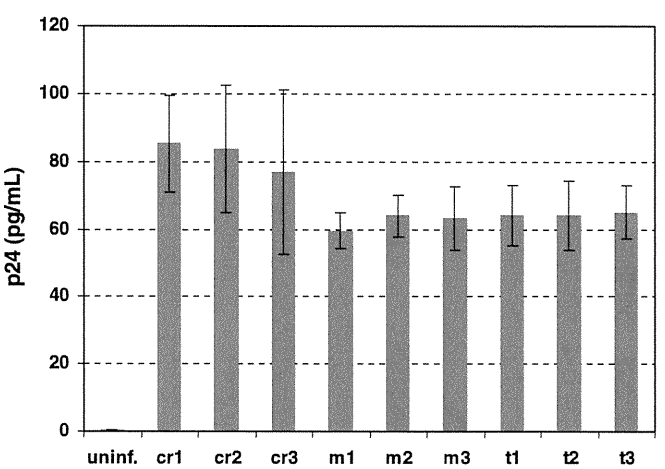

Figure 4. Determination of neutralization activity of the antibodies produced by immunization of C34REG and triC34e antigens. Inhibition of HIV-1 (NL4-3 strain) infection by produced antibodies was evaluated by the p24 assay in MT-4 cells. Y-axis shows the amount of p24 production. Uninf means uninfected 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

This work was supported in part by Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and Health and Labour Sciences Research Grants from Japanese Ministry of Health, Labour, and Welfare. C.H. was supported by JSPS Research Fellowships for Young Scientists.

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Pharmacophore-based small molecule CXCR4 ligands

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ABSTRACT

Low molecular weight CXCR4 ligands were developed based on the peptide T140, which has previously been identified as a potent CXCR4 antagonist. Some compounds with naphthyl, fluorobenzyl and pyridyl moieties as pharmacophore groups in the molecule showed significant CXCR4-binding activity and anti-HIV activity. Structure–activity relationships were studied and characteristics of each of these three moieties necessary for CXCR4 binding were defined. In this way, CXCR4 ligands with two types of recognition modes for CXCR4 have been found.

The chemokine receptor CXCR4 is classified into a family of G protein-coupled receptors (GPCRs), and transduces signals of its endogenous ligand, CXCL12/stromal cell-derived factor-1 (SDF-1).[1] The CXCR4-CXCL12 axis plays a physiological role in chemotaxis,[2] angiogenesis[3] and neurogenesis[4] in embryonic stages. The CXCR4 receptor is linked to many disorders including HIV infection/AIDS,[5] metastasis of cancer cells,[6] leukemia cell progression,[7] rheumatoid arthritis.[8] Since CXCR4 is an important drug target in these diseases, it is thought that effective agents directed to this receptor may be useful leads for therapeutic agents. To date, we and others have developed several potent CXCR4 antagonists. A highly potent antagonist, T140, a 14-mer peptide with a disulfide bridge, and its downsized analogue, FC131, with a cyclic pentapeptide scaffold, and several other related compounds have been reported.[9] Based on T140 and FC131, small-sized linear anti-HIV agents such as ST34 (**1**) have been developed (Fig. 1).[10] AMD3100,[11] KRH-1636,[12] Dpa–Zn complex (**2**)[13] and other azamacrocyclic compounds such as **3**,[14] which like **1**, contain benzylamine and electron-deficient aromatic groups, have also been reported as nonpeptidic antagonists. Compound **1** possesses significant anti-HIV activity but does not have high CXCR4 binding affinity. In the present study, more effective linear CXCR4 antagonists derived from compound **1** have been examined, and structure–activity relationship studies of these compounds have been performed.

Initially, three segments of compound **1** were selected for structural modification to support the design of new synthetic compounds: replacement of the 4-trifluoromethylbenzoyl group (Fig. 2, R^1), modification of the stereochemistry of the 1-naphthylethylamine moiety (R^2) and introduction of pyridine moieties on the nitrogen atom (R^3). In a previous study of T140 analogues, 4-fluorobenzoyl was found to be superior to 4-trifluoromethylbenzoyl as an N-terminal moiety. Thus, 4-fluorobenzyl, 4-fluorobenzoyl and 4-fluorophenylethyl groups were used as substitutes for the 4-trifluoromethylbenzoyl group (R^1) in **1**. The (*R*)-1-naphthylethylamine moiety in **1** is also present in KRH-1636 where it has the (*S*)-stereochemistry and thus both the (*R*) and (*S*)-stereoisomers were investigated in the present study. Several CXCR4 antagonists such as KRH-1636,[12] Dpa-Zn complex (**2**)[13] and Dpa-cyclam compound (**3**),[14] contain pyridyl rings. Thus, 2, 3, or 4-pyridylmethyl and 2, 3, or 4-pyridylethyl groups were introduced on the nitrogen atom of the 4-aminomethylbenzoyl group (R^3). With these modifications, a total of $3 \times 2 \times 6 = 36$ compounds (**12–47**) were designed (Fig. 2).

The synthesis of the structural fragment, Unit 1 is shown in Scheme 1. N-nosylation of 4-amino-methylbenzoic acid (**4**) with 2-nitrobenzenesulfonyl chloride and subsequent esterification gave the *t*-butyl ester **5**. Introduction of an R^3 moiety by means of a Mitsunobu reaction followed by removal of the Ns group yielded amines **6A–F**. Introduction of either 4-fluorobenzyl or 4-fluorophenylethyl groups by reductive amination of **6A–F** produced amines **7Ai–Fi** or **7Aiii–Fiii**, respectively. Conversion of **6A–F** to the appropriate amide (**7Aii-Fii**), and subsequent deprotection of the *tert*-butyl group yielded Unit 1, **8Ai–Fiii**.

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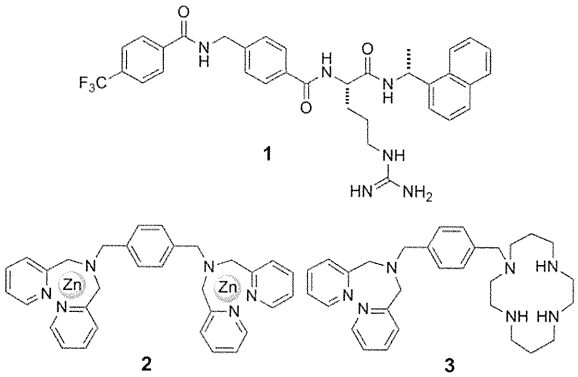


Figure 1. The structures of **1** (ST34), Dpa-Zn complex (**2**) and Dpa-cyclam compound (**3**).

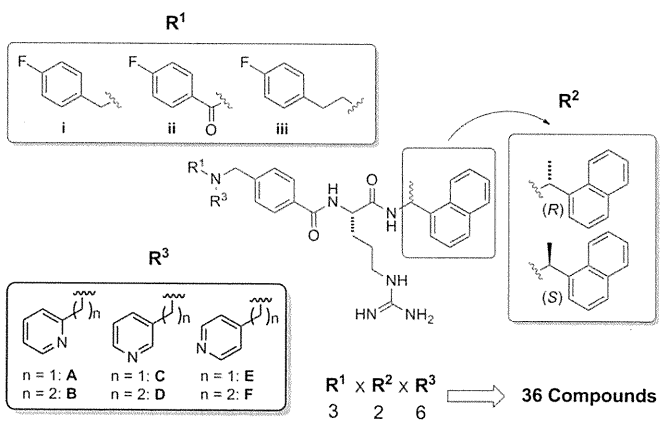
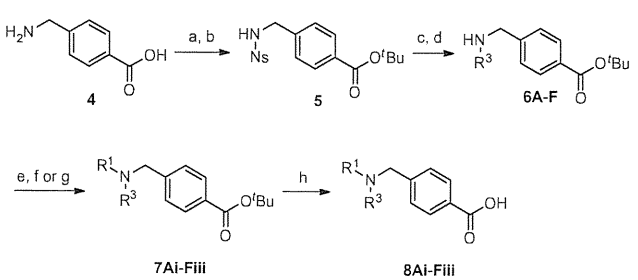


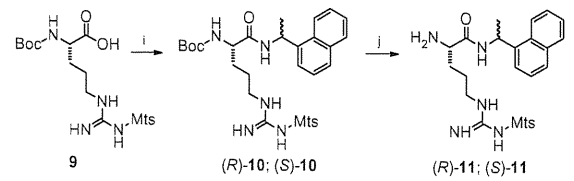
Figure 2. The structures of substituents for three parts of compound **1** in the design of new compounds.



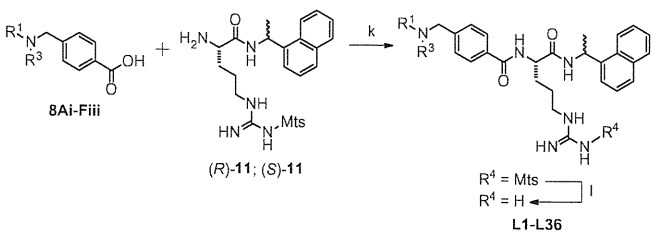
Scheme 1. The synthetic scheme of Unit 1, compounds **8Ai–Fiii**. Reagents and conditions and yields: (a) NsCl, Et_3N, THF/H_2O (1/1); (b) isobutene, THF/H_2SO_4 (10/1), 39% (2 steps); (c) PPh_3, DEAD, R^3OH, THF; (d) PhSH, K_2CO_3, DMF, 42–92% (2 steps); (e) $NaBH(OAc)_3$, 4-fluorobenzaldehyde, CH_2Cl_2; (f) $NaBH(OAc)_3$, (4-fluorophenyl)acetaldehyde, CH_2Cl_2; g) 4-fluorobenzoyl chloride, Et_3N, CH_2Cl_2, 51-94%; (h) TFA then 4 M HCl/EtOAc, quantitative; The structures of R^1 and R^3 are shown in Fig. 2 as i–iii and **A–F**, respectively. Ns = 2-nitrobenzenesulfonyl, ^{t}Bu = *tert*-butyl, DEAD = diethyl azodicarboxylate.

The synthesis of Unit 2 is shown in Scheme 2. Condensation of Boc-Arg(Mts)-OH (**9**) and (*R*)-1-naphthylethylamine or its (*S*) isomer produced amides (*R*)-**10** or (*S*)-**10**. Removal of the Boc group gave Unit 2, (*R*)-**11** and (*S*)-**11**.

Compounds **12–47** were synthesized by amide condensation of Unit 1, **8Ai–Fiii**, with Unit 2, (*R*)-**11** and (*S*)-**11**, and subsequent deprotection of the Mts group, as shown in Scheme 3.[15] All the synthetic compounds were purified by preparative reverse phase HPLC. In cases where peaks derived from side products appeared around the target peaks on the HPLC profile, the precise analysis was accomplished, giving rise to lower yields (Scheme 3, l).



Scheme 2. Synthetic schemes of Unit 2, compounds (***R/S*)-11**. Reagents and conditions: (i) EDCI·HCl, $HOBt·H_2O$, Et_3N, (*R/S*)-(+/−)-1-(1-naphthyl)ethylamine, CH_2Cl_2, 83–97%; (j) TFA then 4 M HCl/EtOAc, quantitative; EDCI·HCl = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, $HOBt·H_2O$ = 1-hydroxybenzotriazol monohydrate, Mts = 2,4,6-trimethylphenylsulfonyl, Boc = *tert*-butoxycarbonyl.



Scheme 3. Synthetic schemes of compounds **12–47**. Reagents and conditions: (k) EDCI·HCl, $HOBt·H_2O$, Et_3N, DMF, 36-95%; (l) TMSBr, *m*-cresol, 1,2-ethanedithiol, thioanisole, TFA, 4–54%. The structures of R^1 and R^3 are shown in Figure 2 as i–iii and **A–F**, respectively.

The CXCR4-binding activity of synthetic compounds was assessed in terms of the inhibition of [^{125}I]-CXCL12 binding to Jurkat cells, which express CXCR4.[16] The percent inhibition of all the compounds at 10 μM is shown in Table 1. Several of the compounds showed significant binding affinity. In general, compounds in which the 1-naphthylethylamine moiety (R^2) has the (*S*)-stereochemistry, as in KRH-1636, are more potent than the (*R*)-stereoisomers. Ten compounds (**26–28**, **30**, **33**, **36**, **39**, **44**, **45** and **47**, Table 1) were found to induce at least 30% inhibition and compounds **26**, **27** and **33**, which have a pyridyl group with a nitrogen atom at the β-position, showed more than 60% inhibition. It is noteworthy that compounds **26** and **27** in which R^2 is a (*R*)-1-naphthylethylamine moiety, are both more potent than the corresponding (*S*)-stereoisomers **44** and **45**. Compounds **26**, **27** and **33**, have a 4-fluorobenzyl or 4-fluorophenylethyl group, which rather than an amide, is a reductive alkyl type (R^1). As can be seen from Table 1, there is a tendency for compounds with a pyridyl group with a nitrogen atom at the β-position (R^3: C or D), to be more potent in terms of CXCR4-binding activity than the corresponding compounds, which have a pyridyl group with a nitrogen atom at the α- or γ- position (R^3: A, B, E or F), and those with a reductive alkyl 4-fluorobenzyl or 4-fluorophenylethyl group (R^1: i or iii), to be more potent in CXCR4-binding activity than the corresponding compounds, with a 4-fluorobenzoyl group (R^1: ii).

Compounds were next evaluated for anti-HIV activity and cytotoxicity. CXCR4 is the major co-receptor for the entry of T-cell line-tropic (X4-) HIV-1.[5] Accordingly, inhibitory activity against X4-HIV-1 (NL4-3 strain)-induced cytopathogenicity in MT-4 cells (anti-HIV activity), and reduction of the viability in MT-4 cells (cytotoxicity) were assessed[16] and are shown in Table 1. Compounds **26** and **33–35** showed significant anti-HIV activity with EC_{50} values in the micromolar range. Compounds **26** and **33** showed both potent CXCR4-binding activity (79% and 60% inhibition at 10 μM, respectively) and anti-HIV activity (EC_{50} = 11 and 13 μM, respectively), the two activities being highly correlated. Compounds **34** and **35** have significant anti-HIV activity with EC_{50} values of 8 and 10 μM, respectively, which is higher than CXCR4-binding activities, which are 16% and 20% inhibition at 10 μM, respectively. Compound **27**, which showed relatively high CXCR4-binding activity (69% inhibition at 10 μM), failed to show

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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Expert Opinion

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The successes and failures of HIV drug discovery

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Introduction: To date, several anti-human immunodeficiency virus (HIV) drugs, including reverse transcriptase inhibitors and protease inhibitors, have been developed and used clinically for the treatment of patients infected with HIV. Recently, novel drugs have been discovered which have different mechanisms of action from those of the above inhibitors, including entry inhibitors and integrase (IN) inhibitors; the clinical use of three of these inhibitors has been approved. Other inhibitors are still in development.

Areas covered: This review article summarizes the history of the development of anti-HIV drugs and also focuses on successes in the development of these entry and IN inhibitors, along with looking at exploratory approaches for the development of other inhibitors.

Expert opinion: Currently used highly active antiretroviral therapy can be subject to a loss of efficacy, due to the emergence of multi-drug resistant (MDR) strains; a change of regimens of the drug combination is required to combat this, along with careful monitoring of the virus and CD4 in the blood, by methods such as cellular tropism testing. In such a situation, entry inhibitors such as CCR5/CXCR4 antagonists, CD4 mimics, fusion inhibitors and IN inhibitors might be optional agents for an expansion of the drug repertoire available to patients at all stages of HIV infection.

Keywords: AIDS, CCR5, CD4 mimic, chemokine receptor, CXCR4, fusion, HIV, integrase

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1. Introduction

The human immunodeficiency virus (HIV) is the cause of acquired immunodeficiency syndrome (AIDS), which was discovered by Montagnier and colleagues in 1983 [1]. HIV infects human host cells and destroys immune systems, subsequently causing immunodeficiency. To date, the number of people worldwide infected with HIV is certainly in excess of 30 million.

Several anti-HIV drugs have been reported in the last 25 years (**Figure 1A**). HIV is a retrovirus, which is an RNA virus that is replicated in a host cell via the enzyme reverse transcriptase to produce DNA from its RNA genome. This DNA is then incorporated into the host genome by an integrase (IN) enzyme. These inhibitors block the action of reverse transcriptase, and include nucleoside reverse transcriptase inhibitors (NRTIs) such as azidothymidine (AZT) and non-nucleoside reverse-transcriptase inhibitors (NNRTIs). The class of anti-HIV drugs that were initially approved for clinical use is reverse transcriptase inhibitors such as AZT [2]. The class of drugs that were subsequently approved is protease inhibitors, which prevent the cleavage of HIV precursor proteins into active proteins, a process that normally occurs in viral replication. This family of drugs includes Saquinavir (Invirase/Fortovase, Roche, Basel, Switzerland) and Ritonavir (Norvir, Abbott Laboratories, IL, USA), which have been used clinically in HIV/AIDS treatment. These drugs are usually administered as part of a two- or three-drug cocktail, accompanied by one or more

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Article highlights.

- The highly active anti-retroviral therapy (HAART) involving use of reverse transcriptase inhibitors and protease inhibitors has led to great success in clinical treatment of human immunodeficiency virus (HIV)-infected patients.
- Brand-new drugs with different action mechanisms have been discovered to date.
- Enfuvirtide, a fusion inhibitor, Maraviroc, a co-receptor CCR5 antagonist and Raltegravir, an integrase (IN) inhibitor have successively been approved for clinical use.
- The potential of new inhibitors from novel drug categories such as entry inhibitors including CCR5/CXCR4 antagonists and CD4 mimics, fusion inhibitors and IN inhibitors might be critical.
- Optional agents are valuable for an expansion of the drug repertoire available to patients because in case of loss of efficacy of HAART, change of regimens of the drug combination in HAART is required.

This box summarizes key points contained in the article.

NRTIs. Such cocktail therapies are known as highly active anti-retroviral therapy (HAART), which has brought great success and hope in the clinical treatment of HIV-infected patients [2]. HAART is capable of lowering the HIV level in the blood until it cannot be detected with current methods. Side effects associated with protease inhibitors include a lipodystrophy syndrome in which abnormal distribution of fat occurs and the face, arms and legs become thin but the therapy involves more serious clinical problems such as the emergence of multi-drug resistant (MDR) HIV-1 strains, and considerable expense. These drawbacks encouraged us to develop brand-new drugs with novel mechanisms of action.

Recently, the molecular mechanism of HIV-1 replication has been elucidated in detail. A dynamic supramolecular mechanism is associated with HIV entry/fusion steps. First, an HIV envelope protein gp120 interacts with a host-cell surface protein CD4, which causes gp120 to undergo a conformational change subsequently binding to the second cellular receptors, a chemokine receptor such as CCR5 [3-7] and CXCR4 [8]. The binding triggers the exposure of another envelope protein gp41 whose N-terminus penetrates the cell membrane. This is followed by the formation of the gp41 trimer-of-hairpins structure, which leads to fusion of HIV/cell membranes, completing the infection process [9]. The description of this dynamic molecular machinery has encouraged researchers to develop inhibitors which block the HIV entry/fusion steps targeting the receptors, CD4, CCR5 and CXCR4, and the viral protein gp41. In 2003, the Food and Drug Administration (FDA) approved Enfuvirtide (Fuzeon/T-20, Roche, Basel, Switzerland/Trimeris, Durham, NC, USA) as the first 'fusion inhibitor' for use in combination with other anti-HIV drugs to treat advanced HIV-1 infection [10]. In 2007, the FDA approved a CCR5 co-receptor antagonist, Maraviroc (Pfizer, New York, NY, USA), for use in combination with other anti-HIV drugs for the treatment of patients infected with CCR5-tropic HIV-1 [11]. In the same year, the FDA approved Raltegravir (Merck Sharp & Dohme Corp., NJ, USA) for use in combination with other antiretroviral agents in treatment-experienced adult patients who present with evidence of viral replication and HIV-1 strains resistant to multiple HAART agents [12,13]. Subsequently in 2009, the FDA granted expanded approval of Raltegravir for use in all patients. Numerous reviews exist concerning reverse transcriptase and protease inhibitors, and this review will focus on the success of a fusion inhibitor, Enfuvirtide, a CCR5 antagonist, Maraviroc and an IN inhibitor, Raltegravir, as well as the development of other anti-HIV agents including CXCR4 antagonists and CD4 mimics.

2. HIV fusion inhibitors such as Enfuvirtide

The binding of gp120 to CCR5 or CXCR4 triggers the formation of the trimer-of-hairpins structure of gp41 and subsequent fusion of the HIV/cell membranes, as described above. The trimer-of-hairpins structure is a six-helical bundle consisting of a central parallel trimer of the N-terminal helical region (HR1 region) surrounded by the C-terminal helical region (HR2 region) oriented in an antiparallel, hairpin fashion (**Figure 1B**) [9]. A subdomain is composed of two peptides, a 51-mer from the HR1 region and a 43-mer from the HR2 region, designated as N51 and C43, respectively [14]. There have been numerous reports that several HR2 region peptides inhibit bundle formation of six alpha-helices by the binding to the inner three-stranded coiled coils of the HR1 region thereby inhibiting membrane fusion (**Figure 2A**) [15]. An HR2 region peptide, C34, with 34 residues from the native sequence of gp41, has potent inhibitory activity against HIV-1 fusion [16]. In addition, a 36-residue peptide, T-20, which has the native sequence of gp41 and 24 residues in common with C34, shows potent anti-HIV activity, and its clinical use as the first entry/fusion inhibitor [10] in HIV/AIDS treatment was approved by the FDA in 2003 designated as Enfuvirtide. Enfuvirtide, in combination with other antiretroviral agents was approved for the treatment of advanced HIV-1 infection in adults and children aged 6 years or older with evidence of HIV-1 replication notwithstanding current therapy, and of resistance to current anti-HIV drugs. In view of the clinical use of Enfuvirtide, the dynamic supramolecular mechanism involving membrane fusion is a valid and rational target for inhibitors of HIV-1 replication. The success of Enfuvirtide has encouraged the development of entry/fusion inhibitors as a new class of anti-HIV drugs distinct from the first- and second-generation drugs. While reverse transcriptase inhibitors and protease inhibitors work internally in T cells to inhibit functions of viral enzymes, entry and fusion inhibitors work extracellularly, preventing HIV from invading cells. C34 has an exact interface that is capable of interacting with the inner three-stranded coiled coils of the gp41 HR1 region, compared with Enfuvirtide, which has a poorly delineated interface. However, a disadvantage