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Novel anti-HIV peptides containing multiple copies of artificially designed heptad repeat motifs

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ABSTRACT

The peptidic anti-HIV drug T20 (Fuzeon) and its analog C34 share a common heptad repeat (HR) sequence, but they have different functional domains, i.e., pocket- and lipid-binding domains (PBD and LBD, respectively). We hypothesize that novel anti-HIV peptides may be designed by using artificial sequences containing multiple copies of HR motifs plus zero, one or two functional domains. Surprisingly, we found that the peptides containing only the non-natural HR sequences could significantly inhibit HIV-1 infection, while addition of PBD and/or LBD to the peptides resulted in significant improvement of anti-HIV-1 activity. These results suggest that these artificial HR sequences, which may serve as structural domains, could be used as templates for the design of novel antiviral peptides against HIV and other viruses with class I fusion proteins.

Human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein gp160 is proteolytically cleaved into the surface subunit gp120 that is responsible for virus binding to the receptors and the transmembrane fusion protein subunit gp41 that mediates virus fusion and entry [1]. The gp41 molecule contains a cytoplasm domain (CT), a transmembrane domain (TM), and an extracellular domain (ectodomain) which consists of three major functional regions: fusion peptide (FP), N-terminal heptad repeat (NHR), and C-terminal heptad repeat (CHR) (Fig. 1A). Both NHR and CHR regions are composed of 4-3 hydrophobic heptad repeat (HR) sequences which have a tendency to form coiled-coil structure [2].

During HIV infection, gp120 binds to CD4 and a chemokine receptor (CCR5 or CXCR4) on the target cell to trigger gp41 structural rearrangement. This results in the formation of a stable gp41 six-helix bundle (6-HB) core structure, in which three NHR-helices associate to form the central trimeric coiled coil. Three C-helices pack obliquely in an anti-parallel manner into the highly conserved hydrophobic grooves on the surface of the NHR-trimer [3]. In each groove, there is a highly conserved hydrophobic deep pocket formed by the pocket-forming sequence (residues 565–581) in the NHR region. This pocket plays a critical role in viral fusion and maintaining the stability of the 6-HB [3]. The formation of 6-HB is believed to bring both the viral and target cell membranes into proximity, resulting in fusion between the virus and target cell membranes [4,5].

Synthetic peptides derived from the gp41 CHR regions, e.g., T20 and C34, are highly potent in inhibiting HIV fusion [2,6,7], and T20 (brand name: Fuzeon; generic name: Enfuvirtide) is the first HIV fusion inhibitor approved by the US FDA for treatment of HIV/AIDS [8]. Both T20 and C34 share a common HR sequence as a structural domain, but contain different functional domains, i.e., lipid-binding domain (LBD) and pocket-binding domain (PBD), respectively [9]. It is believed that CHR-peptides inhibit HIV fusion by interacting through their HR sequences with the viral gp41 NHR region blocking gp41 6-HB core formation [3,8].

Based on these lines of evidence, we hypothesized that novel anti-HIV peptides could be designed by using artificial sequences containing multiple copies of HR motifs as templates. Here, we designed a series of peptides containing the artificial HR sequence plus zero, one or two functional domains. We found that the peptides consisting of all non-natural sequences that were predicted to form coiled-coil structures exhibited inhibitory activity on HIV-1-mediated cell–cell fusion activity, HIV-1 replication,

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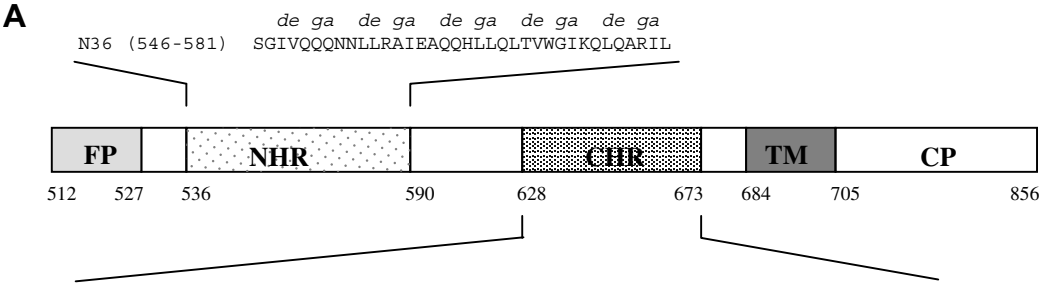

T20 (638-673) **YTSLIHSLIEESQNQQEKNEQELLELDK**WASLWNWF
C34 (628-661) WMEWDREI**NNYTSLIHSLIEESQNQQEKNEQELL**

	a d a d a d a d a d bcdefga	aa No.	MW	pI
5HRa	**(EELAKKA)$_5$**	35	3868.0	6.69
5HRb	**(EEAAKKL)$_5$**	35	3868.0	6.69
5HRc	**(EALAKAA)$_5$**	35	3292.2	6.55
4HRa-LBD	**(EELAKKA)$_4$**WASLWNWF	36	4189.3	6.64
4HRb-LBD	**(EEAAKKL)$_4$**WASLWNWF	36	4189.3	6.64
4HRc-LBD	**(EALAKAA)$_4$**WASLWNWF	36	3728.7	6.50
PBD-4HRa	WMEWDREI**(EELAKKA)$_4$**	36	4244.3	5.02
PBD-4HRb	WMEWDREI**(EEAAKKL)$_4$**	36	4244.3	5.02
PBD-4HRc	WMEWDREI**(EALAKAA)$_4$**	36	3783.8	4.74
PBD-3HRa-LBD	WMEWDREI**(EELAKKA)$_3$**WASLWNWF	37	4565.6	4.90
PBD-3HRb-LBD	WMEWDREI**(EEAAKKL)$_3$**WASLWNWF	37	4565.5	4.90
PBD-3HRc-LBD	WMEWDREI**(EALAKAA)$_3$**WASLWNWF	37	4220.2	4.63

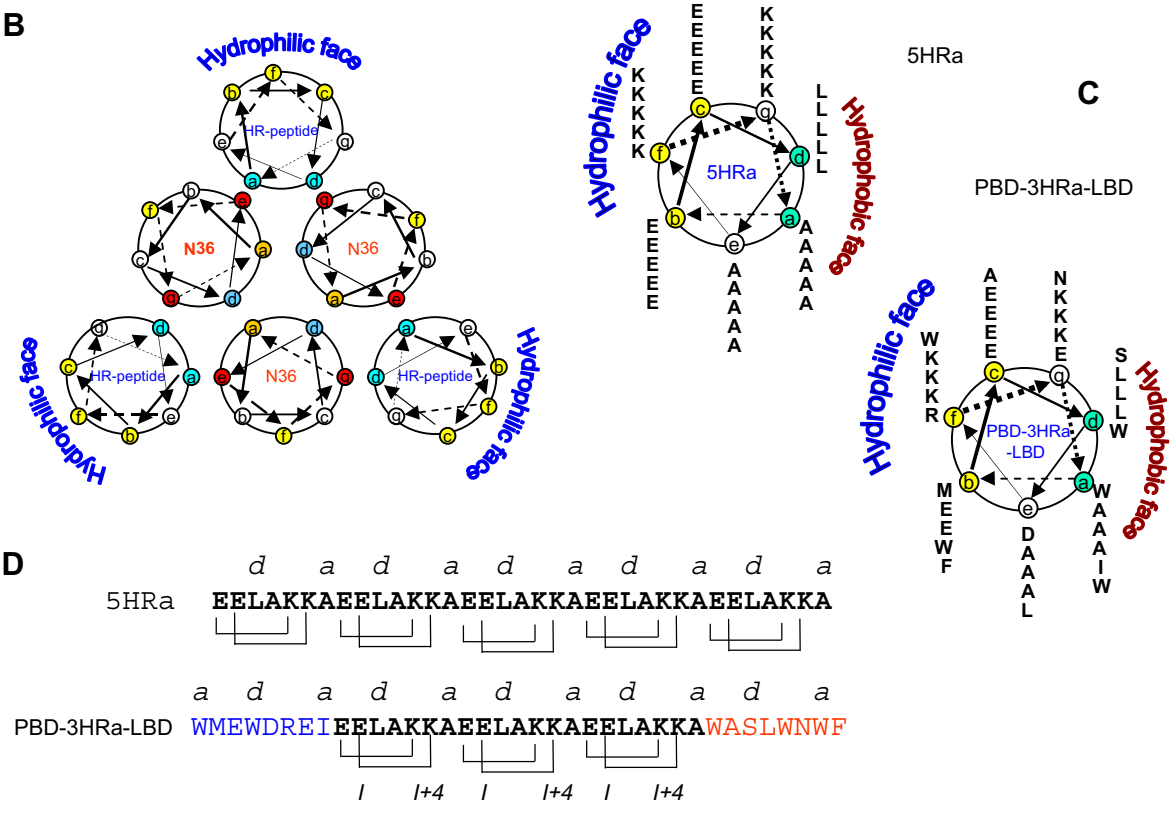

Fig. 1. Rational design of anti-HIV peptides. (A) Schematic representation of the HIV-1 gp41 and sequences of the HR-peptides. NHR, N-terminal heptad repeat; CHR, C-terminal heptad repeat; FP, fusion peptide; TM, transmembrane domain; CP, cytoplasm domain. The residue numbers of each region correspond to their position in gp160 of the HIV-1_{HXB2} (GenBank Accession No. AAB50262). The blue and red sequences in a C-helix are pocket- and lipid-binding domain, respectively. The M_W and p*I* were analyzed using ProtParam at ExPASy Proteomics Server (http://www.expasy.org/tools/protparam.html). (B) Association of the HR-peptides to N36 peptide to form 6-HB. The gp41 6-HB is formed by N36 and HR-peptides through the interaction between hydrophobic amino acid residues localized in the hydrophobic interfaces. The residues at the *a* position (orange) and *d* position (blue) in a N-helix can interact with the *d* position (blue) and *a* position (orange) of other N-helices, respectively, to form an internal N-trimer. The residues at the *a* and *d* positions (green) in an HR-peptide can interact the *e* and *g* positions (red) in the N-helices, respectively, to form 6-HB. (C) The hydrophobic and hydrophilic interfaces of the designed HR-peptides. The residues at the *a* and *d* positions (green) can form the hydrophobic face to interact with the hydrophobic face of N-helices, while those at the *b*, *c*, and *f* positions (yellow) can form a hydrophilic face to interact with the H_2O molecules in solution. (D) Prediction of the ionic interaction between the basic and acidic amino acid residues in the designed HR-peptides 5HRa and PBD–3HRa–LBD. The acidic residues at the *b* and *c* positions (*i*) in an HR-peptide can interact with the basic residues in the *f* and *g* positions in the same peptide (*i* + 4), respectively, to form intramolecular salt-bridges to stabilize the α-helical conformation.

and gp41 6-HB formation, while addition of LBD and/or PBD led to a significant increase of the anti-HIV-1 activity. Therefore, this study lays the theoretical/empirical groundwork for the rational design of novel antiviral peptides against HIV and other viruses with class I fusion proteins.

Materials and methods

Peptide synthesis. Peptides were synthesized on 4-methyl benzhydrylamine (MBHA) resin by using the Fmoc strategy. They were cleaved from the resin by hydrogen fluoride. The N-termini of the peptides were acetylated, and their C-termini were amidated. All the peptides were purified by reverse phase high performance liquid chromatography to >90% homogeneity. The molecular weight of the peptides was confirmed by MALDI-TOF-MS (Autoflex III, Bruker Daltonics).

HIV-1-mediated cell–cell fusion. HIV-1-mediated cell–cell fusion was determined by a dye transfer assay as previously described [10] using Calcein AM-labeled HIV-1_{IIIB} chronically infected H9 (H9/HIV-1_{IIIB}) cells as effector cells and MT-2 cells as target cells. The % inhibition of cell fusion by the HR-peptides was calculated as previously described [10], and 50% inhibitory concentration (IC_{50}) was calculated using the CalcuSyn software [11].

Detection of HIV-1 replication. The inhibitory activity of the HR-peptides on HIV-1_{IIIB} replication was determined as previously described [12,13]. Briefly, MT-2 cells were infected with HIV-1_{IIIB} in the presence or absence of the HR-peptides at graded concentrations. On the fourth day post-infection, culture supernatants were collected and assayed for p24 antigen using ELISA as previously described [13]. On the sixth day post-infection, an indicator XTT tetrazolium dye (PolySciences, Inc., Warrington, PA) was added to the cells for detection of cytopathic effect (CPE).

ELISA for detection of the gp41 6-HB formation. The inhibitory activity of the designed HR-peptides on gp41 6-HB formation was determined by a sandwich ELISA as previously described [14]. Briefly, the peptide N36 (2 μM) was pre-incubated with an HR-peptide at graded concentrations at 37 °C for 30 min, followed by addition of C34 (2 μM). After incubation at 37 °C for 30 min, the mixture was added to wells of ELISA plates pre-coated with purified rabbit anti-6-HB IgG. Murine anti-6-HB monoclonal antibody (mAb) NC-1, biotin-labeled goat-anti-mouse IgG, Streptavidin-conjugated horseradish peroxidase (SA-HRP), and the substrate 3,3′,5,5′-tetramethylbenzidine (TMB) were then added sequentially. Absorbance at 450 nm (A_{450}) was measured using an ELISA reader (Ultra 384, Tecan, Durham, NC).

Prediction of the secondary and tertiary structures of the designed HR-peptides. Secondary structure prediction of designed HR-peptides was carried out on the NPS@ Web server (http://npsa-pbil.ibcp.fr/) using the HNN Secondary Structure Prediction Method [15]. According to the crystal structure of the gp41 core formed by the NHR-peptide N36 and CHR-peptide C34 [4] and the predicted tertiary structure of the designed HR-peptides, the interaction between the HR-peptides and N36 was modeled by using the PyMOL program (http://pymol.sourceforge.net).

Results

Principles of peptide design

We designed four sets of HR-peptides with 35 or more amino acid residues: (i) peptides containing only an artificial sequence consisting of five copies of an identical 4-3 HR motif that has the tendency of forming coiled-coil structure, e.g., 5HRa—(**EELAKKA**)$_5$; (ii) peptides containing four copies of an HR motif plus a LBD, such as 4HRa–LBD—(**EELAKKA**)$_4$WASLWNWF; (iii) peptides containing four copies of an HR motif plus a PBD, e.g., PBD–4HRa—WMEWDREI (**EELAKKA**)$_4$; and (iv) peptides containing three copies of an HR motif plus both PBD and LBD, such as PBD–3HRa–LBD—WMEWDREI(**EELAKKA**)$_3$WASLWNWF (Fig. 1A).

The design of these peptides was based on the following principles. First, each peptide contains 3–5 copies of an artificially designed HR motif, which is totally different from the current anti-HIV peptides T20 and C34 that share a natural gp41 CHR sequence consisting of three different HR motifs (i.e., YTSLIHS-LIEESQN-QQE-KNEQ) [7,16]. Second, the residues at the *a* and *d* positions in the helical wheel are hydrophobic amino acids, e.g., Leu (L) or Ile (I), which can form a hydrophobic face for interaction with the residues at the *e* and *g* positions in the helical wheels of the N-helices. Third, the residues at the *b* and *c* positions in the helical wheel are negatively charged residues, e.g., Glu (E) and those at the *f* and *g* positions are positively charged residues, such as Lys (K). These amino acid residues can form a hydrophilic face to interact with water for increasing peptide solubility (Fig. 1B and C). Furthermore, the acid residues at the *b* and *c* positions in the helical wheel can interact with those at the *f* and *g* positions (*i* and *i* + 4), respectively, to form salt-bridges, in order to increase the helical structure stability of the peptides [17] (Fig. 1D). Five, the functional domains, PBD and LBD, are added, respectively, to the N- and C-termini of the HR sequence, mimicking the CHR-peptides C34 and T20, respectively [9,18].

All the designed HR-peptides were predicted to display mainly α-helical conformation and to interact with the NHR-peptide N36

As shown in Table 1, 86–94% of the amino acid residues were in α-helix conformation. Only about 6–14% of the residues mainly localized in the N- or C-terminal regions were in random coil conformation. These results suggest that all these designed HR-peptides have a tendency to form α-helical structure.

It has been shown that mixing of equimolar concentration of the gp41 NHR-peptide N36 and the CHR-peptide C34 results in formation of highly stable 6-HB [16], and the tertiary structure of the 6-HB has been determined by X-ray crystallography [4] (Fig. 1B). While modeling the helix bundles formed by the designed HR-peptides with N36 by the PyMOL program, we found that all the designed HR-peptides had a tendency to interact with the NHR-peptide to form 6-HB. As shown in Fig. 2, four amino acid residues located at the *d* positions (magenta) in 5HRa (L3, L10, L17, and L24) may interact with the four amino acid residues at the *g* positions (yellow) in N36 (G572, L565, A558, and Q551), respectively. However, the binding and the complementarities between the residues of 5HRa and N36 are weaker than those of the residues between

Table 1
Prediction of the secondary structure of the designed HR-peptides

Peptides	α-Helix	Random coil
5HRa	91.43	8.57
5HRb	91.43	8.57
5HRc	91.43	8.57
4HRa–LBD	94.44	5.56
4HRb–LBD	94.44	5.56
4HRc–LBD	94.44	5.56
PBD–4HRa	86.11	13.89
PBD–4HRb	86.11	13.89
PBD–4HRc	86.11	13.89
PBD–3HRa–LBD	89.19	10.81
PBD–3HRb–LBD	89.19	10.81
PBD–3HRc–LBD	89.19	10.81
T20	85.29	14.71
C34	91.67	8.33

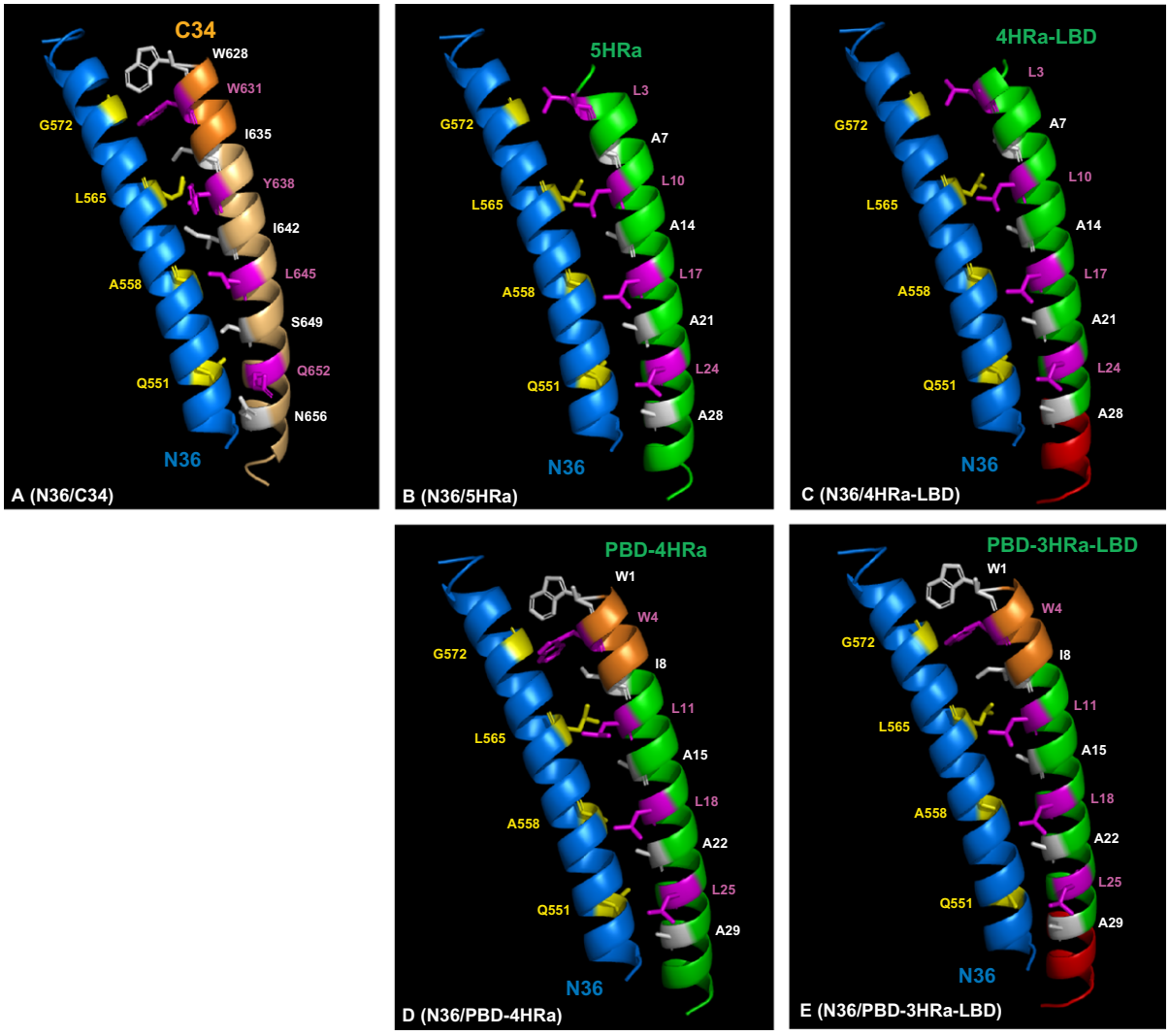

Fig. 2. Molecular modeling analysis of the interaction between the NHR-peptide N36 and the CHR-peptide C34 (A), and the HR-peptides 5HRa (B), 4HRa–LBD (C), PBD–4HRa (D), and PBD–3HRa–LBD (E), respectively. An HR-peptide is expected to interact with two N36 molecules. However, for clarity, only one N36 peptide (blue) is shown. The 4-3 HR sequences in C34 and the designed HR-peptides are in light orange and green, respectively. The PBD and LBD are in orange and red, respectively. The amino acid residues at *d* position (magenta) in HR-peptides interact with those at *e* position (yellow) in one N36 molecule, while residues at the *a* position (white) in the HR-peptides would bind to those at the *g* position in another N36 helix, which is not shown.

C34 and N36. When the LBD was introduced to 4HRa, it appeared from Fig. 2C that most of the critical interactions remained the same. However, addition of this hydrophobic part may have added hydrophobic interactions which are not evident from the currently available N36/C34 structure. On the contrary, the introduction of PBD in 4HRa made the most critical hydrophobic domain of C34 available to bind to the pocket region, probably in a similar fashion as C34. Addition of both PBD and LBD to the HR sequence (e.g., PBD–3HRa–LBD) may have more dramatic effect on the overall interactions of these of peptides with the N-trimer.

The designed HR-peptides exhibited varying inhibitory activity on the HIV-1-mediated cell–cell fusion dependent on the presence of functional domain(s)

The inhibitory activity of the designed HR-peptides on the HIV-1 mediated cell–cell fusion was determined by a dye transfer assay [10]. Surprisingly, all three peptides containing only the artificial HR sequences exhibited inhibitory activity in a dose-dependent manner. 5HRa was somewhat more effective than 5HRb and 5HRc. However, addition of LBD or PBD to the C- or N-terminus, respectively, of the 4HR sequence resulted in significant increase of the fusion inhibitory activity. For example, the inhibitory activity of 4HRa–LBD or PBD–4HRa on the HIV-1-mediated cell–cell fusion was about 1- and 2-fold higher than that of 5HRa. Addition of both PBD and LBD to the N- and C-termini, respectively, of the 3HR sequence led to further improvement of the membrane fusion inhibitory activity, e.g., PBD–3HRa–LBD was about 11-fold more potent than 5HRa (Table 2).

The designed HR-peptides possessed different antiviral activity against HIV-1 replication

The inhibitory activity of the designed HR-peptides on HIV-1_{IIIB} replication was assessed by measuring p24 production and CPE [13]. Consistent with their inhibitory activity on HIV-1-mediated cell–cell fusion, all the three HR-peptides containing only the artificial HR sequences exhibited moderate inhibitory activity on HIV-1 replication, while addition of the functional domain LBD and/or PBD resulted in remarkable enhancement of the antiviral activity. For instance, the inhibitory activity of PBD–3HRa–LPD on p24 production and CPE was higher than that of 5HRa by 61- and

Table 2
Inhibition of the designed HR-peptides on HIV-1-mediated cell–cell fusion

Peptides	Sequences	Inhibition of cell–cell fusion IC_{50} (μg/ml)*	Relative potency
5HRa	(**EELAKKA**)$_5$	189.44 ± 11.47	1.00
5HRb	(**EEAAKKL**)$_5$	219.12 ± 10.41	0.87
5HRc	(**EALAKAA**)$_5$	257.38 ± 11.79	0.74
4HRa–LBD	(**EELAKKA**)$_4$WASLWNWF	92.16 ± 4.03	2.06
4HRb–LBD	(**EEAAKKL**)$_4$WASLWNWF	120.64 ± 5.95	1.57
4HRc–LBD	(**EALAKAA**)$_4$WASLWNWF	154.29 ± 6.36	1.23
PBD–4HRa	WMEWDREI(**EELAKKA**)$_4$	66.29 ± 2.37	2.86
PBD–4HRb	WMEWDREI(**EEAAKKL**)$_4$	89.36 ± 4.89	2.12
PBD–4HRc	WMEWDREI(**EALAKAA**)$_4$	137.64 ± 4.72	1.38
PBD–3HRa–LBD	WMEWDREI(**EELAKKA**)$_3$WASLWNWF	15.75 ± 0.58	12.02
PBD–3HRb–LBD	WMEWDREI(**EEAAKKL**)$_3$WASLWNWF	21.38 ± 0.79	8.86
PBD–3HRc–LBD	WMEWDREI(**EALAKAA**)$_3$WASLWNWF	86.49 ± 2.62	2.19

* Each sample was tested in quadruplicate, and the data were presented in means ± SD.

167-fold, respectively. Notably, the peptide PBD–3HRa–LBD was about 15- to 55-fold more potent than PBD–3HRb–LBD and PBD–3HRc–LBD in inhibiting HIV-1 replication (Table 3). These results suggest that that the antiviral activity of HR-peptides is dependent on both HR sequences and functional domains.

The inhibitory activity of the designed HR-peptides on gp41 6-HB formation was correlated with their anti-HIV-1 activity

T20 and C34 are believed to inhibit HIV fusion with the target cell by interacting with the viral gp41 NHR region to block the gp41 6-HB core formation [3,8]. Here, we compared the potential inhibitory activity of the designed HR-peptides on the gp41 6-HB formation by a sandwich ELISA using a gp41 6-HB-specific mAb NC-1 [14]. As shown in Fig. 3A, all the peptides containing only the HR sequence or an HR sequence plus LBD exhibited marginal inhibitory activity. Addition of the PBD, or both PBD and LBD, to the HR sequence led to a significant increase of inhibitory activity against 6-HB formation. Interestingly, the 6-HB formation inhibitory activity of the designed HR-peptides is correlated with their inhibitory activity on the HIV-1-mediated cell–cell fusion (Fig. 3B). These results suggest that these designed HR-peptides inhibit HIV fusion by interfering with viral gp41 fusogenic core formation.

Discussion

Discovery of anti-HIV-1 peptides has created new avenues for developing viral fusion inhibitors against other viruses with class I membrane fusion proteins. This results from the fact that all these proteins contain the unique NHR (or HR1) and CHR (or HR2) sequences and use similar mechanisms to mediate viral fusion and entry [19]. It has been reported that peptides derived from the CHR regions of class I membrane fusion proteins are also effective against corresponding viruses, such as simian immunodeficiency viruses (SIV) [20], respiratory syncytial virus (RSV) [21], Ebola virus [22], Nipah and Hendra viruses [23], and severe acute

Table 3
Inhibition of the designed HR-peptides on HIV-1_{IIIB} replication

Peptides	Inhibiting p24 production		Inhibiting CPE	
	IC_{50} (μg/ml)*	Relative potency	IC_{50} (μg/ml)*	Relative potency
5HRa	1013.72 ± 42.37	1.00	1284.31 ± 59.43	1.00
5HRb	1245.08 ± 56.14	0.81	1336.79 ± 60.27	0.96
5HRc	1442.35 ± 41.92	0.70	1660.22 ± 51.38	0.77
4HRa-LBD	638.35 ± 22.84	1.59	345.97 ± 10.03	3.71
4HRb-LBD	919.56 ± 30.03	1.10	487.13 ± 21.42	2.69
4HRc-LBD	977.26 ± 28.72	1.04	772.17 ± 27.51	1.66
PBD-4HRa	341.48 ± 9.46	2.97	229.32 ± 11.85	5.60
PBD-4HRb	722.37 ± 16.28	1.40	346.88 ± 64	3.70
PBD-4HRc	878.97 ± 20.19	1.15	614.25 ± 31.77	2.09
PBD-3HRa-LBD	16.34 ± 1.73	62.04	7.61 ± 0.22	168.77
PBD-3HRb-LBD	260.13 ± 9.47	3.90	125.07 ± 7.14	10.27
PBD-3HRc-LBD	482.95 ± 18.46	2.10	429.87 ± 12.79	2.99

* Each sample was tested in triplicate, and the data were presented in means ± SD.

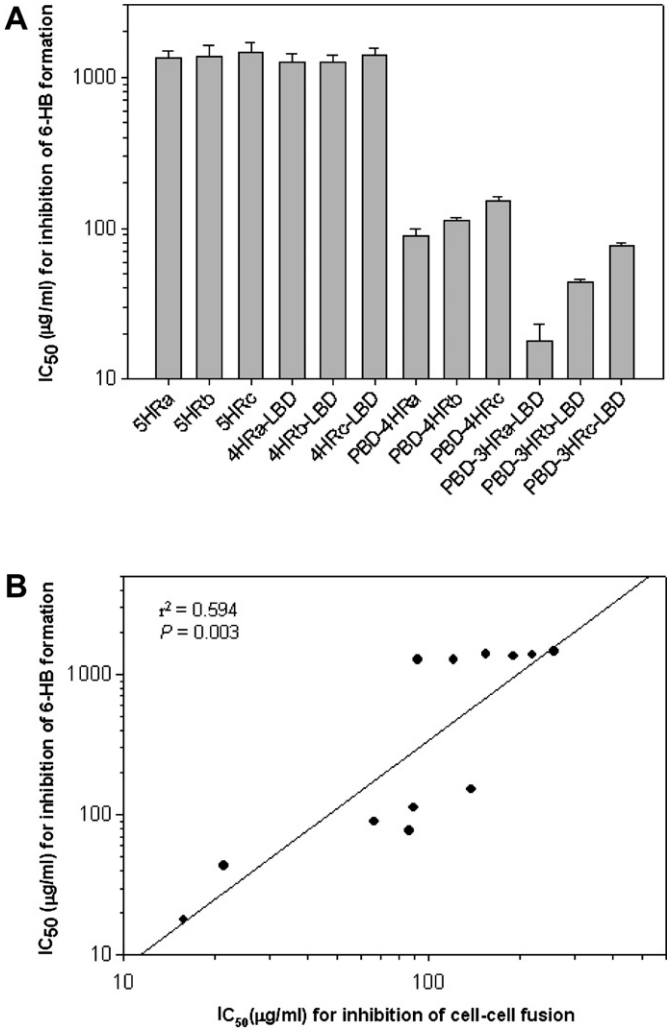

Fig. 3. Inhibition of the HR-peptides on gp41 6-HB formation. (A) IC_{50} values of the designed HR-peptides for inhibition of 6-HB formation. Formation of the 6-HB between N36 and C34 was determined by a sandwich ELISA. Each sample was tested in triplicate, and the data were presented in means ± SD (bars). (B) Correlation between the inhibitory activities of the designed HR-peptides on gp41 6-HB formation and HIV-1-mediated cell–cell fusion.

respiratory syndrome-associated coronavirus (SARS-CoV) [24]. We thus questioned whether novel antiviral peptides could be designed by using a universal HR sequence consisting of multiple artificially designed leucine-zipper motifs as a template. To test this hypothesis, we first designed and synthesized three peptides, each of which contains five copies of a unique HR motif (5HRa, 5HRb, and 5HRc), and then assessed their potential anti-HIV-1 activity. Strikingly, all these peptides exhibited inhibitory activity on HIV-1-mediated cell–cell fusion and on HIV-1 replication, although the potency was moderate (Tables 1 and 2). Our previous studies have shown that both anti-HIV CHR-peptides, T20 and C34, contain a common 4-3 HR sequence. However, the peptide containing only this HR sequence (e.g., CHR-3) had weak anti-HIV-1 activity while addition of PBD or LPD to the N- or C-terminus of this HR sequence (e.g., C34 and T20, respectively) resulted in significant increase of HIV fusion inhibitory activity [9,18]. Therefore, we postulated that addition of PBD and/or LBD to the peptides containing multiple copies of an HR motif (e.g., HRa) might also improve their anti-HIV-1 activity. Following this logic, we designed and synthesized three sets of peptides by adding PBD or LBD to the N- or C-terminus of the HR sequence and tested their anti-HIV-1 activity. Indeed, all these peptides containing an artificial HR sequence plus one functional domain (e.g., PBD–4HRa or 4HRa–LBD) displayed improved inhibitory activity on HIV-1-mediated cell–cell fusion and HIV-1 replication. Interestingly, addition of both PBD and LBD to the N- and C-termini of an HR sequence (e.g., PBD–3HRa–LBD) resulted in further improvement of the anti-HIV-1 activity. Although the functional domain is critical for anti-HIV-1 activity, the HR sequence in the structural domain is also an important determinant for the antiviral activity of the designed HR-peptides. For example, all the HR-peptides containing HRa motif are more potent than those containing HRb or HRc motif in inhibiting HIV-1 replication (Table 3).

To study the mechanism by which these designed HR-peptides inhibit HIV fusion and replication, we compared their inhibitory activity on the gp41 6-HB formation between N36 and C34. We found that the inhibitory activity of these HR-peptides on 6-HB formation is correlated with their potency in blocking HIV-1-mediated cell–cell fusion (Fig. 3B). This indicates that these peptides inhibit HIV fusion with the target cell by interfering with the virus gp41 6-HB core formation, a critical step for the HIV fusion process [2,3,25].

In conclusion, the results from this study suggest that the HR sequence in an anti-HIV peptide may serve as a structural domain that can support the shape and the size of the peptide responsible for its interaction with the viral gp41 NHR domain. Here, we have identified several artificial HR sequences consisting of multi-copies of 4-3 HR motifs that could be used as templates, in conjunction with addition of the unique functional domain(s) from the corresponding viruses, for designing novel antiviral peptides against HIV and other viruses with class I fusion proteins.

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