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PAPER

A multi-functional peptide as an HIV-1 entry inhibitor based on self-concentration, recognition, and covalent attachment†

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HIV entry is mediated by the envelope glycoproteins gp120 and gp41. The gp41 subunit contains several functional domains: the N-terminal heptad repeat (NHR) domains fold a triple stranded coiled-coil forming a meta-stable prefusion intermediate. The C-terminal heptad repeat (CHR) subsequently folds onto the hydrophobic grooves of the NHR coiled-coil to form a stable 6-helix bundle, which juxtaposes the viral and cellular membranes for fusion. A conserved salt bridge between Lys^{574} in NHR and Asp^{632} in CHR plays an essential role in the formation of the six-helix bundle. A multi-functional peptide inhibitor for anti-HIV derived from the CHR of gp41 has been designed. It bears a cholesterol group (Chol) at the C-terminal through which the inhibitor can anchor in the cell membrane, and carries an isothiocyanate (NCS) group at the side chain of Asp^{632} through which the inhibitor can bind to target covalently at Lys^{574} in NHR. The dual functionalized peptide (NCS-C34-Chol) shows high antiviral activity *in vitro* and *in vivo*. The inhibitor reacts specifically and rapidly to NHR from gp41. In addition, it exhibits better stability under the digestion of the Proteinase K than C34 and T20.

Introduction

The human immunodeficiency virus type 1 (HIV-1) trimeric envelope protein complex consisting of gp41 and gp120 is non-covalently associated on the virus surface.[1,2] When HIV-entry occurs, the ectodomain of gp41 undergoes conformational changes resulting in the formation of a six-helix bundle that mediates membrane fusion.[3,4] The six-helix bundle is a trimer and each monomer contains an N-terminal helical region (NHR) and a C-terminal helical region (CHR).[5,6] The NHR features a hydrophobic groove targeted by CHR residues Trp^{628}, Trp^{631}, and Ile^{635} located in the pocket binding domain (PBD). A conserved salt bridge between Lys^{574} in NHR and Asp^{632} in CHR plays an essential role in the formation of the six-helix bundle and, in turn, promotes virus infectivity[7,8] (Fig. 1A). An effective strategy for the design of inhibitors that block HIV entry has been to modify amino acids in the NHR and CHR sequence,[9–15] particularly the residues involved in formation of the salt bridge. The Blumenthal group first reported a covalent inhibitor that targeted the NHR sequence by modifying the side chain of Asp^{632} with maleimide propionic acid chemically coupled to a long linker (aminoethoxy ethoxy acetic acid).[16] This covalently modified inhibitor displayed great promise in inhibiting HIV-1 entry since it functioned as an irreversible inhibitor during the protein–protein interaction.[16–18] However, only a limited number of HIV entry inhibitors have featured covalent functionalization of amino acid residues. The synthesis of more rapid and specific covalent inhibitors that prevent the formation of the six-helix bundle remains a promising means of combating the spread of the HIV virus.

Herein, we describe a series of multi-functional peptide inhibitors based on the C34 sequence (Table 1). An analysis of the crystal structure of the N36 and C34 complex[5] led us to propose the substitution of an isothiocyanate (NCS)-modified lysine for Asp^{632} in the C34 sequence (Fig. 1B). We selected the NCS group instead of the previously used maleimide group because NCS is small in size and selectively modifies target amino groups in a rapid and complete manner.[19] Introduction of the NCS-Lys at position 632 would allow the replacement of the salt bridge between Lys^{574} and Asp^{632} by a covalent thiourea bond formed by the NCS group and the ε-amine of Lys^{574} (Fig. 1C). Moreover, the addition of a membrane anchor group to the inhibitor can increase its concentration near the cell surface.[20,21] Therefore, we also incorporated a cholesterol group into our peptide inhibitor.

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We envisaged that after being anchored onto the cell membrane, this anti-fusogenic peptide NCS-C34-Chol could selectively bind to NHR, and then form a covalent thiourea bond through reaction with the ε-amino group of Lys^{574} of NHR.

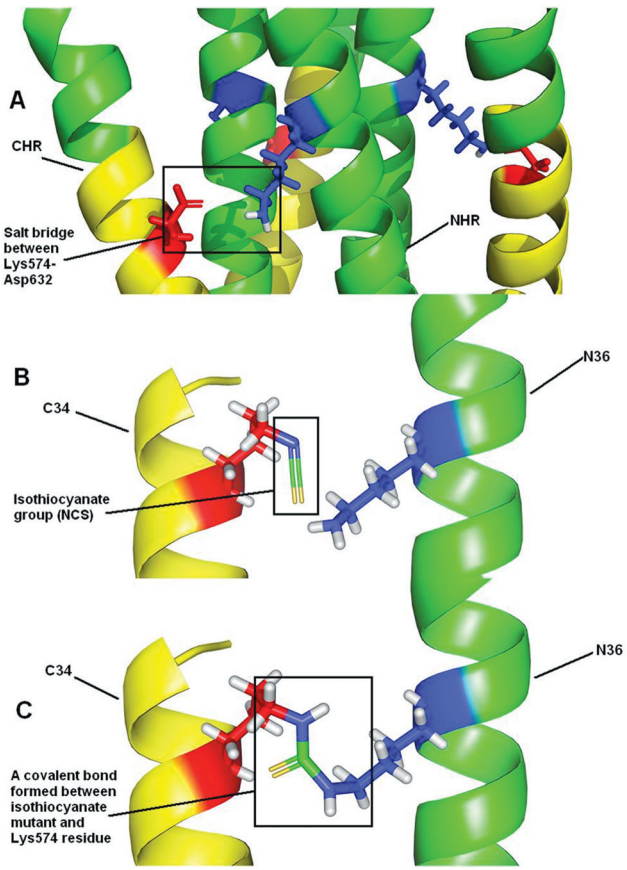

Fig. 1 Model of the HIV gp41 ectodomain in the six helix bundle conformation. These models were created from the theoretical model 1IF3,[22] and 1AIK[5] using PyMOL. The Lys^{632} mutant of C34 was created by the Swiss-Model. (A) Salt bridge between Lys^{574} and Asp^{632}. (B) An isothiocyanate group was attached to the lysine residue at site 632. (C) The isothiocyanate group and NH_2 of Lys^{574} formed a covalent bond. The C34 portion of the CHR sequence is highlighted in yellow. NH_2-Terminal residue Lys^{574} is shown in blue while C-terminal residue Asp^{632} is shown in red.

Results and discussion

Design and synthesis of peptide inhibitors

Based on previous analysis, we synthesized several peptide inhibitors and a control peptide sequence as shown in Table 1 (the synthesis of the cholesterol modified amino acid building block is described in the supporting information†). Lys^{655} has the potential to react with the NCS group within the C34 sequence. Therefore, peptides containing Arg^{655} instead of Lys^{655} were also synthesized to prevent possible self assembly.

Covalent binding of NCS-C34/NCS-C34-Chol with N36 detected by HPLC

NCS-C34 was firstly tested for its potency of binding to N36 by analytical HPLC using C34 as a control. NCS-C34 and C34 were individually incubated with N36 and then monitored by reversed-phase analytical HPLC. We found that NCS-C34 displayed a very high affinity with N36. Because of the non-covalent association between N36 and C34, these two peptides from the complex eluted with the same retention times as observed in studies where they were injected individually (Fig. 2A). After the incubation of N36 with NCS-C34 (Fig. 2B), a new peak with a retention time of 19 min was observed and proved to be the adduct of N36 with NCS-C34 by MALDI-TOF (data shown in ESI†), while the peaks corresponding to free N36 and NCS-C34 disappeared. Analytical HPLC confirmed the association of NCS-C34-Chol and N36 (Fig. 2C) and this association reaction was completed within 1.5 hours. Isothiocyanate modification was not only useful for labeling the amino group, but it also could stabilize the α-helical structure of C34 peptide (Fig. 2D). The CD spectrum showed that NCS-C34 formed more α-helical structure than C34 based on the values of $[\theta]_{222}$. The presence of more α-helical structure may expose the binding sites of the C34 peptide and make them more easily accessible to the temporarily exposed NHR target.[23]

The interaction between N36 and covalent inhibitors tested by N-PAGE and SDS-PAGE

We investigated the interaction between N36 and NCS-C34 as well as NCS-C34-Chol by native-PAGE. After 0.5 h incubation at 37 °C, as shown in Fig. 3(A), N36 exhibited no band,

Table 1 HIV entry inhibitors in this study

Inhibitor[a]	Sequence[b]
C34	^{628}WMEW**D**REINNYTSLIHSLIEESQNQQEKNEQELL
NCS-C34	^{628}WMEWK(**NCS**)REINNYTSLIHSLIEESQNQQEKNEQELL
C34-Chol	^{628}WMEWDREINNYTSLIHSLIEESQNQQEKNEQELLGSGN-Chol
NCS-C34-Chol	^{628}WMEWK(**NCS**)REINNYTSLIHSLIEESQNQQEKNEQELLGSGN-Chol
NCS-C34-CholK655R	^{628}WMEWK(**NCS**)REINNYTSLIHSLIEESQNQQE**R**NEQELLGSGN-Chol
N36	^{546}SGIVQQQNNLLRAIEAQQHLLQLTVWGIKQLQARIL

[a] Abbreviated names of the control and inhibitory peptides. Letters in parenthesis indicate the lysine residue modified with NCS in the inhibitory peptide. [b] All the peptides without a cholesterol group are modified with N-terminal acetylation and C-terminal amidation.

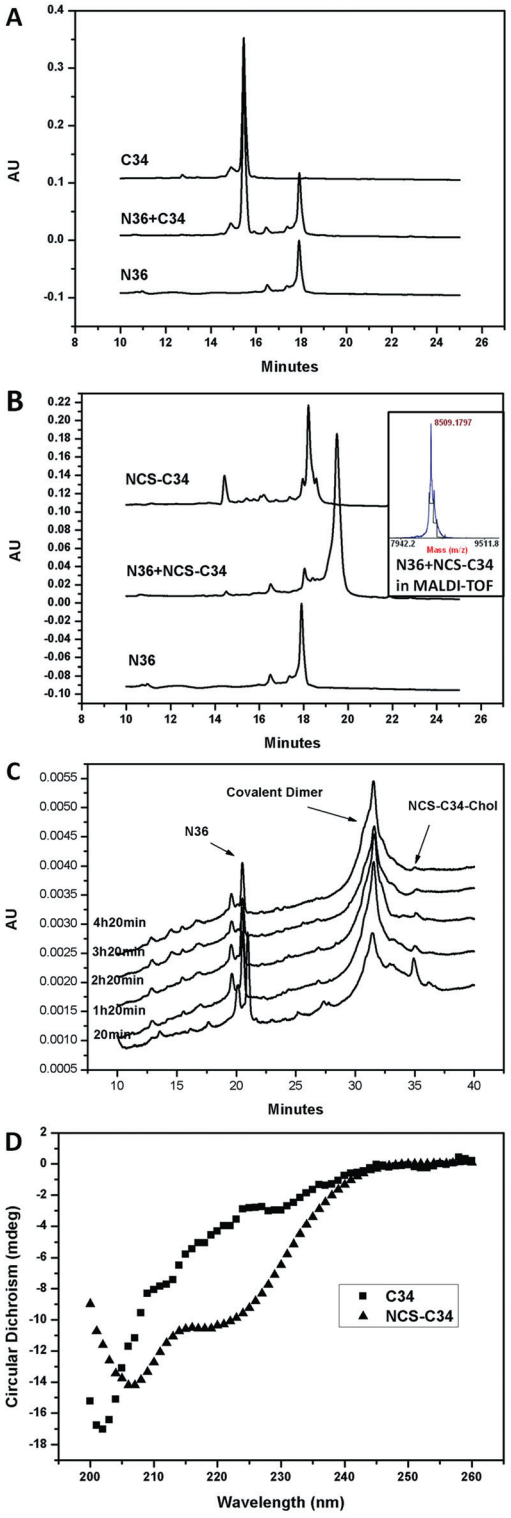

Fig. 2 NHR peptides and the covalent entry inhibitors from CHR peptides form covalent dimers. Panels A–C show HPLC analyses after co-incubation of peptides. (A) Traces of C34 or N36 alone, and N36 incubated with C34. (B) Traces NCS-C34 or N36 alone, and N36 incubated with NCS-C34. Mass of the covalent dimer is shown inset. (C) N36 incubated with NCS-C34-Chol for 20 min (low trace). The upper traces show the progressive effect of additional incubation time, with each trace indicating one additional hour of exposure. (D) α-helical conformations of the peptide inhibitors analyzed by CD spectroscopy. ▲ NCS-C34; ■ C34.

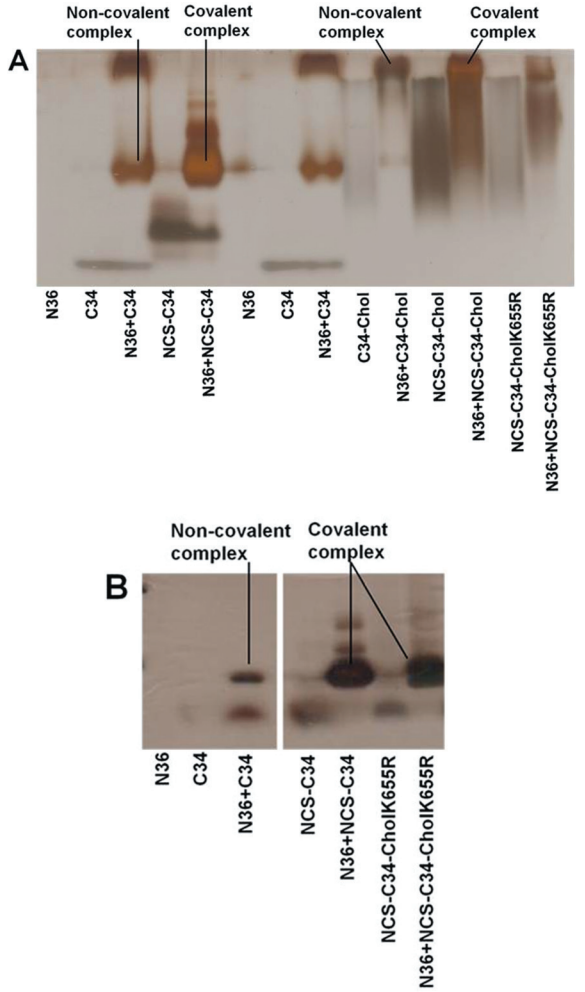

Fig. 3 (A) Determination of the six helical bundle formation between N36 and covalent inhibitors or C34 peptides by 12% Acr-Bis native PAGE. (B) Determination of the formation of covalent complexes between N36 and the peptide inhibitors by 4%–12% gradient SDS-PAGE.

probably because it was positively charged and migrated off the gel. C34 and NCS-C34, respectively, appeared as single bands in the lower part of the gel. Like C34, NCS-C34 was also able to form the 6-helical bundle with N36 as reflected by the lesser migration into the gel of the band formed from the mixture of NCS-C34 and N36. C34-Chol alone showed a very slow migration rate in the native gel, probably because the cholesterol interacted strongly with the native gel. In contrast, the position of the band corresponding to the mixture of C34-Chol with N36 was shifted upward, indicating the presence of a larger peptide, consistent with the formation of a complex. As expected, NCS-C34-Chol and NCS-C34-CholK655R alone showed bands with migration values similar to C34-Chol, while bands from the mixture of NCS-C34-Chol with N36 showed a stronger upward shift as compared with the mixture of C34-Chol with N36. These results were in accordance with the reversed-phase HPLC results which indicated the formation of a complex characterized by covalent binding. To further investigate the covalent interactions between NCS-34 and N36, we conducted electrophoresis studies under denaturing conditions (SDS-PAGE). After 48 h

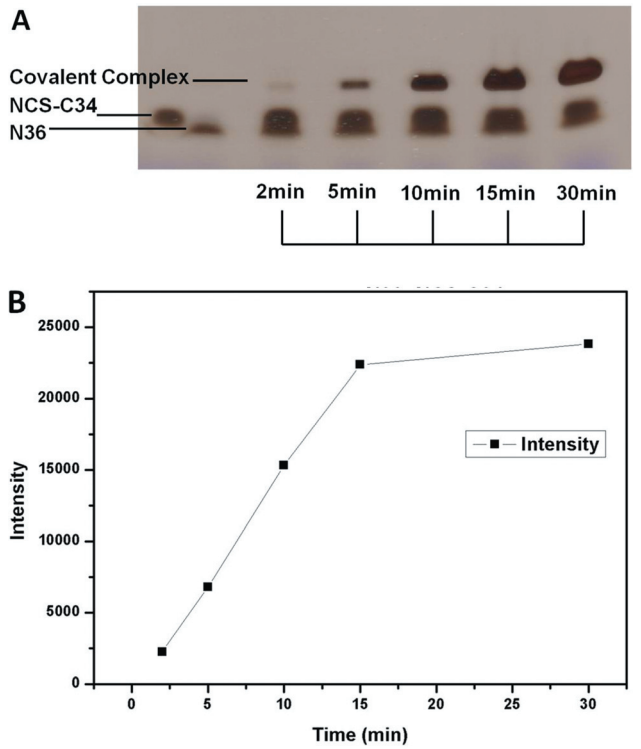

Fig. 4 (A) 4%–12% gradient SDS-PAGE was used in detecting the reaction rate of N36 and NCS-C34. (B) The intensity of the amount of covalent complex shown in SDS-PAGE which was calculated by ImageJ.

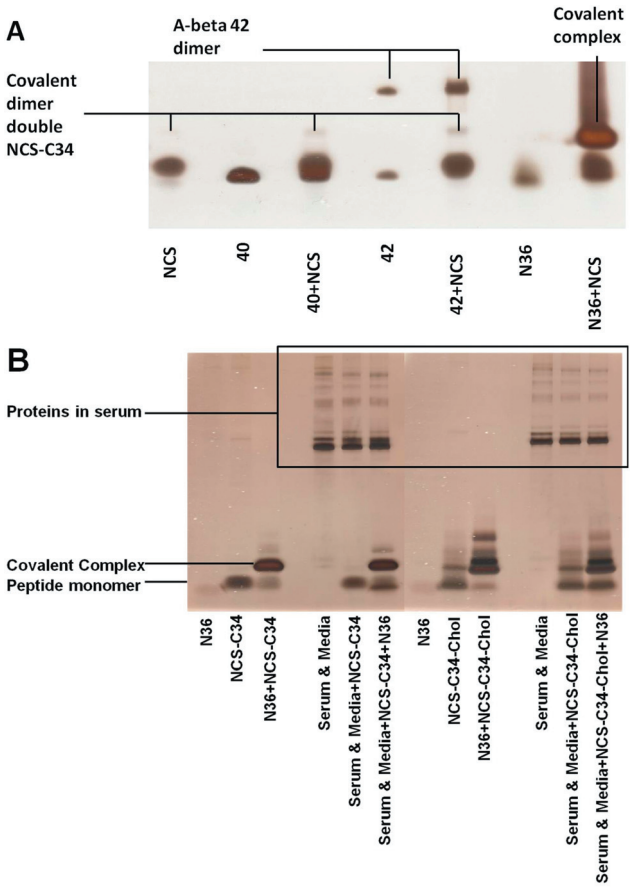

Fig. 5 4%–12% gradient SDS-PAGE was used for detecting the specificity of NCS-C34 peptide recognition. (A) Specific binding test with Aβ. (B) Serum was used in the test of specificity of isothiocyanate and cholesterol modification.

incubation, the complex of NCS-34 with N36 still displayed a strong band even though the peptides had been denatured and lost conformation completely or partially in SDS-PAGE. In contrast, the complex of C34 with N36 displayed a much weaker band (Fig. 3B). These results were consistent with our observations from native-PAGE studies that NCS-C34 formed a covalent bond with N36, much stronger than the non-covalent interaction between C34 and N36. Both electrical gel and HPLC did not show the clear covalent complex founded by NCS-C34 or NCS-C34-Chol themselves in 0.5 h incubation. Thus, the K655R mutation allowed us to rule out the possibility that our observations were attributed to self-assembly between K655 and NCS-K632.

The association rate of N36 and NCS-C34

We sought to determine the association rate of N36 and NCS-C34 *in vitro* and thus to evaluate the activity of isothiocyanate modification that covalently bind to target. To this end, we envisaged an SDS-PAGE test model (Fig. 4A) to monitor the reaction process. We operationally define the time of the reaction state as 2, 5, 10, 15 and 30 min.

The rate of the covalent binding reaction was denoted by the ratio of the covalent complex at each time length. The covalent complex band clearly appeared after 5 min. The reaction rate was accelerated during 5–15 min with an increased concentration of the covalent complex (Fig. 4B). Our model readily describes direct interaction between N36 and NCS-C34. In the HIV-1 entry process, the gp41 refolding proceeds culminating in the formation of a helical bundle structure which is blocked by peptide inhibitors targeting the complementary domains of gp41. The exposure time of gp41 was more than 1 h.[24]

The specificity of the covalent inhibitors to N36

To investigate the specificity of the covalent inhibitors, we chose other proteins to co-incubate with the new inhibitor and then detected the covalent complex by SDS-PAGE. From the previous results of SDS-PAGE and CD spectra, NCS-C34 which shows an α-helix structure in PBS buffer did not show the obvious band of covalent dimer even though they also have lysine in their sequence. To study the specificity of NCS-C34, we chose Aβ(1-40) and Aβ(1-42) as target molecules. Aβ is a peptide derived from amyloid precursor protein. The conformation of Aβ contains α-helix, β-turn, and random coil, these conformations will change during Aβ aggregation.[25,26] There are three free amino groups in Aβ, from Lys[16] and Lys,[28] and N-terminus. We chose different types of Aβ (40 or 42) as target molecules to detect the specificity of NCS-C34. The specificity of NCS-C34 was detected by SDS-PAGE (Fig. 5A). We incubated Aβ 40, Aβ 42, and N36 to co-incubate with NCS-C34 at

37 °C for 0.5 h separately. The final peptide concentration was 100 μM. Then the samples were measured by SDS-PAGE to find the covalent complex. It was clearly showed that Aβ 40 and 42 had no reaction with NCS-C34, while N36 showed a dark band which represented the covalent complex. The extreme differences in covalent complex formation with N36, Aβ 40, and Aβ 42 indicated that NCS-C34 had excellent selectivity in binding to N-peptide.

Moreover, the isothiocyanate group is small but quite reactive. To mimic the real complex disease environment model *in vivo*, we also tested the specificity of isothiocyanate modification in serum. To investigate whether cholesterol might synergize with the isothiocyanate group to give nonspecific interaction, we also used NCS-C34-Chol to interact with serum protein (Fig. 5B). We used two different inhibitors NCS-C34 and NCS-C34-Chol, and 5% FBS (Fetal Bovine Serum) in DMEM (Dulbecco's Modified Eagle Medium) for serum protein. After 24 h co-incubation with peptide inhibitors and diluted (1/100 in PBS) serum protein, N36 was added in the mixture with extended incubation for 0.5 h. Then the samples were loaded to SDS-PAGE to find the covalent complex. The result clearly showed that there was no new band obtained by the addition of the serum protein, the bands of the inhibitor with or without the serum protein were almost same. This indicated that the serum protein had no reaction with the isothiocyanate group, while N36 can covalently bind to the peptide inhibitor after 0.5 h followed by 24 h co-incubation of peptide inhibitor and serum protein. Obviously, these results proved the high specificity of covalent peptide inhibitors.

C-peptide inhibitors resistant to Proteinase K digestion

One of the major disadvantages of T20 is its short half-life *in vivo* and high sensitivity to the proteolytic enzymes in blood.[27] We speculated that the isothiocyanate and cholesterol modifications may change the conformation and resistance of inhibitors to proteolytic enzymes of C34. Therefore, the stability of NCS-C34-Chol under the digestion of the Proteinase K (a broad-spectrum serine proteinase) was determined (Fig. 6).

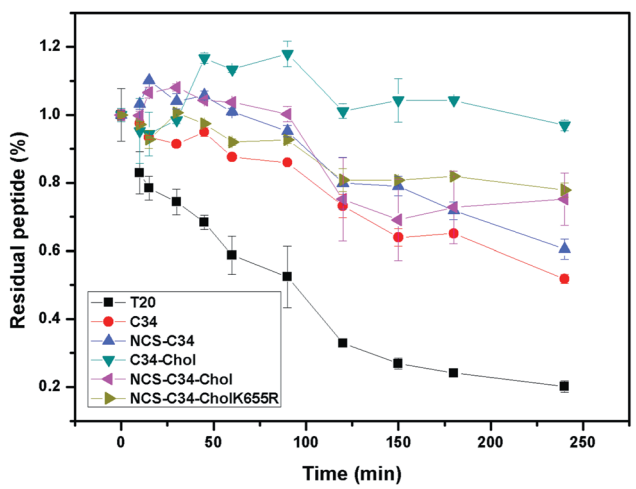

Fig. 6 The digestion of C-peptide inhibitors in Proteinase K test by ELISA.

After the treatment with Proteinase K in PBS for 4 h, NCS-C34-Chol maintained 75% of the original amount detected by ELISA. Under the same conditions, T20 and C34 retained only 20% and 52% of the original amount. These results suggest that the dual functionalized peptide is more resistant to Proteinase K than T20 and C34.

Cholesterol modification detached from the covalent complex after long time period incubation

During the covalent attachment test *in vitro*, the degradation of the covalent dimer has been observed after 48 h incubation. N36 was incubated with C-peptide inhibitors at 37 °C for 48 h. The isolated N- and C-peptides were also incubated. The final concentration of N-peptide and C-peptide were 100 μM (in 50 mM sodium phosphate and 150 mM NaCl, pH = 7.4). After the incubation, peptide samples were loaded to analytical HPLC and Native-PAGE (Fig. 7). Compared with 0.5 h incubation (Fig. 7A), it was clear that after 48 h incubation, NCS-C34 monomer was self-covalently assembled to form dimer, and the covalent complex formed by N36 and NCS-C34-CholK655R was degraded (Fig. 7B). The product of degradation had a similar transferred rate as covalent complex without cholesterol. Analytical HPLC also displayed a fragment peak at a retention time of 23 min (Fig. 7C). Obviously, the covalent dimer of N36 and NCS-C34-Chol/NCS-C34-CholK655R had been degraded. The cholesterol group was detached from the covalent complex. MALDI-TOF and N-PAGE results show the molecular weight of new compounds.

Cholesterol modification dramatically increased antiviral activity in pseudovirus test

The peptides and controls were analyzed for their anti-fusogenic activity using a standard pseudovirus fusion inhibition assay and prime wash inhibition assay[21] and their IC_{50} values were determined (Table 2). Of the two modifications to C34, the addition of NCS and of cholesterol, the cholesterol modification in the C-peptides had the most dramatic effect, improving the IC_{50} as much as ~200-fold. These results showed that in the pseudovirus system, cholesterol could enhance the anti-viral affinity remarkably because cholesterol had a strong binding affinity with the cell membrane. In contrast, the isothiocyanate group modifications in NCS-C34 had no obvious impact upon the binding affinity of the C34 peptide to the NHR region of gp41 in pseudovirus fusion inhibition assays.

The function of cholesterol and isothiocyanate modification studied by prime wash assay in pseudovirus and temperature-arrest state prime wash assay in cell–cell membrane fusion

Despite the clear preference for the cholesterol modification in the studies of the pseudovirus fusion inhibition assays, we could not overlook the HPLC and SDS-PAGE results that showed the NCS group displayed strong covalent binding affinity with N36 from NHR of gp41. We next examined the effect of the isothiocyanate group in an anti-virus assay, temperature-arrest state prime wash inhibition based on the cell–cell membrane fusion

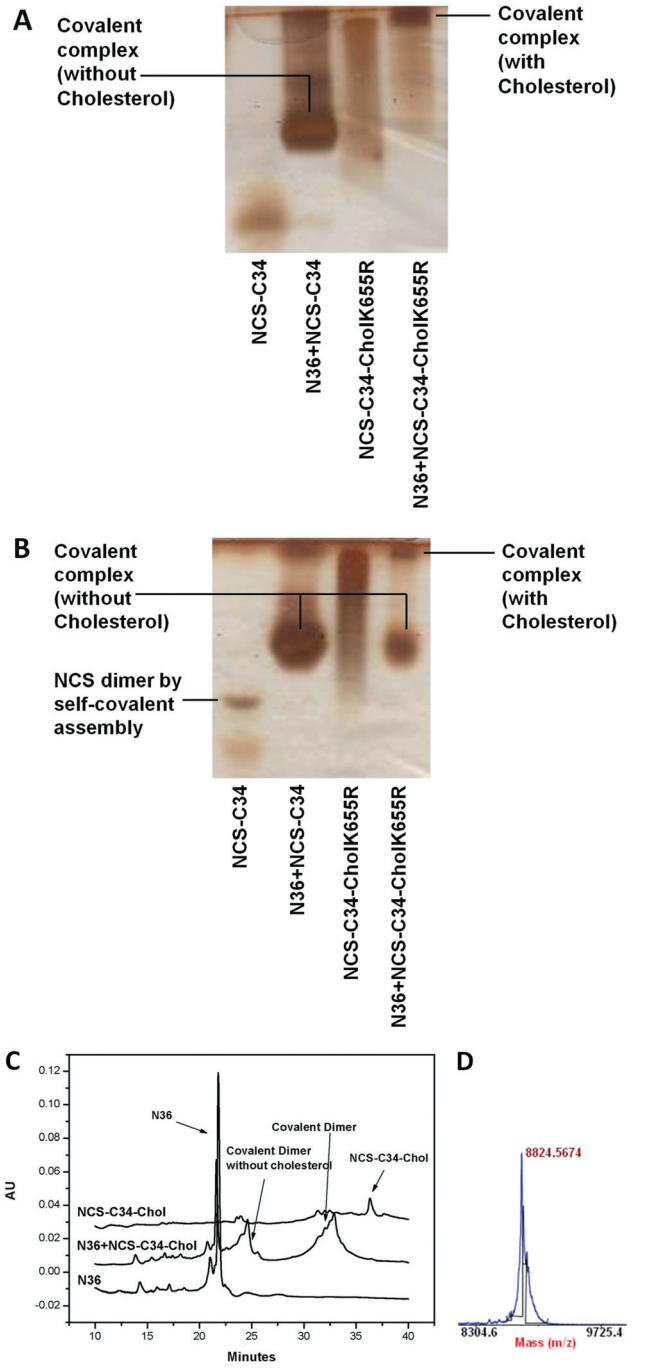

Fig. 7 Detection of cholesterol degraded product after long time incubation. 12% Native-PAGE detected the covalent complex with an incubation time (A) 0.5 h and (B) 48 h. (C) Analytical HPLC by using polar CN column. After 48 h incubation, the degraded compound was shown as retention time 23 min. (D) MALDI-TOF result of N36/NCS-C34-Chol covalent dimer with cholesterol degradation.

assay which was able to prolong the membrane fusion process so that the inhibitors could interact directly with the target protein for a longer time.[16] The cell–cell fusion results are shown in Table 3. In contrast to the results obtained for NCS-C34 in the pseudovirus system, in the temperature-arrest state prime wash assay, NCS-C34 exhibited ~10-fold improved anti-viral activity as compared to C34 alone. These results indicated that NCS-C34 strongly interacted with the gp41 ectodomain in the temperature-arrest state. In the previous study, isothiocyanate or cholesterol modification could increase the antiviral activity of C34 individually. However, the combination NCS-C34-Chol did not exhibit more antiviral activity than the C34-Chol control, indicating that these two groups counteract each other in the process of inhibition. Nevertheless, NCS-C34-Chol has some advantages over C34-Chol in that it can bind the target protein irreversible and play as a "bridge" molecule between the virus and the target cell. In addition, similar antiviral activity of NCS-C34-CholK655R demonstrated that K655 does not play a key role in the activity of this peptide inhibitor and that self-assembly between C34 peptides does not occur to any important extent.

Conclusions

In summary, we report a peptide inhibitor modified with the isothiocyanate group and cholesterol. This novel inhibitor increases the anti-virus potency of the C34 peptide dramatically by (a) first anchoring the inhibitor in the membrane lipid raft quasi-irreversibly; (b) the peptide then binds to gp41 rapidly, specifically and permanently. The two modifications of C34 serve as a bridge molecule between the virus and the target cell. Moreover, this multifunctional inhibitor has a range of potential applications beyond the amelioration of anti-virus inhibitors, such as for imaging the HIV entry process or in binding and isolating specific proteins after they are labeled with fluorophores or affinity tags.

Experimental

General considerations

Chemicals were obtained from Acros, Aldrich, GL Biochem or Alfa Aesar and used without further purification. Reverse-phase HPLC separation was run by Waters 2487 (600E) and eluted by solution A (80% acetonitrile–water with 0.06% trifluoroacetic acid) and B (100% water with 0.06% trifluoroacetic acid). Detection of the peptide was achieved with a dual absorbance UV detector at a wavelength of 215 nm and254 nm. Separation for the peptides was performed by preparative HPLC using a C-18 column (10 × 250 mm) with a flow rate of 7 mL^{-1} min^{-1}. The peptides with a cholesterol group were separated by preparative HPLC on a polar CN column (10 × 250 mm) with a flow rate of 7 mL^{-1} min^{-1}. Analytical HPLC data were recorded with C-8 or polar CN column with a flow rate of 0.8 mL min^{-1}. MALDI-TOF-MS spectra were recorded by using cyano-4-hydroxy cinnamic acid (CHCA) or 2,5-dihydroxybenzoic acid (DHB) on an Applied Biosystems 4700 Proteomics Analyzer 283. ^{1}H, ^{13}C NMR spectra were recorded on JEOL-300 spectrometers. ESI-QTOF-MS spectra were obtained on micrOTOF TM mass spectrometers (Bruker).

Synthesis of Fmoc-Asp(OH)-OChol building block

Fmoc-Asp(O*t*Bu)-OH 3.0 g and cholesterol 4.2 g (1.5 equiv) were dissolved in DCM. After stirring for 10 min, DCC (2.3 g,

Table 2 IC_{50} values of antiviral inhibitors evaluated by pseudovirus assay

Inhibitor	SF162[a] (nM)	CNE28[a] (nM)	JRFL[a] (nM)	JRFL[b] (μM)
C34	16.83 ± 1.66	3.52 ± 3.013	24.58 ± 7.70	32.17 ± 4.91
NCS-C34	22.68 ± 3.96	10.83 ± 1.04	40.35 ± 7.30	49.59 ± 29.66
C34-Chol	0.26 ± 0.16	0.45 ± 0.02	0.26 ± 0.06	0.15 ± 0.14
NCS-C34-Chol	0.40 ± 0.03	1.31 ± 1.42	0.81 ± 0.27	0.16 ± 0.012
NCS-C34-CholK655R	0.99 ± 0.25	0.90 ± 0.23	1.13 ± 0.22	0.14 ± 0.06

[a] Pseudovirus infection of the ghost cell by routine virus test protocol. [b] Pseudovirus infection of the ghost cell by prime wash inhibition assay.

Table 3 IC_{50} values of antiviral inhibitors evaluated by membrane fusion assay

Inhibitor	IC_{50}[a] (μM)	IC_{50}[b] (μM)
C34	6.13 ± 1.46	13.42 ± 0.93
NCS-C34	3.13 ± 1.40	1.30 ± 0.27
C34-Chol	0.0038 ± 0.00056	0.0098 ± 0.0016
NCS-C34-Chol	0.0084 ± 0.0022	0.22 ± 0.038
NCS-C34-CholK655R	0.011 ± 0.0039	0.26 ± 0.025

[a] Membrane fusion by routine protocol. [b] Membrane fusion by temperature-arrested state prime wash assay.

1.5 equiv) in DCM was added dropwise. The mixture was stirred at RT for 3 h monitored by TLC. After 3 h reaction, the mixture was cooled down to −20 °C for 30 min. After filtration and concentration, the crude reaction mixture was purified by column chromatography (silica, ethyl acetate–cyclohexane (1 : 4)) to yield a white solid (R_f = 0.6). All the product was dissolved in DCM–TFA–H2O (1 : 1 : 0.1, v/v/v). After stirring for 30 min (reaction monitored by TLC), the mixture was concentrated under high vacuum. The crude mixture was purified by column chromatography (silica, ethyl acetate–cyclohexane (1 : 4)) and dried under high vacuum to yield yellow solid (R_f = 0.15, 2.7 g, 51.1% total yield).

General procedure for the synthesis of peptides with or without cholesterol group

Commercially available Rink Amide HMBA resin was used for all the solid-phase reactions during the synthesis of peptides. The loading of the resin in this study was 0.23 mmol g^{-1}, the scale used for each peptide was 0.1 mmol. All cycles of solid-phase peptide synthesis were performed by using a microwave peptide synthesizer (Liberty, CEM Corporation). All Fmoc amino acid building blocks were coupled by using HATU. Typically, coupling reactions of amino acids were conducted under microwave irradiation for 10 min, with a power of 20 W at 50 °C. This process was repeated twice for the coupling of Fmoc-Arg(Pbf)-OH. The amino acids (5 equiv), and HATU (5 equiv), which were dissolved in DMF and DIEA (10 equiv), were then dissolved in NMP. All of the above solutions were added automatically. For the peptides including cholesterol at C-terminal, a flexible spacer Gly-Ser-Gly has been chosen for linking the peptide and cholesterol group.[21] The first building block Fmoc-Asp(OH)-OChol as a side chain anchor was added manually. This coupling reaction was conducted under room temperature for 2 h, with the amino acids (4 equiv), HATU (4 equiv), and DIEA (8 equiv) which were dissolved in DMF–DCM (1 : 1). The side chain anchoring reaction was performed twice followed by capping reagent treatment. The unreacted free amino groups were capped by acetylation using HOBt (0.013 m) in Ac_2O–DIEA–DMF (4.75 : 2.25 : 93.0, v/v/v), and the capping was carried out under microwave irradiation at 65 °C for 30 s and 30 s again with power of 40 W. A solution of piperidine (20%)–HOBt (0.1 m) in DMF was used for Fmoc removal, which was conducted under microwave irradiation at 75 °C for 180 s and 180 s again with power of 48 W. After coupling all the building blocks, the resin was transferred from the peptide synthesizer to a flask. Then, a mixture of TFA–TIS–H_2O (95 : 2.5 : 2.5, v/v/v) was applied for 2 h to detach the peptide from the resin.

General synthesis of isothiocyanate modified peptides

Peptides were firstly prepared by solid-phase peptide synthesis following the general procedure. For isothiocyanate modification Fmoc-Lys(Alloc)-OH was used as building block instead of the original amino acid. After assembly, the Alloc group was deprotected using a catalytic amount of palladium(0) ($Pd(Ph_3P)_4$ 0.1 equiv) in DCM in the presence of phenyl silane (24 equiv) as a scavenger for the allyl group.[28,29] After the deprotection, the resin was washed by DCM 5 times then DMF 5 times. To obtain the isothiocyanate derivative, the resin was treated with thiocarbonyldiimidazole (TCD) (4 equiv) in a minimum amount of DMF (3 mL) for 4 h.[30] To obtain the isothiocyanate modified peptides, the cleavage cocktail (following the general procedure) was applied for 0.5 h to detach the peptide from the resin. The purified dry peptide was stored at −20 °C. The isothiocyanate modified peptides can also be stored in DMSO for less than a week. The isothiocyanate group is stable in the aqueous phase for approximately 2 days.

Circular dichroism studies

CD spectra were recorded on an Applied Photophysics Pistar π-180 CD spectrometer equipped with a temperature controller using 1 mm length cells, 10 mm pathlength, and a scan speed of 1.0 nm/2 s at 298 K. The spectra were averaged over 3 scans with the baseline subtracted from analogous conditions as those for the samples. The samples were prepared in 1 mM phosphate buffered saline with the final peptide concentration 40 μM.

The concentrations of unfolded peptides were determined by measuring the peptide solid using high precision balance.

Native polyacrylamide gel electrophoresis (N-PAGE)

N-PAGE was carried out to determine the 6-helical bundle formation between the N- and C-peptides.[31] Tris-glycine gel (12%) was used for N-PAGE. Low molecular weight markers were obtained from Thermo PageRuler product# 26632. The N-peptide was incubated with the C-peptide at 37 °C for 30 min (the final concentration of N-peptide and C-peptide was 100 μM in PBS). After the incubation, the sample was mixed with Tris-glycine native sample buffer (glycerol 5 mL, H_2O 2.7 mL, Tris-HCl 0.5 M, pH = 6.8, bromophenol blue 0.05%) at a ratio of 3 : 1 and then loaded to a native gel (20 μL each well). Gel electrophoresis was carried out with 120 V constant voltage at room temperature for 1.5 h in N-PAGE running buffer (Tris 1.5 g, glycine 6.5 g, H_2O 500 mL). The gel was then stained with silver staining (Beyotime, fast silver stain kit, China).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out to determine the covalent complex formed between N-peptide and covalent inhibitors and the specification of inhibitor interaction with other proteins. NuPAGE gels (4%–12%) were produced by Invitrogen Co. Low molecular weight markers were obtained from Invitrogen Co. The incubation of peptide samples were followed by N-PAGE protocol. The final concentration of N-peptides and C-peptides was 100 μM in PBS. After the incubation, the samples were mixed with SDS-PAGE sample buffer at a ratio of 3 : 1 followed by boiling in a water bath for 10 min. The samples were loaded on to SDS gel (15 μL each well), and gel electrophoresis was carried out with 150 V constant voltage at room temperature for 1.5 h. The gel was then stained with silver staining (Beyotime, fast silver stain kit, China).

Measurement of anti-HIV activity of C34 mutants by pseudovirus test

Plasmids, including pNL4-3.LucR2E2, Env-expressing plasmid pHXB2, pJRFL, pSF162, pCNE28, as well as target cells (GFP-transduced human osteosarcoma cells, Ghost cells) which express CD4 and both coreceptors CCR5 and CXCR4 were kindly provided by Dr Linqi Zhang at Tsinghua University. HEK293T (obtained from NIH) were used to package pseudovirus through cotransfecting pNL4-3.LucR2E2 and Env-expressing plasmid. In pseudovirus prime wash assay, the peptides and controls were incubated with ghost cells at 37 °C for 2 h, followed by washing with phosphate-buffered saline (PBS) three times to remove unbound peptide, and by addition of HIV-1 pseudovirus, to initiate infection. After 48 h, the antiviral activities of the remaining peptides that survived the washing steps were measured by detecting the luciferase activity using a plate reader (Molecular Devices SpectraMax M5).

Cell–cell fusion assay

HL2/3 cells were gifts from Dr Barbara Felber and Dr George Pavlakis at NIH.[32] TZM-bl cells were kindly provided by Dr John C. Kappes, Dr Xiaoyun Wu, and Tranzyme Inc at NIH.[33–35] HL2/3 and TZM-bl cells were assessed for cell–cell fusion activity as previously described.[16] Briefly, HL2/3 cells were added to a 96-well plate (Corning Costar) with 50 μl of 2×10^6 cells per ml per well. After 24 h incubation, 20 μl peptide and control together with 50 μl of 5×10^5 cells per ml per well TZM-bl cells were added to HL2/3 cells at 27 °C for 2 h. This step was to establish the temperature-arrested state. And then TZM-bl cells and inhibitors were washed before cocultivation with equal amount of TZM-bl cells, and the plates were moved immediately to 37 °C to initiate cell–cell fusion. After 7 h, fusion was measured by detecting the luciferase activity.

Assay for stability against Proteinase K digestion

The stability of C-peptide inhibitors against Proteinase K was tested and detected by ELISA as previously described.[36] Peptide (10 μg mL^{-1}) was incubated at 37 °C in PBS containing 1 μU mL^{-1} Proteinase K-Acrylic Beads (Sigma). Samples were collected at different times. The residual peptide concentration was detected by ELISA. The protocol to detect the residual peptides followed previous reports.[37]

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