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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

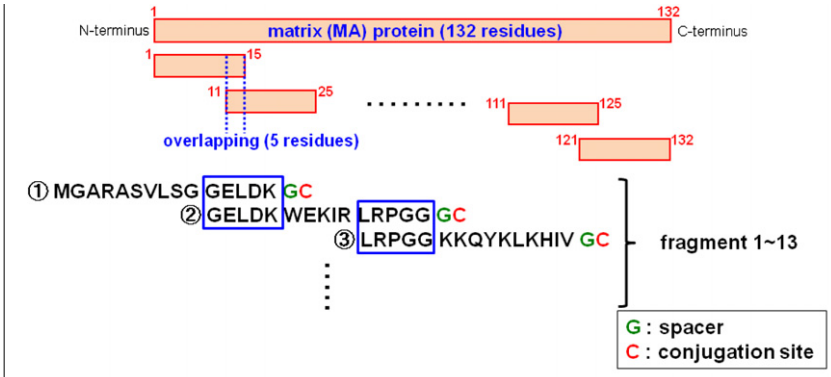
CD spectra were recorded on a JASCO J-720 spectropolarimeter at 25 °C. The measurements were performed using a 0.1 cm path length cuvette at a 0.1 nm spectral resolution. Each spectrum represents the average of 10 scans, and the scan rate was 50 nm/min. The concentrations of samples 8L and 9L were 28.2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

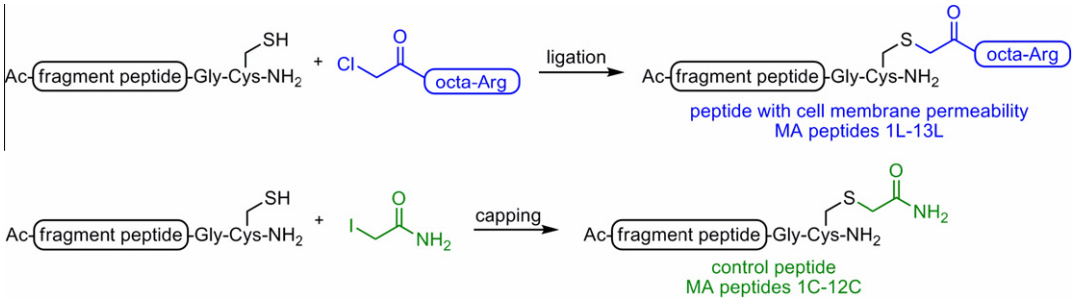


Figure 2. The design of MA peptides with cell membrane permeability (upper) and their control peptides (lower).

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

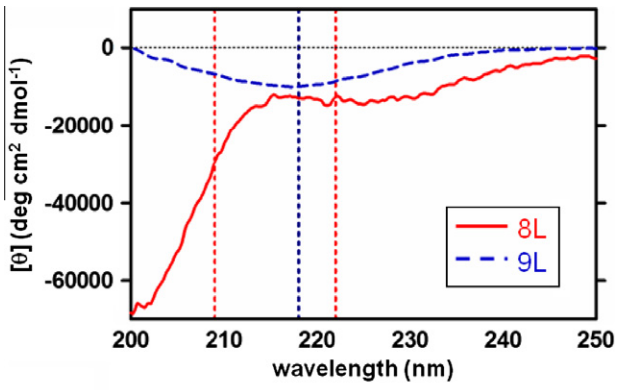


Figure 3. CD spectra of MA peptides 8L (28 μM) and 9L (65 μM) in PBS buffer, pH 7.4 at 25 °C.

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

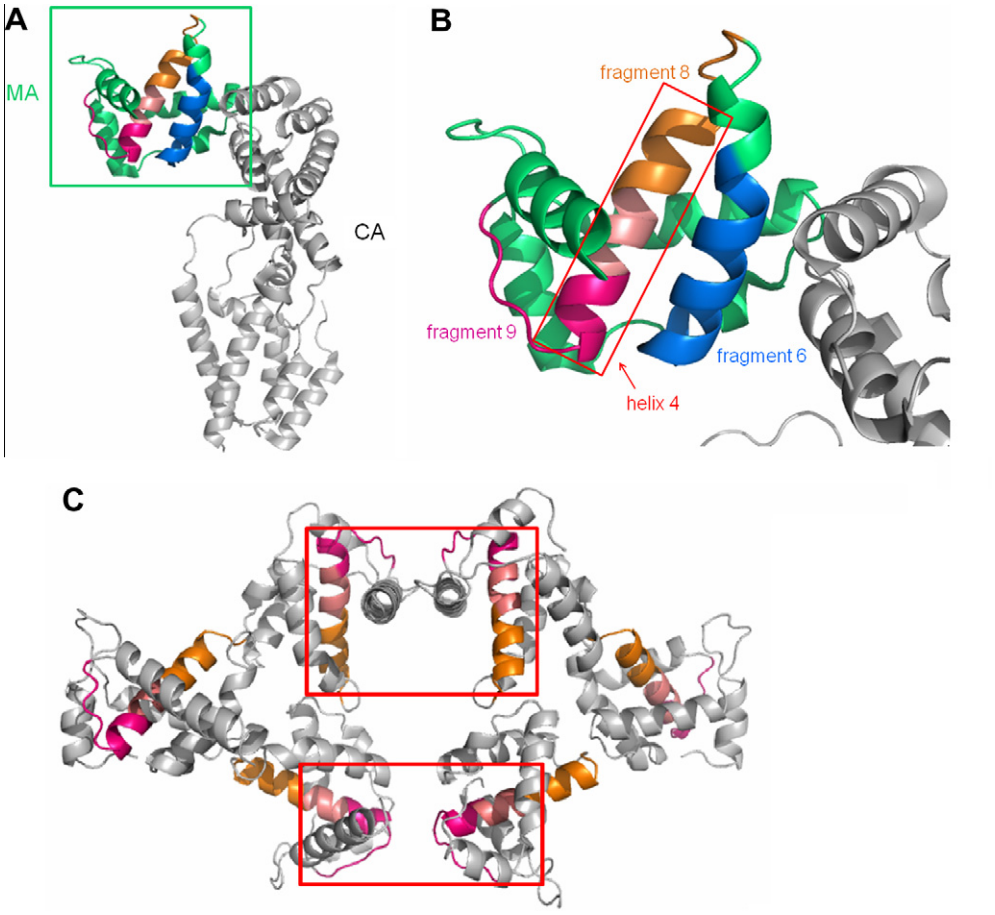


Figure 4. (A) The complete structure of MA and CA proteins (PDB ID: 2gol). (B) The enlarged structure of the highlighted region of (A). (C) The structure of an MA hexamer. Red-colored squares show interfaces between two MA trimers (PDB ID: 1hiw). Orange- and pink-colored helical ribbons represent fragments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

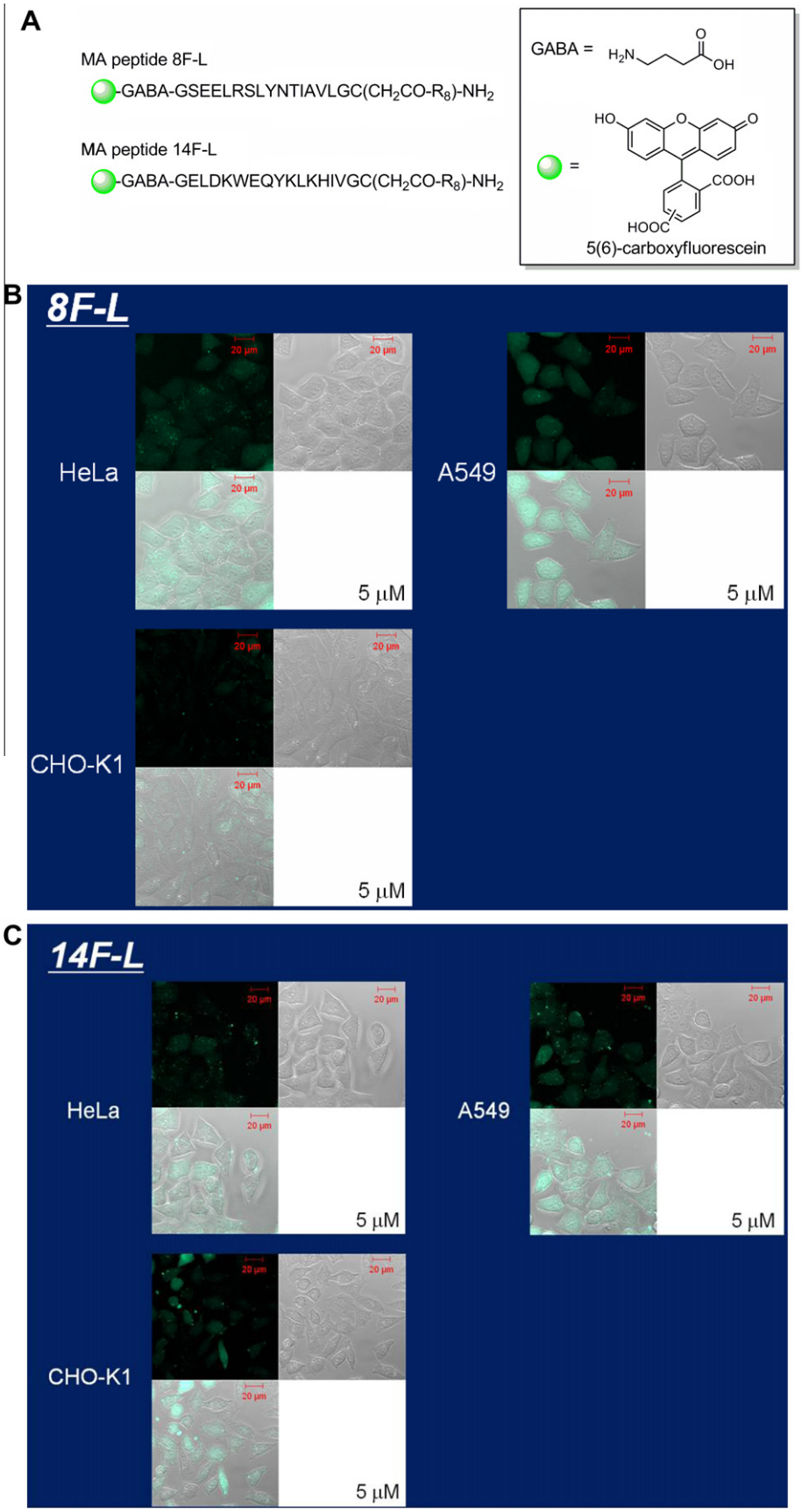


Figure 5. (A) The structures of fluorophore-labeled MA peptides 8F–L and 14F–L. (B) The fluorescent imaging of live cells HeLa, A549 and CHO-K1 by 8F–L. (C) The fluorescent imaging of live cells HeLa, A549 and CHO-K1 by 14F–L.

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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