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Rational design of highly potent HIV-1 fusion inhibitory proteins: Implication for developing antiviral therapeutics

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Abstract

Recombinant protein containing one heptad-repeat 1 (HR1) segment and one HR2 segment of the HIV-1 gp41 (HR1–HR2) has been shown to fold into thermally stable six-helix bundle, representing the fusogenic core of gp41. In this study, we have used the fusogenic core as a scaffold to design HIV-1 fusion inhibitory proteins by linking another HR1 to the C terminus of HR1–HR2 (HR121) or additional HR2 to the N terminus of HR1–HR2 (HR212). Both recombinant proteins could be abundantly and solubly expressed and easily purified, exhibiting high stability and potent inhibitory activity on HIV-1 fusion with IC₅₀ values of 16.2 ± 2.8 and 2.8 ± 0.63 nM, respectively. These suggest that these rationally designed proteins can be further developed as novel anti-HIV-1 therapeutics.

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The fusion and entry of the human immunodeficiency virus type I (HIV-1) are mediated by its envelope (env) glycoprotein [1]. The env protein is synthesized as a precursor polypeptide gp160 which is then proteolytically cleaved into two non-covalently attached proteins, gp120 and gp41 [1]. Gp120 is responsible for binding to cellular receptors, CD4 and certain chemokine co-receptors, while gp41 possesses membrane fusion activity. The N terminus of gp41 contains a hydrophobic fusion peptide, followed by two well-conserved heptad-repeat regions 1 and 2 (HR1 and HR2). HR1 and HR2 play a crucial role in membrane fusion. Upon gp120 binding to cellular receptors, gp41 undergoes a series of conformational changes to convert from a native, non-fusogenic conformation to fusogenic conformation [2]. The fusogenic core of gp41 is a trimer-of-hairpins structure

(a six-helix bundle) in which three HR2 helices pack against a central trimeric coiled-coil formed by three HR1 helices [3,4]. Formation of the trimer-of-hairpins brings the viral and cellular membranes into the close proximity necessary for membrane fusion to occur. During the fusion process, the native gp41 converts to a transiently populated prehairpin intermediate prior to formation of the fusogenic trimer-of-hairpins structure when the fusion peptide of gp41 is inserted into the target cell membrane, and both HR1 and HR2 are transiently accessible to inhibitory compounds [5]. Peptides derived from HR1 and HR2 regions can inhibit gp41-mediated membrane fusion by binding to the prehairpin intermediate to block the proper formation of the trimer-of-hairpins, thus preventing membrane fusion [6,7]. Recombinant proteins that contain the HR1 and HR2 segments of HIV-1 gp41 separated by a short, hydrophilic, six-residue linker (HR1–HR2) have been produced and shown to fold into thermally stable six-helix bundles [8]. Based on these, we design two proteins:

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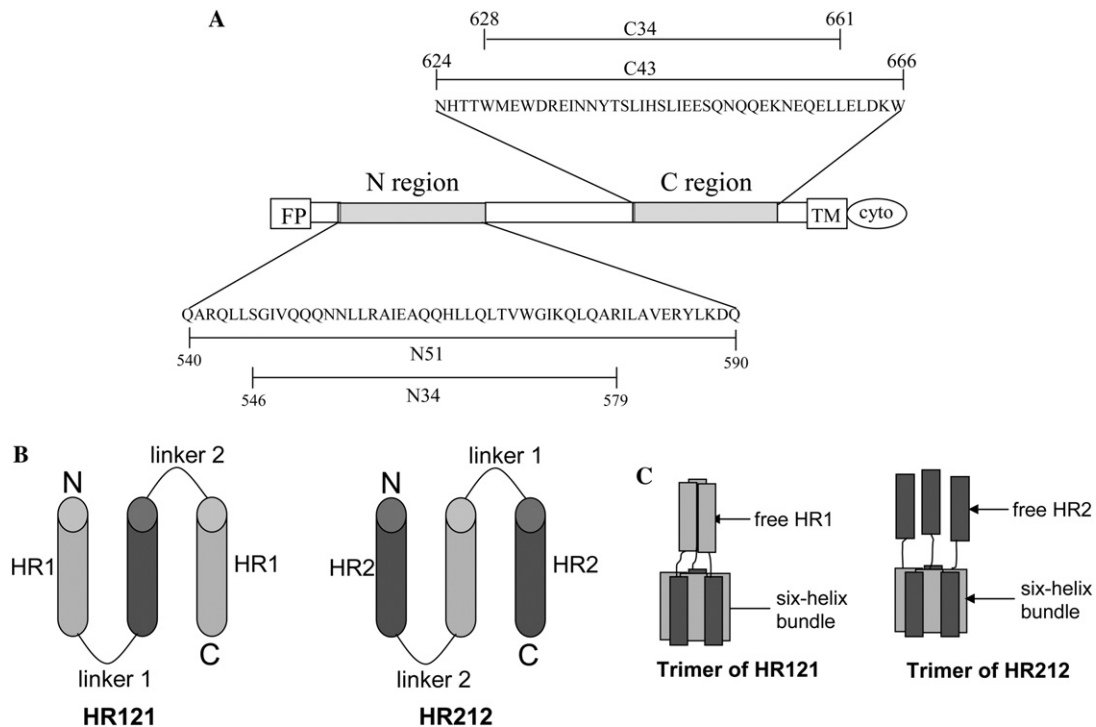


Fig. 1. Schematic diagram of HIV-1 gp41 and the constructs used in this study. (A) Schematic representation of HIV-1 gp41 sequence. The fusion peptide (FP), the N region (HR1), the C region (HR2), the transmembrane segment (TM), and the cytoplasmic tail (cyto) are all shown. The segments for N51, N34, C43, and C34 are also indicated. (B) HR121 contained two HR1 segments and one HR2 segment alternatively linked with the short Gly/Ser peptide sequences, while HR212 consisted of two HR2 segments and one HR1 segment. The sequences of HR1 and HR2 for constructing HR121 and HR212 were peptides N34 and C34, respectively; linker 1, GSSGG; linker 2, GGSGG. (C) Schematic models of the trimeric HR121 (left) and HR212 (right). Both HR121 and HR212 assembled into six-helix bundles with free HR1s or HR2s.

HR121, in which one HR1 (N34) peptide is linked to the C terminus of HR1–HR2 (HR1–HR2–HR1), and HR212, in which one HR2 (C34) peptide is linked to the N terminus of HR1–HR2 (HR2–HR1–HR2). The rationale for this design is that three heptad-repeats (two HR1 and one HR2 or one HR1 and two HR2) are linked by flexible linkers so that HR1 and HR2 in each protein can associate to form a hairpin structure. As a consequence, three molecules of the proteins can form a stable six-helix bundle with three free heptad-repeats (HR1 or HR2) exposed (Fig. 1). These free HR1 or HR2 can bind to the counterpart regions in the viral gp41, thereby blocking gp41-mediated membrane fusion. Here, we showed that HR121 and HR212 were well expressed as soluble proteins and could be easily purified. The two proteins inhibited HIV-1 membrane fusion in nanomolar concentrations.

Materials and methods

Gene construction. Genes encoding the viral gp41 HR1 fragments N51 (residues 540–590) and N34 (residues 546–579), and HR2 fragments C43 (residues 624–666) and C34 (residues 628–661) were amplified by PCR from gp160 expression vector pMT3-HXB2. HR121 (N34–C34–N34) consisted of two N34 segments and one C34 segment alternatively linked with the short peptide se-

quences, while HR212 (C34–N34–C34) consisted of two C34 segments and one N34 segment. The sequences of the C to N linker (linker 1) were GSSGG while those of the N to C linker (linker 2) were GGSGG. The genes encoding the proteins were then subcloned into *Escherichia coli* expression vector pGEX-6P-1 (Pharmacia) by two restriction enzyme sites of *EcoRI* and *XhoI*. A stop codon was introduced before the *XhoI* site. This cloning strategy yielded glutathione *S*-transferase (GST) fusion proteins, designated GST-HR121 and GST-HR212, respectively. N51 and C43 were subcloned into the *BamHI/XhoI* site of pET-30a and yielded the proteins with N-terminal 6 \times His-tag, thrombin, and enterokinase cleavage sites, which were named after N51-30a and C43-30a, respectively.

Protein expression and purification. The HR121 and HR212 proteins were expressed in *E. coli* strain BL21 (DE3) and purified by glutathione–Sepharose 4B affinity column according to the method described earlier [9]. The N108, N44, and C38 were applied on Ni-chelated Sepharose affinity column [10].

CD spectroscopy. CD spectra were performed on a Jasco J-715 spectrophotometer with proteins in PBS. Wavelength spectra were recorded at 25 °C using a 0.1 cm pathlength cuvette. The protein concentration used for this was 10 μ g/ml.

GST pull-down assay. Excess N51-30a and C43-30a in classified bacterium supernatants were mixed with GST-HR121 and GST-HR212, respectively. The mixtures were incubated for 1 h at room temperature before glutathione–Sepharose 4B affinity gel was added. The gel with the protein mixtures was then incubated with gentle agitation at room temperature for 30 min. After centrifugation at 500g for 5 min, the supernatants were removed and the gel with adsorbed fusion protein complexes was loaded into a column, which was washed with 10-bed volumes of PBS and eluted with

reduced L-glutathione. The eluted samples were analyzed by SDS-PAGE.

Inhibition of HIV-1 env glycoprotein-mediated virus-cell fusion by HR121 and HR212. Inhibition of virus-cell fusion was determined using a recombinant luciferase reporter assay. Virus pseudotyped with the HIV-1 env glycoproteins was produced by cotransfecting 293T cells with an HIV-1 genome containing a frame-shift mutation in *env* and a luciferase gene replacing *nef* (pNL43LucE⁻R⁻) and pMT3-HXB2. The cellular debris was removed by low-speed centrifugation. The supernatants containing pseudovirus particles were then mixed with serially diluted proteins to be tested. The virus/protein mixture was then transferred to 24-well plates seeded with GHOST-CXCR-4 cells. Three hours later, the medium was replaced. After incubation at 37 °C for 2 days, the cells were harvested and lysed in lysis buffer, followed by the addition of luciferase substrate. The luciferase activity was measured for 10 s in a TD-20/10 luminometer. The IC₅₀ values were calculated by fitting the HR121 and HR212 titration data Langmuir function {luciferase activity % = 100/(1 + C/IC₅₀), where C is the concentration of protein inhibitors}.

Results

Both HR121 and HR212 were expressed and purified as soluble proteins

To produce HR121 and HR212, we used the recombinant *E. coli* expression system which was able to provide protein products in large quantities at low cost.

The two designed proteins (GST-HR121 and GST-HR212) expressed as GST fusion proteins were soluble and readily eluted from the glutathione–Sepharose column by the normal elution buffer (10 mM reduced glutathione, 50 mM Tris–HCl, pH 8.0). Moreover, the two GST fusion proteins could be easily cleaved by GST-3C protease (Figs. 2A and B). Every liter of cells could yield 30 mg of HR121 and HR212, respectively. These suggested that the soluble recombinant proteins, HR121 and HR212, can be efficiently expressed and purified in large quantities at low cost.

The purified HR121 and HR212 were then analyzed in gel filtration for estimation of the molecular weight and the assembly character. The elution peaks of both HR121 and HR212 proteins were just after the peak position corresponding to 52 kDa (Figs. 2C and D). In SDS-PAGE, both HR121 and HR212 showed major bands with molecular weight 13 kDa (insets in Figs. 2C and D), in agreement with the calculated molecular weights of HR121 and HR212 (13.1 and 13.5 kDa, respectively). These data suggest that both HR121 and HR212 could form trimeric structure (i.e., six-helix bundle) in PBS. However, unlike the six-helix bundles formed by HR1–HR2, those formed by HR121 and HR212 were expected to express three unassociated HR1 or HR2 helices (Fig. 1C).

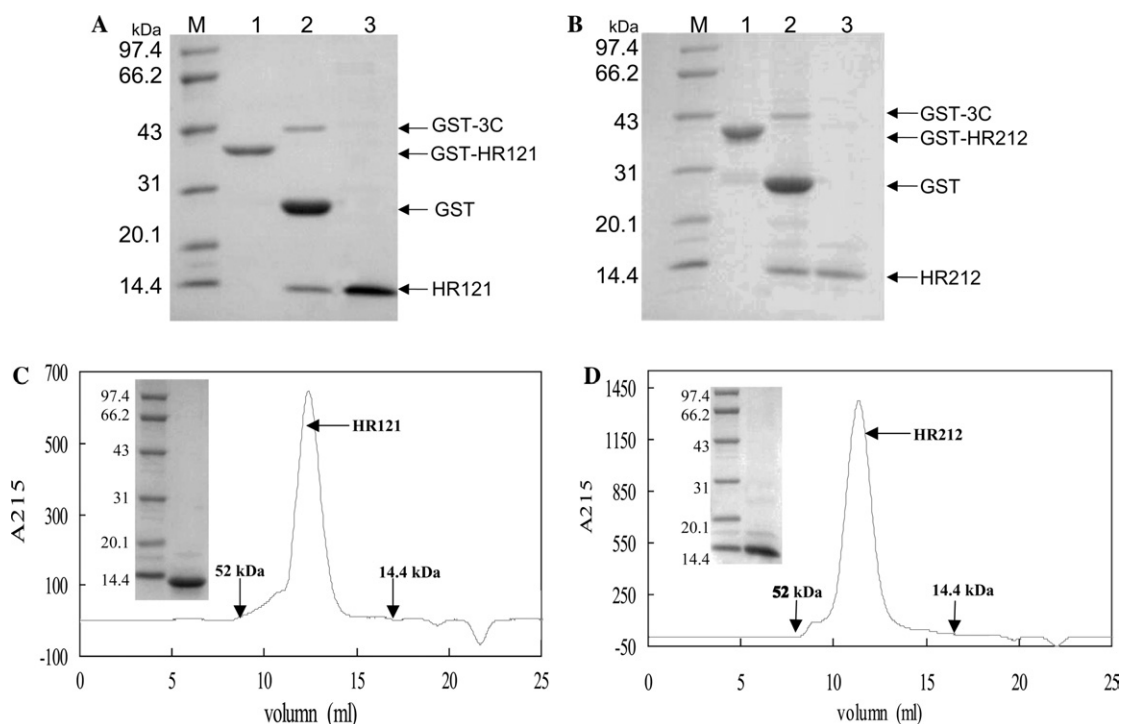


Fig. 2. SDS-PAGE and gel-filtration analysis of the purified proteins. Both HR121 (A) and HR212 (B) were expressed as GST fusion proteins and cleaved by GST-3C. Lane M, protein markers (in kDa); lane 1, GST fusion protein with HR121 or HR212; lane 2, the GST fusion proteins digested after GST-3C; and lane 3, the free HR121 and HR212. The free HR121 and HR212 were then loaded on Superdex G75 column. The relative positions of the standard protein markers were indicated (kDa). (C) and (D) represent the gel-filtration profiles of HR121 and HR212, respectively. The inset pictures were SDS-PAGE analysis of the sample from the peaks; left, protein markers (kDa); right, the proteins from the peaks.

HR121 and HR212 exhibited α -helical structure

The biophysical properties of the individual proteins concerned were tested by CD spectrometry as described in Materials and methods. HR121 and HR212 had a CD spectrum exhibiting double minima at 208 and 222 nm, while C43-30a had not. These indicated that HR121 and HR212 showed a salient α -helix character and C43-30a was unordered (Fig. 3A). N51-30a had a strong tendency to aggregate. It was not soluble and liable to precipitate in PBS but was partially soluble in clarified bacterium supernatants, so we could not acquire the purified N51-30a and did not analyze its structure. However, based on Kliger's study [5], N51 was partially α -helix. Accordingly, the two designed proteins were more stable than single HR1 or HR2.

HR121 and HR212 bound to C43-30a and N51-30a, respectively

GST-HR121 bound to C43-30a, while it did not bind to N51-30a. GST-HR212 bound to N51-30a, while it did not pull down C43-30a (Figs. 3B and C). These implied

that GST as well as the fusion partner of 50 extra amino acids of N51-30a and C43-30a did not affect the binding activity of HR121 and HR212. These results suggested that HR121 and HR212 were analogous to the monomers HR1 and HR2, respectively, and might bind to their counterpart regions in the viral gp41 to block fusion between the viral and target cell membranes.

HR121 and HR212 potently inhibited HIV-1 env glycoprotein-mediated virus-cell fusion

HR121 and HR212 were found to have anti-viral activity (Fig. 4), while GST alone could not inhibit the virus-cell fusion mediated by HIV-1 env glycoprotein, even up to 20 μ M. The IC_{50} values of HR121 and HR212 in this assay were 16.2 ± 2.8 and 2.8 ± 0.63 nM, respectively.

The monomer C43-30a was also tested for its inhibitory activity of HIV-1 env glycoprotein-mediated virus-cell fusion assay. The IC_{50} value of C43-30a was 1.7 nM, which was lower than that of HR212. It was possible that the folding of HR212 was not as good as the 2-Helix (HR1–HR2) and the binding sites of HR1 or HR2

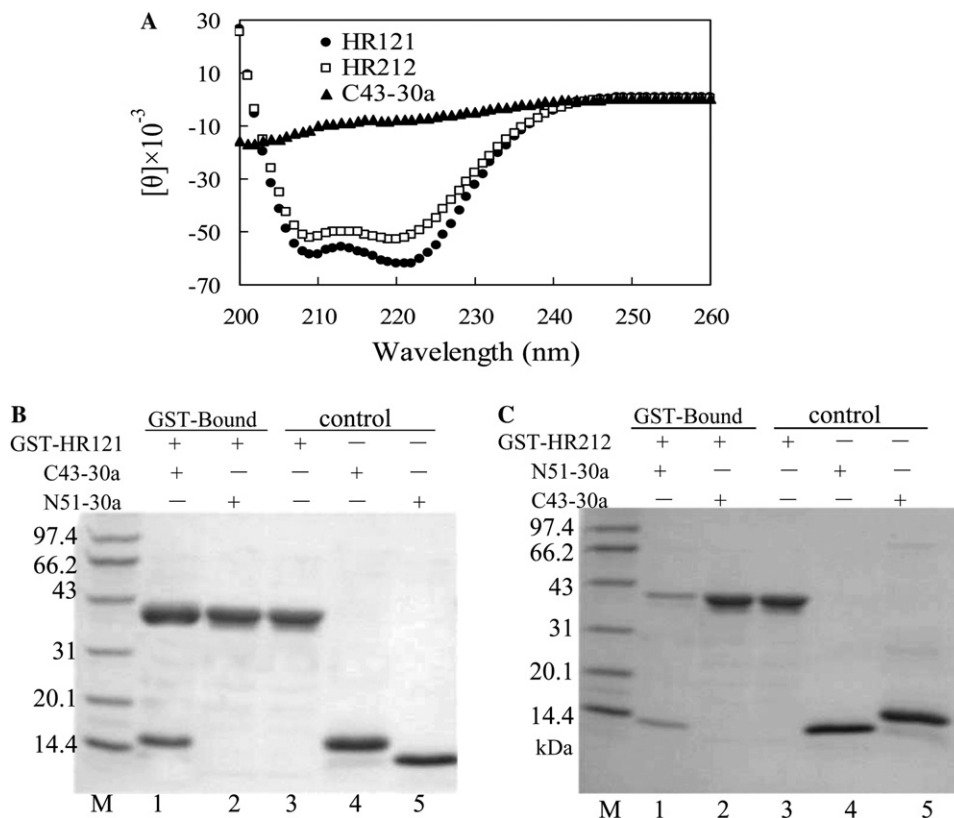


Fig. 3. (A) CD spectra of HR121 (filled circles), HR212 (open squares), and C43-30a (filled triangles) in PBS at 25 °C. (B) C43-30a was pulled down by GST-HR121. The results showed that GST-HR121 could interact with C43-30a (lane 1), while GST-HR121 could not pull down N51-30a (lane 2); lane 3, purified GST-HR121; lane 4, purified C43-30a; lane 5, purified N51-30a; and lane M, protein markers (kDa). (C) GST-HR212 could interact with N51-30a (lane 1), while GST-HR212 could not pull down C43-30a (lane 2); lane 3, purified GST-HR212; lane 4, purified N51-30a; lane 5, purified C43-30a; and lane M, protein markers (kDa).

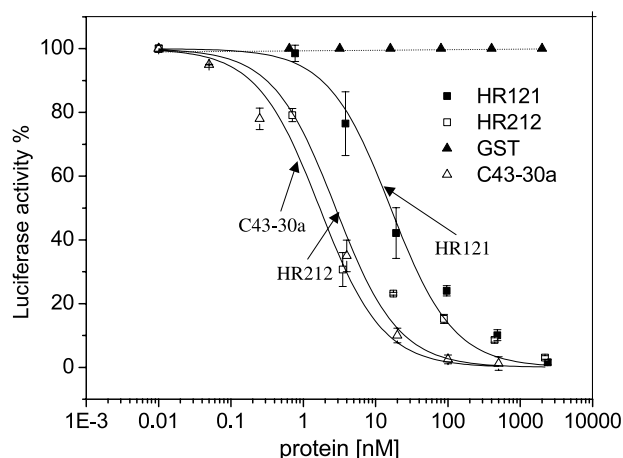


Fig. 4. Titration of virus-cell fusion inhibitory activities of HR121 (filled squares), HR212 (open squares), C43-30a (open triangles), and GST (filled triangles). The IC_{50} values of HR121, HR212, and C43-30a in this assay were 16.2 ± 2.8 , 2.8 ± 0.63 , and 1.7 ± 0.3 nM, respectively. GST protein itself was used as control and no inhibitions were observed. The data represented means \pm SEM of three separate experiments.

were not exposed very well. Since N51-30a had a strong tendency to aggregate and we could not acquire the purified N51-30a, its inhibitory activity was not tested.

Discussion

Peptides derived from HR2 region of gp41 are potent inhibitors of HIV-1 entry, with IC_{50} values in the low nanomolar range. One of these peptides, T-20 (brand name: Fuzeon; generic name: Enfuvirtide) [11], has been approved by the US Food and Drug Administration (FDA) and European Commission for treatment of HIV-1 infection in adults and children who have failed to respond to the current antiretroviral drugs. The inhibitory activity of T-20 provides compelling evidence that the prehairpin intermediate is a useful target for anti-HIV-1 therapy. However, although T-20 is effective in blocking in vitro HIV-1 infection at nanomolar level, large amounts of this peptidic drug (about 200 mg/day) are required to maintain the in vivo anti-HIV-1 efficacy in humans. Therefore, treatment with T-20 is priced about \$20,000 and \$25,000 per patient per year in the United States and Europe, respectively. One of the major limitations of T-20 is the difficulty of peptide synthesis, resulting in high cost of production and insufficiency of supplies [12]. Peptides derived from HR1 region of gp41, which are thought to either bind to the HR2 region of the prehairpin intermediate or hinder the formation of the internal trimeric coiled-coil [13,14], possess anti-HIV-1 activity about three orders of magnitude lower than those of HR2 peptides, presumably because of aggregation and their inability to form the trimeric coiled-coil in the absence of HR2 peptides [15]. There-

fore, it is necessary to design a new class of molecules targeting HR1 or HR2 of the viral gp41 with potent anti-HIV-1 activity, high stabilities, and low cost of production.

Lu's study has showed that N34-L6-C28 (HR1-HR2), which was constructed by covalent attachment of peptide N34 (HR1, residues 546–579) and C28 (HR2, residues 628–655) by a short flexible linker, could form a highly thermostable, α -helical trimer that represented the smallest stable folded gp41 core [3]. Lu also showed that the linker in N34-L6-C28 stabilized the folded gp41 core for entropic reasons, without perturbing its structure [3]. Based on these, we made use of the fusogenic core as a scaffold to link another HR1 to its C terminus and additional HR2 to its N terminus to design two proteins denoted HR121 (N34-linker-C34-linker-N34) and HR212 (C34-linker-N34-linker-C34), respectively, which were constructed by N34 and C34 (residues 628–661). C34 peptide is extended six residues from the C-terminal sequence of the C28 segment in N34-L6-C28. We selected C34 segment not C28 segment mainly because C34 peptide inhibited HIV-1 entry with the lowest IC_{50} values of 2–5 nM [14].

HR121, in which the solvent-exposed trimeric coiled-coil of gp41 is stabilized by directly linking it to the C terminus of a thermostable core fragment of gp41 comprising a six-helix bundle with a flexible linker, is a highly potent inhibitor of HIV-1 env glycoprotein-mediated cell fusion with an IC_{50} value of 16.2 nM. HR121 sustains the HR1 peptide coiled-coil conformation and, strikingly, it has dramatically increased antiviral potency over the corresponding HR1 peptides, which have a tendency to aggregate. The inhibitory activity of HR121 is comparable with those of 5-Helix, Nccg-gp41, IQN17, and IQN23 [14,16–18]. 5-Helix (HR1-HR2-HR1-HR2-HR1), five of six helices that constitute the gp41 trimer-of-hairpins, is linked covalently into a single polypeptide. 5-Helix lacks a third HR2 segment and this vacancy creates a high-affinity binding site for HR2 peptides. 5-Helix is a potent and broad-spectrum inhibitor of HIV-1 membrane fusion, with IC_{50} values in the low nanomolar range. However, 5-Helix is expressed in *E. coli* as inclusion bodies that results in difficulties to purify it. Nccg-gp41 (HR1-HR1-HR2), which is constructed by grafting one HR1 to N terminus of N34-L6-C28 core, inhibits membrane fusion at nanomolar concentration. But it is also extracted from inclusion bodies. Both IQN17 and IQN23 are synthesized, which is at high cost. Compared with these inhibitors targeting the HR2 region of the prehairpin intermediate of gp41, HR121 is expressed in *E. coli* as soluble proteins and can be easily purified. On the other hand, the inhibitory activity of HR121 further tests the hypothesis that the weak inhibition of HR1 peptide results from their tendency to aggregate in the absence of HR2 peptide.

HR212, in which three C34 peptides are stabilized by linking them to a six-helix bundle of gp41, is also a highly potent inhibitor of HIV-1 env glycoprotein-mediated cell fusion. Its inhibitory activity is comparable to that of T20, but the stability of HR212 is improved with the production at low cost. This design has already been applied to SARS-CoV. Both HR121 and HR212 inhibit HIV/SARS pseudotyped viruses entry in micromolar range [19].

In a word, the designed proteins, HR121 and HR212 can be easily expressed and purified with the production at low cost, exhibiting typical α -helical structure. They were potentially used as therapeutic agents, in a manner analogous to peptides derived from HR1 and HR2 of gp41.

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