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Swapped domain constructs of the glycoprotein-41 ectodomain are potent inhibitors of HIV infection

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Abstract

The conformational rearrangement of N- and C-heptad repeats (NHR, CHR) of the HIV-1 glycoprotein-41 (gp41) ectodomain into a trimer of hairpins triggers virus – cell fusion by bringing together membrane-spanning N- and C-terminal domains. Peptides derived from the NHR and CHR inhibit fusion by targeting a prehairpin intermediate state of gp41. Typically, peptides derived from the CHR are low nM inhibitors, while peptides derived from the NHR are low μ M inhibitors. Here we describe the inhibitory activity of swapped domain gp41 mimics of the form CHR-loop-NHR, which were designed to form reverse hairpin trimers exposing NHR grooves. We observed low nM inhibition of HIV fusion in constructs that possessed the following properties: an extended NHR C-terminus, an exposed conserved hydrophobic pocket on the NHR, high helical content and trimer stability. Low nM activity was independent of CHR length. CD studies in membrane mimetic dodecylphosphocholine micelles suggested that bioactivity could be related to the ability of the inhibitors to interact with a membrane-associated prehairpin intermediate. The swapped domain design resolves the problem of unstable and weakly active NHR peptides, and suggests a different mechanism of action from that of CHR peptides in inhibition of HIV-1 fusion.

Introduction

Inhibition of HIV-1 fusion is an important therapeutic option for individuals infected with HIV, especially in cases of resistance or intolerance to standard antiretroviral drugs in the HAART regimen.¹ Peptides derived from the ectodomain of HIV transmembrane glycoprotein-41 (gp41) inhibit fusion by a dominant negative mechanism of action, in which they associate with the viral protein as it undergoes one or more conformational transitions, thereby halting progression of fusion.² Conformational transitions of gp41 terminate in a six-helix bundle,³ containing a 50-residue N-heptad repeat (NHR) coiled coil trimer and

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Supporting Information Available: Dose response titrations of hairpin constructs in a HXB2 viral infectivity assay. This material is available free of charge via the Internet at <http://pubs.acs.org>.

three antiparallel 39-residue C-heptad repeat (CHR) helices connected by a 34-residue loop.⁴ A schematic of the primary structure is shown in Figure 1. The FDA approved fusion inhibitor T20 (Enfuvirtide®) is a 36-residue segment of the CHR and membrane-proximal external region (MPER) of gp41.⁵ T20 and other CHR-peptides have high affinity for a prehairpin intermediate state of gp41⁶ and have low nM inhibitory activity against fusion. Increasing the helicity of CHR peptides through salt bridge substitutions is correlated with increased potency.^{7, 8} Potency is also a function of peptide length, and can be enhanced by addition of a hydrophobic moiety such as cholesterol or fatty acids, which are believed to concentrate inhibitor peptide at the membrane boundary.^{9, 10}

In contrast, NHR-peptides are typically μ M inhibitors of fusion,¹¹ presumably due to their propensity to aggregate and their failure to adopt a discrete trimeric state when excised from the full length protein.^{12, 13} Several constructs have been designed to stabilize NHR segments of the ectodomain, following the discovery of fusion inhibitory activity by gp41 peptides. These have included construction of 5-helix, a single polypeptide with alternating NHR and CHR segments and missing the CHR along one groove;¹⁴ fusion of NHR peptides to the thermostable six-helix bundle ectodomain¹⁵ or to a GCN4 trimer^{16, 17}; addition of interhelical disulfide bonds,¹⁸ or mutation of residues involved in the trimerization domain.¹² These various NHR constructs demonstrated increased potency, inhibiting HIV fusion with IC₅₀'s in the 15–50nM range. Low nM potency was also achieved by conjugating NHR peptides with fatty acids to orient them in the membrane.^{19, 20}

In this study, we have investigated the potency and mechanism of engineered swapped domain constructs in inhibition of HIV-1 fusion. These constructs contain an N-terminal CHR domain connected to a C-terminal NHR domain by a short loop (i.e. CHR-loop-NHR). The topology enables increasing lengths of the NHR groove to be exposed by successive truncation of the CHR. In a recent study, we described three constructs with CHR domains of length 39, 28 and 21 residues, and characterized their solution behavior and ability to bind to ligands directed to the exposed NHR segments. The results showed that the proteins spontaneously formed trimers and were excellent receptors for ligand-detected NMR experiments. Here we have made additional constructs and investigated the importance of the exposed pocket and the length of the CHR and NHR segments in antiviral potency. We have determined that constructs with a full length 50-residue NHR and exposed hydrophobic pocket are highly potent, low nM inhibitors. CD spectroscopy and equilibrium analytical ultracentrifugation (AUC) were used to evaluate the association between structural integrity and biological potency. In particular, the effect of a membrane mimetic on structure and stability was evaluated, given previous observations of the interaction between lipids and NHR, CHR or core domain structures.^{21–24} Our results are consistent with a mechanism in which the NHR is the primary inhibitory determinant, unlike forward hairpin constructs of the form NHR-loop-CHR, which had moderate activity dependent on transient exposure of the CHR.²⁵ Loss of the trimeric hairpin conformation in lipids may explain the reversibility of NHR-loop-CHR hairpin formation in the milieu of the fusion reaction. We will show that several of the swapped domain structures retain much of their helical content in lipids under physiological conditions and that transient CHR exposure is not the origin of their potency.

Results

Design of swapped domain constructs

To represent gp41, we prepared swapped domain constructs in which the CHR-domain precedes the NHR domain with a short connecting loop (Walsh et al, submitted). Figure 1 shows the primary structure, sequences and topology of the reverse hairpins in the context of full-length gp41. They present elements of the gp41 ectodomain in a manner similar to previously described core structures N36(L6)C34 and N34(L6)C28,^{26–28} but have the constraining loop at the opposite end. This topology was first demonstrated by Stewart et al as a way to expose the hydrophobic pocket for studies of drug interactions.^{29, 30} The constructs are named for the lengths of the gp41 CHR and NHR domains, and the intervening loop. C39(L4)N50, C28(L4)N50 and C21(L4)N50 all contain a full-length 50-residue NHR domain, and an increasingly pared back CHR domain exposing more of the hydrophobic groove of the NHR. Included in this groove is the well-known hydrophobic pocket (HP), which is a target for small molecules,^{31, 32} D-peptide inhibitors^{17, 33} and monoclonal antibodies.^{34, 35} The segment C39 contains the HP binding domain (HPbd) at its N-terminus. Constructs containing an abbreviated CHR (C28 and C21) were both designed to expose the HP. Two additional constructs C39(L4)N40 and C28(L4)N40 were made in which the NHR was terminated immediately after the HP, similar to many previous constructs used to evaluate gp41 activity and structure,^{14, 17, 36} including hairpins N34(L6)C28 and N36(L6)C34,^{27, 28} and the previously described reverse hairpin structure Protein-1.^{29, 30} N40-containing constructs contained 12 fewer residues at the C-terminus, including NHR residues A582-A591. We^{37, 38} and others³⁹ have determined that this 10 residue segment C-terminal to the pocket mediates important inter-domain interactions, and we therefore included it in our longer constructs. We additionally made C28(L4)N45, to probe this C-terminal segment while reducing fraying of the NHR helix at the hydrophobic pocket (unpublished results).

In the sequences shown in Figure 1, there are several helix stabilizing salt-bridge and alanine substitutions^{7, 40} on the outer solvent-exposed face of the helical CHR. All N50-containing constructs also contain two additional C-terminal amino acids KR, which are not part of the HIV sequence. Formic acid cleavage was used in C28- and C21-containing constructs to remove an N-terminal segment that was included to improve overexpression yields and solubility during purification, leaving an N-terminal proline. (Walsh et al, submitted) In the previous studies, we determined the trimeric state of C39(L4)N50, C28(L4)N50 and C21(L4)N50, and showed that HP and C-terminal binding grooves designed to be exposed were accessible to binding by peptides and small molecules in NMR studies.

Anti-fusion activity of swapped domain constructs

Activity testing in cell – cell (CCF) and virus – cell (VCF) fusion assays (Figure 2A, Table 1, Figure S1, Supplementary Information) revealed 10 nM inhibition by C28(L4)N50 and C21(L4)N50, and no evidence of inhibition by C39(L4)N50 or C39(L4)N40 up to 2000 nM, the highest concentration tested. In C28-containing constructs C28(L4)N50, C28(L4)N45 and C28(L4)N40, the successive truncation of the C-terminal end of the NHR resulted in loss of activity, with IC₅₀'s of 10 nM, 44 nM and 121 nM, respectively, against CCF, and 9

nM, 409 nM and > 2000 nM against VCF. None of the constructs tested exhibited any toxicity in cell viability assays. The reduced activity of C28(L4)N45 and C28(L4)N40 in VCF assays was attributed to a lack of stability over time, since VCF assays were conducted about two weeks after preparation of the samples. Diminished activity was also observed in later cell – cell fusion assays. CD results indicating poorly folded structure for C28(L4)N45 and C28(L4)N40 are in keeping with this observation (see below). By contrast C28(L4)N50 and C21(L4)N50 were stable over several months at pH 5.5, with reproducible fusion inhibitory activity and CD spectra.

Potent fusion inhibitory activity correlated with hydrophobic pocket exposure as determined by an ELISA with mAb D5, a known hydrophobic pocket binding monoclonal antibody.³⁴ Dose response titrations yielded concentration dependent signals for C28(L4)N50 and C21(L4)N50 but no response with C39(N4)L50 or with BSA used as a negative control. Results of the ELISA experiment are shown in Figure 2B, confirming that C28(L4)N50 and C21(L4)N50 were folded and contained the required HP binding surface. The absence of reactivity of D5 with C39(L4)N50 indicated that the CHR of the extended hairpin did not unfold to expose the hydrophobic pocket under the conditions of the assay.

Further confirmation of an exposed pocket under physiological conditions was obtained from observed antagonism between D-peptide PIE7⁴¹ and C28(L4)N50 when used in combination in a cell-cell fusion assay. The two inhibitors can bind to each other in addition to *in situ* prehairpin gp41. In the presence of D-peptide at 100 times the concentration of hairpin, IC₅₀^{CCF} of C28(L4)N50 increased by a factor of 2 (Fig 2C). Using the method of Chou and Talalay,⁴² we calculated the combination index to assess the synergistic, additive or antagonistic behavior of the inhibitors. A combination index > 1 was obtained (Figure 2D), indicating antagonism between C28(L4)N50 and PIE7. The concentration of PIE7 present at the halfway point caused minimal inhibition of fusion on its own (~10%), limiting the competing reaction of fusion inhibition by PIE7 itself. This suggests that PIE7 binding directly to C28(L4)N50 reduced the effective concentration of inhibitory protein, and points to the exposed NHR as the active inhibitory component.

Inactivity of C39(L4)N50 contrasts with results obtained for the hairpin constructs N36(L6)C34 and N34(L6)C28, which had IC₅₀'s of 217nM and 1500nM, respectively, in a cell-cell fusion assay.^{26, 43} Mutagenesis studies on N36(L6)C34 suggested that the observed potency was due to transiently exposed CHR under physiological conditions, despite the high thermal stability of the hairpin (T_M=76°C) in PBS. Later, observation of hairpin destabilization in the presence of lipids^{19, 23, 44} suggested a mechanism by which CHR exposure could occur in the conditions of cell culture, given the close association of gp41 with membranes. It is likely that the strong HP - HPbd interactions in C39(L4)N50 clasp the two ends of the hairpin together and prevent unraveling of the CHR. In support of this premise, CD and AUC findings presented below and in Walsh et al (submitted) demonstrated an exclusively trimeric and highly stable structure (T_m > 100°C), with enhanced helicity in lipids.

Oligomeric state and secondary structure of swapped domain constructs

We explored whether secondary structure determined by CD spectroscopy and oligomeric state determined by AUC correlated with the biological studies indicating NHR as the functional inhibitor, with high potency requiring a 28- or 21-residue CHR and 50-residue long NHR.

C39-containing constructs, which exhibited no measurable bioactivity, were found to be highly helical, extremely stable and fully trimeric in CD and AUC experiments. Truncating the NHR from 50 to 40 residues did not alter the trimer state. We previously examined C39(L4)N50 in AUC experiments, and established the trimeric structure (Walsh et al, submitted). Here we examined C39(L4)N40 under similar conditions at three concentrations (48, 24 and 12 μ M) at pH 5.5. Figure 3 shows the AUC data for C39(L4)N40 at 12 μ M; a global fit of the data over 5 speeds gave a molecular weight of 29.8 kDa for C39(L4)N40 (calc. 15 N-labeled trimer 29694). Similar results at 24 and 48 μ M gave no evidence of aggregation. Thus a 40-residue long NHR appears to be sufficient to stabilize the trimer state.

CD studies of C39(L4)N50 at pH 5.5 in 50 mM MES, 50 mM KCl buffer (Figure 4A, Table 2) revealed a helical content of 85%, which increased to 100% in the presence of 10mM dodecylphosphocholine (DPC), a membrane mimetic solvent forming micelles at a CMC $\sim$ 1mM.⁴⁵ In PBS at pH 7.4, both C39(L4)N50 and C39(L4)N40 were 97% helical, and both increased to 100% helix upon addition of 10mM DPC to the PBS. Earlier studies on gp41 hairpins were conducted at both pH 4 and pH 6 with no significant differences observed.²³ The pH sensitivity observed here could be attributed to greater stability of salt bridges built into the CHR at neutral pH, or to aggregation at the higher pH.

No unfolding transition was observed up to 90°C for C39(L4)N50 in a CD melt (Figure 4B, Table 3), in either the presence or absence of DPC, with helix stabilization by DPC maintained throughout. The extreme stability of the helix bundle is likely to account for the inability of C39(L4)N50 and C39(L4)N40 to inhibit fusion. Stabilization of C39(L4)N50 by DPC micelles differed from the 23% loss of helicity observed for N34(L6)C28 in DPC at 37°C.²³ Absence of a measurable melting transition was in contrast to N34(L6)C28 and N36(L4)C34 which unfolded at 70°C and 79°C, respectively.^{25, 28} These factors may explain the ability of the “forward” hairpins to inhibit fusion. Biophysical²³ and mutagenesis²⁵ data pointed to the formation of a monomeric unfolded state of N34(L6)C28 and N36(L4)C34 under physiological conditions or in lipids. In the unfolded state, C-peptide becomes exposed and can inhibit fusion. Unzippering of C-peptide from its C- to N-terminal end is prevented in C39(L4)N50 by the constraining loop, and fraying from the N-terminal end is not observed, as NMR data shows hydrophobic-pocket covering residues to be helical (unpublished results). Fraying is likely restricted by the strong HP – HPbd interactions.

C28- and C21-containing constructs were dependent on NHR length and independent of CHR length for both bioactivity and helical structure. C28(L4)N50 and C21(L4)N50 were 81% and 80% helical at 20°C and pH 5.5, confirming that high potency was correlated to a well-formed structure and exposed HP (Figures 5A, 6A, Table 2). The ratio of $\theta_{222}/\theta_{208}$ in the spectra was $\sim$ 1, a measure of coiled coil and helix bundle stability.^{46, 47} Structural

stability is compatible with the evidence of trimeric NHR as the active fusion inhibitory component. AUC data presented previously (Walsh et al, submitted) also confirmed the trimer state. C21(L4)N50 was 87% helical at pH 7.4, but 20 μ M C28(L4)N50 was insoluble at pH 7.4. Melting curves are shown in figure 5B, and 6B for C28(L4)N50 and C21(L4)N50 in MES buffer at pH 5.5, and in buffer containing 10mM DPC. A very similar melting curve was obtained for C21(L4)N50 in PBS. The curves were fit to equation 3 (Materials and Methods), which describes a simple two state transition⁴⁹ and assumes no change in heat capacity between the folded and unfolded states, based on previous observations for gp41 hairpin unfolding.⁵⁰ Calculated data were adjusted for the observed linear dependence on temperature before and / or after the transition, due to gradual fraying of existing structure. Neither construct underwent a complete unfolding transition in the range 20 – 90°C in buffer, although C21(L4)N50 reached close to the halfway point of the transition and could be fit well assuming a value between 0 and –5000 deg.cm².dmol^{–1} for the mean residue ellipticity of the unfolded state, $\theta_{U,MRE}$. C28(L4)N50 was more stable than C21(L4)N50 with both peptides having transition midpoints (T_M) over 90°C. T_M 's of ~112 and ~97°C were estimated (Table 3). The high enthalpy of unfolding $H_F = -30 - -37$ kcal/mol was indicative of a fairly stable folded state.⁵¹

DPC had an deceptively small effect on helical content of these highly bioactive constructs at 20°C, causing a 6% increase in C28(L4)N50 at pH 5.5 and a 1% or 9% decrease in C21(L4)N50 at pH 7.4 or pH 5.5 respectively. But the static single temperature measurement masked the very large effect that DPC had on the structural stability of these proteins, which was demonstrated in temperature melts. 10mM DPC caused a significant reduction in T_M , which occurred at 74°C for C28(L4)N50 and 45°C for C21(L4)N50, clear evidence of a strong interaction between the surfactant and hairpins. The melting transition of C21(L4)N50 was complete, allowing for fitting of the beginning and end points in addition to T_M and the enthalpy of folding. It was not possible to fit the melting curves using an end point of $\theta_{U,MRE} = 0 - -5000$ deg.cm².dmol^{–1}. Clear evidence of residual structure was obtained, both from the calculated ellipticity after the transition (Table 3) and from the continued linear dependence of ellipticity on temperature after the transition (Figure 6B). From the calculated ellipticity, two thirds of the helical structure remained after the transition in both proteins. It is possible that the trimeric NHR is still intact in DPC,⁴⁴ although an alternative explanation is that monomeric NHR and CHR exist in a helical conformation in DPC micelles.²³

The helical state, and correspondingly the bioactivity, was significantly impaired with NHR truncation of swapped domain constructs containing a 28-residue CHR. C28(L4)N45 and C28(L4)N40 were 22% and 18% helical respectively. Since C39(L4)N40 was 97% helical and trimeric, suggesting that a 40-residue NHR interface could maintain structural integrity, it is evident that full length CHR was required to stabilize the structure. Absence of the HPbd resulted in unfolding of the oligomeric state. It is likely that this interaction in *in situ* gp41 helps to stabilize the six helix bundle at the end of the fusion reaction.

Discussion

We have described swapped domain gp41 ectodomain proteins with the CHR preceding the NHR in sequence, separated by a short loop. Biophysical characterization indicated that the constructs formed reverse hairpins with varying degrees of stability, which correlated to their potency as HIV-1 fusion inhibitors. The reverse hairpin design allowed us to probe the contributions of NHR, CHR and structural integrity in fusion inhibition. C39(L4)N50 contained full length NHR and CHR domains, while C39(L4)N40 was missing the 10-residue C-terminal end of the NHR, a region which we consider to be very important in the thermodynamics of six helix bundle formation.³⁷ C28(L4)N50, C28(L4)N45 and C28(L4)N40 contained a CHR shortened at the N-terminal end, which would expose the highly conserved hydrophobic pocket formed upon trimerization. Differing lengths of NHR were probed to evaluate the importance of its C-terminal end. C21(L4)N50 contained a CHR further pared back at its N-terminus, exposing more of the hydrophobic groove of the trimer. We have found that these constructs behave very differently from isolated NHR or CHR peptides or from “forward” hairpin mimics of the gp41 ectodomain. Uniquely, C28(L4)N50 and C21(L4)N50 were low nM inhibitors of fusion, directly consequent to their stability and trimeric state, and to exposure of the conserved hydrophobic groove.

Hairpin trimerization depended on both NHR and CHR length

AUC experiments in this and a previous publication demonstrated >90% trimer state for C39(L4)N50, C39(L4)N40, C28(L4)N50 and C21(L4)N50. In CD experiments C39(L4)N50 and C39(L4)N40 were between 85 – 97% helical at pH 5.5 and pH 7.4, with higher helicity observed at the higher pH; C28(L4)N50 was 81% helical at pH 5.5 and C21(L4)N50 was 80 or 87% helical at pH 5.5 or 7.4. There was no evidence of an unfolding transition for C39(L4)N50 up to 90°C. C28(L4)N50 and C21(L4)N50 had unfolding transitions with calculated $T_M \sim 112^\circ\text{C}$ and $\sim 97^\circ\text{C}$, respectively. High helicity and trimer stability have been observed previously for the forward hairpins N36(L4)C34 and N34(L4)C28.^{26, 28, 52} Unfolding transitions were at 79°C and 70°C . The stability of the reverse hairpin structure was significantly impacted by a reduction in the length of the NHR, unless the full length C39 CHR was retained. Thus we observed minimal secondary structure for C28(L4)N45 and C28(L4)N40, while in C39(L4)N40, 97% helix and 100% trimer state was retained. It is therefore evident that HP – HPbd binding interactions had a significant effect on trimer and six helix bundle stability. The interactions occurring in the hydrophobic pocket are vital for stabilizing the conformation of post-fusion gp41, as it transitions from an open conformation with exposed NHR and CHR into the six-helix bundle. This confirms the hydrophobic pocket as a very important target for inhibition of six helix bundle formation.

Domain-swapped constructs uniquely presented NHR as a key determinant of antiviral potency

The requirement for high potency against cell – cell fusion and viral infectivity was existence of stable trimer in which the CHR was pared back to expose the highly conserved hydrophobic pocket region. This structure was achieved in reverse hairpins C28(L4)N50 and C21(L4)N50. In addition to the biophysical evidence of trimer stability and exposed hydrophobic grooves, presented above and in NMR studies of ligand binding interactions

(Walsh et al, submitted), HP epitope presentation was confirmed by an ELISA with monoclonal antibody D5. Furthermore, antagonism between C28(L4)N50 and the hydrophobic pocket binding D-peptide PIE7 in a fusion assay confirmed that these two entities were associating with each other during the fusion reaction. C28(L4)N50 and C21(L4)N50 were 10 nM inhibitors of HIV fusion. C39(L4)N50 and C39(L4)N40, which contain a full length CHR (C39) covering the hydrophobic pocket, were inactive up to the highest measured concentration of 2000 nM. C28(L4)N45 and C28(L4)N40 were mid- to high nM inhibitors which lost potency over time, in accordance with their lack of well-defined secondary structure.

These observations set the reversed hairpins apart from isolated NHR and CHR peptides or forward hairpins N36(L4)C34 and N34(L6)C28, as well as from previous constructs designed to present NHR as the inhibitory determinant. Moderate activity and length dependence was found for isolated N-peptides, with N36, N44 and N51 having IC_{50} 's of 16 μ M, 0.7 μ M and 0.5 μ M respectively^{12, 15, 53}. C28(L4)N50 and C21(L4)N50 are more than an order of magnitude more potent than N51. Isolated CHR peptide DP,⁵⁴ encompassing the same C-peptide region as C28(L4)N50 had an IC_{50} = 2.9mM, two orders of magnitude less potent than C28(L4)N50. A shorter C-peptide, such as that in C21(L4)N50 would be expected to be even weaker than DP as an isolated peptide. Meanwhile, forward hairpins N36(L6)C34 and N34(L6)C28²⁸ were found to have residual activity 0.2 – 1.5 μ M due to transient exposure of the CHR.²⁵ C39(L4)N50 and C39(L4)N40 have no activity, since they do not unfold, even transiently, while C28(L4)N50 and C21(L4)N50 are 20 – 150 times more potent than the forward hairpins.

It is obvious that the combination and topology of N- and C-peptides in the reverse hairpins is critical for the observed potency. The combined effect is greater than the sum of the parts. They demonstrate a new paradigm for inhibition, which appears to rely principally on NHR, but which must involve the CHR to some extent, although truncation of CHR length had no apparent effect on potency. It is possible that the C-terminal domain increases bioavailability or helps to direct the inhibitors to the site of action. It is also possible that weak C-peptide binding to a nearby NHR domain in intact gp41 has a multiplicative effect on the binding affinity and potency of the NHR to *in situ* CHR. Equipotency of C21(L4)N50 and C28(L4)N50 may be a serendipitous outcome of weaker binding of C21 but increased sampling of the active open conformation of C21(L4)N50 in lipids, given its lower T_M .

Exposed NHR interacted strongly with lipids

The structural stability of the protein inhibitors in solution may not adequately reflect properties that are important in the fusion reaction. This is exemplified by the discrepancy between high bundle stability in N36(L6)C34, T_M = 79°C, and N34(L6)C28, T_M = 70°C, and fusion inhibition due to exposed C-peptide.^{25, 26} The transient exposure of C-peptide may be linked to adoption of a monomeric or CHR-dissociated form in lipids,^{23, 55} relevant to the environment of *in situ* gp41. We therefore examined the effect of a lipid environment on the reverse hairpin structures. Indeed, unfolding in lipids was not a factor for C39(L4)N50, which was stabilized into a 100% helical structure in the presence of DPC, and which failed to exhibit any unfolding transition in either the presence or absence of

DPC. This is consistent with the lack of bioactivity of C39(L4)N50, and likely reflects the strong HP – HPbd interactions at one end and the loop at the other end in preventing unraveling of the hairpin. These observations confirm models which suggest that the final six-helix bundle conformation of gp41 is completely stable in lipids,^{21, 22} and that partitioning into the membrane phase during pore formation⁵⁶ is compatible with structural stability.

C28(L4)N50 and C21(L4)N50 displayed T_M values of 74 and 45°C, respectively, when DPC was present, 38°C or 52°C lower than in buffer alone. Spontaneous refolding occurred under all conditions tested, with identical CD spectra at 20°C before and after the melt. Clearly stability in DPC decreased as C-peptide size decreased, even while the NHR length remained the same, although we did not observe a correlation between the T_M in lipids and biological activity. The dramatic effect of DPC suggests that the exposed NHR grooves may interact strongly with lipids, quite possibly reflecting an important physiological property of the inhibitors. Lipophilic interactions may be contributing to inhibitory ability while targeting a gp41 intermediate that is assembled at the membrane interface around the nascent fusion pore.^{23, 55} The observation of 67% residual helicity in C28(L4)N50 and C21(L4)N50 after the thermal transition in DPC micelles suggests that they did not unfold completely, but rather adopted a conformation which may mirror an intermediate in the fusion process. It is possible that this conformation contains an unfolded CHR while NHR remains trimeric, which would explain the T_M dependence on CHR length. The fact that potency correlated with trimer stability suggests that the active biological form of C28(L4)N50 and C21(L4)N50 is a trimer. If inhibition is occurring at the membrane boundary, it implies that these structures maintain trimer integrity in that milieu. The large reduction in T_M with DPC indicates that the active biological form would be sampled much more frequently in lipids, lending support to models in which the conformational rearrangement of gp41 is impacted by the membrane.

The extended C-terminus of the NHR was an essential structural and inhibitory determinant

The extended NHR C-terminal segment, composed of gp41 (HXB2) residues 582–591, is responsible for the added trimer stability, and consequently the potent bioactivity. Previous constructs designed to stabilize the NHR trimer^{14–16, 18} have been successful at improving potency from μM to nM levels, although many are more complex and larger protein inhibitors. In these previous constructs, the extended C-terminal segment was not included.

Conclusion

We have demonstrated that reverse hairpins with exposed hydrophobic pockets, stabilized along an extended trimer interface, are low nM inhibitors of HIV fusion. The results show that the canonical hydrophobic pocket (residues L565-L581) plus a C-terminal extension not normally considered to be part of the pocket (residues A582-Q591) both contribute to potency, and are likely to provide a high affinity binding surface for constituent C-peptide segments of *in situ* gp41. Clear correlations were observed between trimer integrity and bioactivity, and between NHR groove exposure and bioactivity. Evidence for retention of

significant structure in lipids was obtained. For constructs with an exposed NHR groove, no direct correlation was observed between CHR length and bioactivity, or between the midpoint of the melting transition in DPC and bioactivity. However, thermodynamic and kinetic effects associated with CHR length and accessibility of the open NHR groove may have combined to produce this effect. The results show a novel mechanism of fusion inhibition, in which activity of the swapped domain CHR-loop-NHR entities is greater than the sum of their parts.

The reverse hairpins are not artificially stabilized by covalent bonds, interchain disulfides or multidomain constructs, and are smaller than previous NHR constructs designed to target the CHR. They are likely to have a different resistance profile from T20 and other C-peptides that target the NHR, and could prove useful for resistant strains that develop against C-peptides. They are useful as receptors for investigating low molecular weight inhibitors binding to the NHR. Furthermore, the reverse hairpins provide insight into mechanisms of the gp41 conformational changes that occur during fusion, including how hairpins might unravel, the stabilizing effect of the hydrophobic pocket interactions, including in lipids, and the eventual stabilization of the hairpin in its final six-helix bundle form through associations of fusion peptide at the N-terminus of the NHR with the transmembrane domain at the C-terminus of the CHR.⁵⁸ Detailed tertiary structure and dynamics of the proteins in the membrane environment remain to be fully explored, as our understanding of the coupling between protein folding and membrane fusion evolves.

Materials and Methods

Materials—PIE7 was obtained from Biosynthesis Inc. (Lewisville, TX) and oxidized in air at pH 7.0 for 2 days. All chemicals were used as ordered without further purification.

Protein Expression and Purification—Details of protein design, expression and purification are described elsewhere (Walsh et al, submitted). Additionally, several new protein constructs with truncated NHR (N40, N45) are described here. Briefly, proteins were prepared as precursors containing a completely covered hydrophobic pocket, in order to avoid accumulation in the insoluble fraction during overexpression in *E. coli*. Constructs contained an N-terminal His tag for extraction of the desired protein from the supernatant after bacterial cell lysis. The His tag was subsequently cleaved either using TEV protease to produce constructs that included a hydrophobic pocket binding domain, or by chemical cleavage with formic acid to produce constructs with an exposed hydrophobic pocket. Formic acid cleaved specifically after aspartic acid in the sequence -Asp-Pro- resulting in N-terminal proline in the final constructs prepared by this method. Proteins were further purified after cleavage by a second nickel column to remove the His-tagged fragment, concentrated, and purified by size exclusion chromatography over a Superdex 75 1660 column (GE Healthcare). Purity was verified by SDS-PAGE, and protein molecular weight was determined by MALDI or Electrospray mass spectrometry. Concentrated stocks for biological assays were kept at pH 3 in 50mM formate buffer or at pH 5.5 in 10mM MES, 50mM Arginine base, 50mM Glutamic acid, 0.02% NaN₃.

Cell-cell fusion assay—Cell-cell fusion was measured as previously published⁵⁹ using cell lines obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH. Target cells were TZM-bl cells (contributed by J.C. Kappes, X. Wu and Tranzyme Inc.) expressing CD4, CCR5 and CXCR4,⁶⁰ and containing an integrated reporter gene for firefly luciferase under control of HIV-1 LTR.⁶¹ Effector cells were HL2/3 (contributed by B.K. Felber and G.N. Pavlakis) which produce HXB2 Env, Tat and Rev.⁶² 20,000 TZM-bl cells per well were cultured overnight in 96 well plates, followed by addition of serially diluted inhibitors and 40,000 HL2/3 cells per well. Fusion was allowed to proceed for 6 hours in 100µl reduced serum medium (OptiMEM, Gibco, Life Technologies,) per well. The supernatant was aspirated, and cells were lysed with lysis buffer (Luciferase Cell Culture Lysis 5X reagent, Promega). Luciferase expression was measured using Luciferase Assay Reagent (Promega) according to the manufacturer's instructions. Controls without inhibitor and with and without HL2/3 cells were measured for each inhibitor. Experiments were performed in triplicate and repeated 3 – 10 times for each inhibitor.

Cell viability assay—Cell viability was measured using a resazurin cell viability reagent (Alamar Blue or Presto Blue, Invitrogen, Life Technologies,) following the manufacturers' protocols. 10µl of resazurin reagent was added to each well ½ – 1 hour before the end of the experiment. 80µl of supernatant was withdrawn at the end of the experiment, prior to cell lysis, and fluorescence measured (ex 544nm, em 590nm). Decreased fluorescence was indicative of reduced viability. Upper and lower bounds were determined using supernatants from the cell fusion reaction in the absence of inhibitor, and from wells in which 1% Triton-X-100 was added with the HL2/3 cells, respectively.

Virus-cell fusion assay—U87.CD4.CXCR4 cells were obtained through the NIH AIDS Reagent Program, supplied by Dr. HongKui Deng and Dr. Dan R. Littman.⁶³ Cells were maintained and propagated in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Life Technologies) supplemented with 10% fetal bovine serum (FBS, Amresco), 100 I.U penicillin, 100µg/ml streptomycin (Gibco, Life Technologies), 2mM glutamine, 300 µg/ml G418 Sulfate (Gold Bio) and 1 µg/ml puromycin (Sigma). 293T cells were obtained from E. Hermel and propagated in DMEM containing 2mM L-glutamine, 1x MEM non essential amino acids, 20mM HEPES (Gibco, Life Technologies), 10% FBS.

Pseudotyped virus expressing HXB2 Env was prepared by cotransfecting 293T cells with the plasmids pNL-Luc-AM, obtained from Dr. John Moore at Cornell University, and pSM-HXB2-WTgp160, obtained from Dr. Carol Weiss at the FDA, using Fugene HD transfection reagent (Promega), according to the manufacturer's directions. Media was changed at 16 – 18 hours, and pseudovirions in the supernatant were harvested 48 hours post-transfection. The supernatant was passed through a 0.45µm filter, and frozen in 100 – 500 µl aliquots at –80°C until required. Virus was quantified in an ELISA using p24 antigen (Prospec Bio) and Mab to HIV p24 (NIH AIDS Reagent Program). Virus concentrations used in neutralization assays were selected by dose response dilution of virus stock, to obtain a luciferase readout with a dynamic range of at least 200. This typically corresponded to the use of ~ 40 virus particles per target cell.

Virus infectivity assays were performed by plating 1.5×10^4 U87.CD4.CXCR4 cells/well and incubating overnight at 37°C, 5% CO₂. The supernatant was aspirated and replaced with 50µl OptiMEM containing 3-fold serially diluted inhibitors, pseudovirions at the predetermined concentration and 8µg/ml DEAE dextran (Sigma). Cells were incubated overnight at 37°C after which media was replaced with DMEM supplemented as indicated for U87 cells. After 24 hours, cells were lysed with lysis buffer (Luciferase Cell Culture Lysis 5x reagent, Promega), and luciferase activity was assayed using Luciferase Assay System (Promega) according to the manufacturer's directions.

ELISA—ELISA for the hydrophobic pocket epitope was conducted with D5 antibody, obtained from the NIH AIDS Reagent Program. Briefly, a 96-well flat bottomed ELISA plate (Thermo Fisher Scientific) was coated with 50µl/well reverse hairpin protein or BSA starting at 5µg/ml with 3 fold dilutions down the plate, in 50mM carbonate buffer pH 9.6 at 4°C overnight. The plate was washed twice with wash buffer (154mM NaCl, 0.05% Triton X-100) and blocked with PBS+5% milk at RT for 1 h. 50µl/well D5 antibody (0.85ng/µl in PBS+5% milk) was added to the plate and incubated at RT for 1h, followed by six washes. Wells were then incubated with HRP-conjugated goat anti-mouse IgG (0.1ng/µl in PBS+5% milk, 50µl/well), for 1 h at RT followed by six washes. To detect bound antibodies, 50µl HRP substrate 3,3',5,5'-tetramethylbenzidine (TMB, BioFfx) was added and the reaction stopped after 15 minutes with 50µl 1M H₂SO₄. Absorbance at 450 nm was measured on a plate reader (SpectraMax M5, Molecular Devices).

CD studies—Circular dichroism (CD) studies were performed on a Jasco 715 spectropolarimeter, equipped with a Peltier temperature controller, using 20 µM protein samples in a 2mm pathlength cuvette in either PBS at pH 7.4 or 10 mM MES buffer, 50 mM KCl, pH 5.5. Scans were conducted with a 1nm increment and a scan speed of 8 nm/min. CD melts were performed with 0.5°C increments at a rate of 2°C per minute. The data from CD melts were fit assuming a simple two state transition from folded to unfolded form, assuming the heat capacity of folded and unfolded states are equal.⁴⁹ The calculated data were corrected for observed pre- or post-transition linear changes in the ellipticity as a function of temperature.

The folded to unfolded transition $F \rightleftharpoons U$, has an equilibrium folding constant

$$K = [F] / [U] = \exp(-\Delta G_F / RT), \quad (1)$$

where ΔG_F is the folding free energy. Assuming no change in heat capacity,

$$\Delta G_F = \Delta H_F * (1 - T/T_M), \quad (2)$$

where T_M is the temperature at the midpoint of the transition and ΔH_F is the enthalpy of folding. The observed ellipticity as a function of temperature was fit to the equation

$$\theta_{\text{obs}} = \theta_U + k / (1 + K) * (\theta_F - \theta_U). \quad (3)$$

The parameters ΔH_F , T_M , and the ellipticity of folded and unfolded forms, θ_F and θ_U , were obtained from the fit. A pretransition linear correction was applied to the calculation of the

melting curves for C28(L4)N50 both with and without DPC. For C21(L4)N50, a pretransition linear correction was applied to the calculated data in the absence of DPC and a post-transition linear correction when DPC was present.

Analytical Ultracentrifugation—Sedimentation equilibrium studies were performed on C39(L4)N40 at concentrations of 48, 24 and 12 μ M in MES buffer, pH 5.5, containing 50mM arginine base and 50mM glutamic acid, at speeds of 25000, 30000, 35000, 40,000 and 45000 rpm in a Beckman XL-I ultracentrifuge at 25°C. Data from the five speeds for each sample were fit globally to a single species model of equilibrium sedimentation by a non-linear least squares method using Igor Pro.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Gp41	glycoprotein-41
NHR	N-heptad repeat
CHR	C-heptad repeat
HP	Hydrophobic pocket
HPbd	HP binding domain
CCF	cell – cell fusion
VCF	virus – cell fusion

References

1. Sabin CA, Hill T, Lampe F, Matthias R, Bhagani S, Gilson R, Youle MS, Johnson MA, Fisher M, Scullard G, Easterbrook P, Gazzard B, Phillips A. Treatment exhaustion of highly active antiretroviral therapy (HAART) among individuals infected with HIV in the United Kingdom: multicentre cohort study. *Br. Med. J.* 2005; 330:695–699. [PubMed: 15749728]
2. Naider F, Anglist J. Peptides in the treatment of AIDS. *Curr. Opin. Struct. Biol.* 2009; 19:473–482. [PubMed: 19632107]
3. Markosyan RM, Cohen FS, Melikyan GB. HIV-1 envelope proteins complete their folding into six-helix bundles immediately after fusion pore formation. *Mol. Biol. Cell.* 2003; 14:926–938. [PubMed: 12631714]
4. Caffrey M, Cai M, Kaufman J, Stahl SJ, Wingfield PT, Covell DG, Gronenborn AM, Clore GM. Three-dimensional solution structure of the 44 kDa ectodomain of SIV gp41. *EMBO J.* 1998; 17:4572–4584. [PubMed: 9707417]

5. Wild C, Greenwell T, Shugars D, Rimsky-Clarke L, Matthews T. The inhibitory activity of an HIV type 1 peptide correlates with its ability to interact with a leucine zipper structure. *AIDS Res. Hum. Retroviruses*. 1995; 11:323–325. [PubMed: 7786578]
6. Kilgore NR, Salzwedel K, Reddick M, Allaway GP, Wild CT. Direct evidence that C-peptide inhibitors of human immunodeficiency virus type 1 entry bind to the gp41 N-helical domain in receptor-activated viral envelope. *J Virology*. 2003; 77:7669–7672. [PubMed: 12805467]
7. Dwyer JJ, Wilson KL, Davison DK, Freil SA, Seedorff JE, Wring SA, Tvermoes NA, Matthews TJ, Greenberg ML, Delmedico MK. Design of helical, oligomeric HIV-1 fusion inhibitor peptides with potent activity against enfuvirtide-resistant virus. *Proc. Natl. Acad. Sci. U. S. A.* 2007; 104:12772–12777. [PubMed: 17640899]
8. Naito T, Izumi K, Kodama E, Sakagami Y, Kajiwara K, Nishikawa H, Watanabe K, Sarafianos SG, Oishi S, Fujii N, Matsuoka M. SC29EK, a peptide fusion inhibitor with enhanced alpha-helicity, inhibits replication of human immunodeficiency virus type 1 mutants resistant to enfuvirtide. *Antimicrob. Agents Chemother.* 2009; 53:1013–1018. [PubMed: 19114674]
9. Peisajovich SG, Gallo SA, Blumenthal R, Shai Y. C-terminal octylation rescues an inactive T20 mutant: implications for the mechanism of HIV/SIMIAN immunodeficiency virus-induced membrane fusion. *J. Biol. Chem.* 2003; 278:21012–21017. [PubMed: 12646555]
10. Ingallinella P, Bianchi E, Ladwa NA, Wang YJ, Hrin R, Veneziano M, Bonelli F, Ketas TJ, Moore JP, Miller MD, Pessi A. Addition of a cholesterol group to an HIV-1 peptide fusion inhibitor dramatically increases its antiviral potency. *Proc. Natl. Acad. Sci. U. S. A.* 2009; 106:5801–5806. [PubMed: 19297617]
11. Wild C, Oas T, McDanal C, Bolognesi D, Matthews T. A synthetic peptide inhibitor of human immunodeficiency virus replication: Correlation between solution structure and viral inhibition. *Proc. Natl. Acad. Sci.* 1992; 89:10537–10541. [PubMed: 1438243]
12. Dwyer JJ, Wilson KL, Martin K, Seedorff JE, Hasan A, Medinas RJ, Davison DK, Feese MD, Richter HT, Kim H, Matthews TJ, Delmedico MK. Design of an engineered N-terminal HIV-1 gp41 trimer with enhanced stability and potency. *Protein Sci.* 2008; 17:633–643. [PubMed: 18359857]
13. Zhuang M, Wang W, De Feo CJ, Vassell R, Weiss CD. Trimeric, coiled-coil extension on peptide fusion inhibitor of HIV-1 influences selection of resistance pathways. *J. Biol. Chem.* 2012; 287:8297–8309. [PubMed: 22235115]
14. Root MJ, Kay MS, Kim PS. Protein design of an HIV-1 entry inhibitor. *Science*. 2001; 291:884–888. [PubMed: 11229405]
15. Louis JM, Bewley CA, Clore GM. Design and Properties of NCCG-gp41, a Chimeric gp41 Molecule with Nanomolar HIV Fusion Inhibitory Activity. *J. Biological Chemistry*. 2001; 276:29485–29489.
16. Weissenhorn W, Calder LJ, Dessen A, Laue T, Skehel JJ, Wiley DC. Assembly of a rod-shaped chimera of a trimeric GCN4 zipper and the HIV-1 gp41 ectodomain expressed in *Escherichia coli*. *Proc. Natl. Acad. Sci. U. S. A.* 1997; 94:6065–6069. [PubMed: 9177169]
17. Eckert DM, Malashkevich VN, Hong LH, Carr PA, Kim PS. Inhibiting HIV-1 entry: discovery of D-peptide inhibitors that target the gp41 coiled-coil pocket. *Cell*. 1999; 99:103–115. [PubMed: 10520998]
18. Louis JM, Nesheiwat I, Chang L, Clore GM, Bewley CA. Covalent Trimers of the Internal N-terminal Trimeric Coiled-coil of gp41 and Antibodies Directed against Them are Potent Inhibitors of HIV Envelope-mediated Cell Fusion. *J. Biol. Chem.* 2003; 278:20278–20285. [PubMed: 12654905]
19. Wexler-Cohen Y, Shai Y. Membrane-anchored HIV-1 N-heptad repeat peptides are highly potent cell fusion inhibitors via an altered mode of action. *PLoS Pathog.* 2009; 5:e1000509. [PubMed: 19593361]
20. Wexler-Cohen Y, Ashkenazi A, Viard M, Blumenthal R, Shai Y. Virus-cell and cell-cell fusion mediated by the HIV-1 envelope glycoprotein is inhibited by short gp41 N-terminal membrane-anchored peptides lacking the critical pocket domain. *FASEB J.* 2010; 24:4196–4202. [PubMed: 20605950]

21. Lev N, Fridmann-Sirkis Y, Blank L, Bitler A, Epand RF, Epand RM, Shai Y. Conformational stability and membrane interaction of the full-length ectodomain of HIV-1 gp41: implication for mode of action. *Biochemistry (Mosc)*. 2009; 48:3166–3175.
22. Sackett K, Nethercott MJ, Shai Y, Weliky DP. Hairpin folding of HIV gp41 abrogates lipid mixing function at physiologic pH and inhibits lipid mixing by exposed gp41 constructs. *Biochemistry (Mosc)*. 2009; 48:2714–2722.
23. Roche J, Louis JM, Grishaev A, Ying J, Bax A. Dissociation of the trimeric gp41 ectodomain at the lipid-water interface suggests an active role in HIV-1 Env-mediated membrane fusion. *Proc. Natl. Acad. Sci. U. S. A.* 2014; 111:3425–3430. [PubMed: 24550514]
24. Cai L, Gochin M, Liu K. Biochemistry and biophysics of HIV-1 gp41 - membrane interactions and implications for HIV-1 envelope protein mediated viral-cell fusion and fusion inhibitor design. *Curr Top Med Chem*. 2011; 11:2959–2984. [PubMed: 22044229]
25. Markosyan RM, Ma X, Lu M, Cohen FS, Melikyan GB. The mechanism of inhibition of HIV-1 env-mediated cell-cell fusion by recombinant cores of gp41 ectodomain. *Virology*. 2002; 302:174–184. [PubMed: 12429526]
26. Shu W, Liu J, Ji H, Radigen L, Jiang S, Lu M. Helical interactions in the HIV-1 gp41 core reveal structural basis for the inhibitory activity of gp41 peptides. *Biochemistry (Mosc)*. 2000; 39:1634–1642.
27. Lu M, Kim PS. A trimeric structural subdomain of the HIV-1 transmembrane glycoprotein. *J. Biomol. Struct. Dyn.* 1997; 15:465–471. [PubMed: 9439994]
28. Lu M, Ji H, Shen S. Subdomain folding and biological activity of the core structure from human immunodeficiency virus type 1 gp41: implications for viral membrane fusion. *J. Virol.* 1999; 73:4433–4438. [PubMed: 10196341]
29. Stewart KD, Steffy K, Harris K, Harlan JE, Stoll VS, Huth JR, Walter KA, Gramling-Evans E, Mendoza RR, Severin JM, Richardson PL, Barrett LW, Matayoshi ED, Swift KM, Betz SF, Muchmore SW, Kempf DJ, Molla A. Design and characterization of an engineered gp41 protein from human immunodeficiency virus-1 as a tool for drug discovery. *J. Comput. Aided Mol. Des.* 2007; 21:121–130. [PubMed: 17294246]
30. Stewart KD, Huth JR, Ng TI, McDaniel K, Hutchinson RN, Stoll VS, Mendoza RR, Matayoshi ED, Carrick R, Mo H, Severin J, Walter K, Richardson PL, Barrett LW, Meadows R, Anderson S, Kohlbrenner W, Maring C, Kempf DJ, Molla A, Olejniczak ET. Non-peptide entry inhibitors of HIV-1 that target the gp41 coiled coil pocket. *Bioorg. Med. Chem. Lett.* 2010; 20:612–617. [PubMed: 20004576]
31. Zhou G, Sofiyev V, Kaur H, Snyder BA, Mankowski MK, Hogan PA, Ptak RG, Gochin M. Structure-Activity Relationship Studies of Indole-Based Compounds as Small Molecule HIV-1 Fusion Inhibitors Targeting Glycoprotein 41. *J. Med. Chem.* 2014; 57:5270–5281. [PubMed: 24856833]
32. Cai L, Jiang S. Development of peptide and small-molecule HIV-1 fusion inhibitors that target gp41. *ChemMedChem*. 2010; 5:1813–1824. [PubMed: 20845360]
33. Welch BD, Francis JN, Redman JS, Paul S, Weinstock MT, Reeves JD, Lie YS, Whitby FG, Eckert DM, Hill CP, Root MJ, Kay MS. Design of a potent D-peptide HIV-1 entry inhibitor with a strong barrier to resistance. *J. Virol.* 2010; 84:11235–11244. [PubMed: 20719956]
34. Luftig MA, Mattu M, Di Giovine P, Geleziunas R, Hrin R, Barbato G, Bianchi E, Miller MD, Pessi A, Carfi A. Structural basis for HIV-1 neutralization by a gp41 fusion intermediate-directed antibody. *Nat Struct Mol Biol.* 2006; 13:740–747. [PubMed: 16862157]
35. Sabin C, Corti D, Buzon V, Seaman MS, Lutje Hulsik D, Hinz A, Vanzetta F, Agatic G, Silacci C, Mainetti L, Scarlatti G, Sallusto F, Weiss R, Lanzavecchia A, Weissenhorn W. Crystal Structure and Size-Dependent Neutralization Properties of HK20, a Human Monoclonal Antibody Binding to the Highly Conserved Heptad Repeat 1 of gp41. *PLoS Pathog.* 2010; 6:e1001195. [PubMed: 21124990]
36. Chan DC, Fass D, Berger JM, Kim PS. Core structure of gp41 from the HIV envelope glycoprotein. *Cell*. 1997; 89:263–273. [PubMed: 9108481]
37. Gochin M, Cai L. The role of amphiphilicity and negative charge in glycoprotein 41 interactions in the hydrophobic pocket. *J. Med. Chem.* 2009; 52:4338–4344. [PubMed: 19534533]

38. Chu S, Gochin M. Identification of fragments targeting an alternative pocket on HIV-1 gp41 by NMR screening and similarity searching. *Bioorg. Med. Chem. Lett.* 2013; 23:5114–5118. [PubMed: 23932360]
39. He Y, Cheng J, Li J, Qi Z, Lu H, Dong M, Jiang S, Dai Q. Identification of a critical motif for the human immunodeficiency virus type 1 (HIV-1) gp41 core structure: implications for designing novel anti-HIV fusion inhibitors. *J. Virol.* 2008; 82:6349–6358. [PubMed: 18417584]
40. Oishi S, Ito S, Nishikawa H, Watanabe K, Tanaka M, Ohno H, Izumi K, Sakagami Y, Kodama E, Matsuoka M, Fujii N. Design of a Novel HIV-1 Fusion Inhibitor That Displays a Minimal Interface for Binding Affinity. *J. Med. Chem.* 2008; 51:388–391. [PubMed: 18197613]
41. Welch BD, VanDemark AP, Heroux A, Hill CP, Kay MS. Potent D-peptide inhibitors of HIV-1 entry. *Proc. Natl. Acad. Sci. U. S. A.* 2007; 104:16828–16833. [PubMed: 17942675]
42. Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv. Enzyme Regul.* 1984; 22:27–55. [PubMed: 6382953]
43. Markosyan RM, Xiuwen M, Lu M, Cohen FS, Melikyan GB. The Mechanism of Inhibition of HIV-1 Env-Mediated Cell-Cell Fusion by Recombinant Cores of gp41 Ectodomain. *Virology.* 2002; 302:174–184. [PubMed: 12429526]
44. Lakomek NA, Kaufman JD, Stahl SJ, Wingfield PT. HIV-1 envelope protein gp41: an NMR study of dodecyl phosphocholine embedded gp41 reveals a dynamic prefusion intermediate conformation. *Structure.* 2014; 22:1311–1321. [PubMed: 25132083]
45. le Maire M, Champeil P, Moller JV. Interaction of membrane proteins and lipids with solubilizing detergents, *Biochim. Biophys. Acta.* 2000; 1508:86–111.
46. Gochin M. A suite of modular fluorescence assays interrogate the human immunodeficiency virus glycoprotein-41 coiled coil and assist in determining binding mechanism of low molecular weight fusion inhibitors. *Assay Drug Dev Technol.* 2012; 10:407–416. [PubMed: 22897493]
47. Cohen C, Parry DA. Alpha-helical coiled coils and bundles: how to design an alpha-helical protein. *Proteins.* 1990; 7:1–15. [PubMed: 2184436]
48. Lau SY, Taneja AK, Hodges RS. Synthesis of a model protein of defined secondary and quaternary structure. Effect of chain length on the stabilization and formation of two-stranded alpha-helical coiled-coils. *J. Biol. Chem.* 1984; 259:13253–13261. [PubMed: 6490655]
49. Greenfield NJ. Using circular dichroism collected as a function of temperature to determine the thermodynamics of protein unfolding and binding interactions. *Nat Protoc.* 2006; 1:2527–2535. [PubMed: 17406506]
50. Jelesarov I, Lu M. Thermodynamics of trimer-of-hairpins formation by the SIV gp41 envelope protein. *J. Mol. Biol.* 2001; 307:637–656. [PubMed: 11254387]
51. Robertson AD, Murphy KP. Protein Structure and the Energetics of Protein Stability. *Chem. Rev.* 1997; 97:1251–1268. [PubMed: 11851450]
52. Ji H, Bracken C, Lu M. Buried polar interactions and conformational stability in the simian immunodeficiency virus (SIV) gp41 core. *Biochemistry (Mosc).* 2000; 39:676–685.
53. Desmezieres E, Gupta N, Vassell R, He Y, Peden K, Sirota L, Yang Z, Wingfield P, Weiss CD. Human immunodeficiency virus (HIV) gp41 escape mutants: cross-resistance to peptide inhibitors of HIV fusion and altered receptor activation of gp120. *J. Virol.* 2005; 79:4774–4781. [PubMed: 15795263]
54. Wexler-Cohen Y, Shai Y. Demonstrating the C-terminal boundary of the HIV 1 fusion conformation in a dynamic ongoing fusion process and implication for fusion inhibition. *FASEB J.* 2007; 21:3677–3684. [PubMed: 17575260]
55. Kliger Y, Peisajovich SG, Blumenthal R, Shai Y. Membrane-induced conformational change during the activation of HIV-1 gp41. *J. Mol. Biol.* 2000; 301:905–914. [PubMed: 10966795]
56. Klug YA, Ashkenazi A, Viard M, Porat Z, Blumenthal R, Shai Y. Early and late HIV-1 membrane fusion events are impaired by sphinganine lipidated peptides that target the fusion site. *Biochem. J.* 2014; 461:213–222. [PubMed: 24766462]
57. Eckert DM, Malashkevich VN, Kim PS. Crystal structure of GCN4-pIQI, a trimeric coiled coil with buried polar residues. *J. Mol. Biol.* 1998; 284:859–865. [PubMed: 9837709]

58. Lay CS, Ludlow LE, Stapleton D, Bellamy-McIntyre AK, Ramsland PA, Drummer HE, Pombourios P. Role for the terminal clasp of HIV-1 gp41 glycoprotein in the initiation of membrane fusion. *J. Biol. Chem.* 2011; 286:41331–41343. [PubMed: 21976663]
59. Zhou G, Wu D, Snyder B, Ptak RG, Kaur H, Gochin M. Development of indole compounds as small molecule inhibitors of HIV-1 gp41. *J. Med. Chem.* 2011; 54:7220–7231. [PubMed: 21928824]
60. Platt EJ, Wehrly K, Kuhmann SE, Chesebro B, Kabat D. Effects of CCR5 and CD4 cell surface concentrations on infections by macrophagetropic isolates of human immunodeficiency virus type 1. *J. Virol.* 1998; 72:2855–2864. [PubMed: 9525605]
61. Wei X, Decker JM, Liu H, Zhang Z, Arani RB, Kilby JM, Saag MS, Wu X, Shaw GM, Kappes JC. Emergence of resistant human immunodeficiency virus type 1 in patients receiving fusion inhibitor (T-20) monotherapy. *Antimicrob. Agents Chemother.* 2002; 46:1896–1905. [PubMed: 12019106]
62. Ciminale V, Felber BK, Campbell M, Pavlakis GN. A bioassay for HIV-1 based on Env-CD4 interaction. *AIDS Res. Hum. Retroviruses.* 1990; 6:1281–1287. [PubMed: 2078409]
63. Bjorndal A, Deng H, Jansson M, Fiore JR, Colognesi C, Karlsson A, Albert J, Scarlatti G, Littman DR, Fenyo EM. Coreceptor usage of primary human immunodeficiency virus type 1 isolates varies according to biological phenotype. *J. Virol.* 1997; 71:7478–7487. [PubMed: 9311827]

Figure 1.

Schematic representation and sequences of the reverse hairpins used in this study. A. Arrangement of the primary sequence of the reverse hairpin C39(L4)N50 compared to the gp41 ectodomain. Gp41 (HXB2) numbering and construct numbering are indicated, revealing 2 non-native residues at each end and a 4-residue loop. Sequences for all the reverse hairpins are shown, with non-native N- and C-terminal residues underlined. Other sequence modifications are described in the text. B. Sequence of forward hairpins N36(L6)C34 and N34(L6)C28 previously tested in antiviral assays (see text). C. Reverse hairpin folds showing the sections of NHR and CHR selected for the constructs. D. Structure of forward hairpins from the literature. HP = hydrophobic pocket. Note only one hairpin monomer is shown for simplicity. Trimerization occurs along the NHR axis.

Figure 2. Bioactivity of reverse hairpin constructs. A. Fractional cell – cell fusion activity as a function of swapped domain protein concentration: (○) C21(L4)N50, (●) C28(L4)N50, (■) C28(L4)N45, (◇) C28(L4)N40, (◆) C39(L4)N50. B. ELISA with 1.7 $\mu\text{g}/\text{ml}$ protein and 0.85 $\text{ng}/\mu\text{l}$ mAb D5; C. cell – cell fusion inhibition by C28(L4)N50 alone (●) and in the presence of 100X D-peptide PIE7_{ox} (◇); D. Combination index calculated according to the method of Chou and Talalay, assuming mutually exclusive (●) or mutually non-exclusive (□) inhibitors.

Figure 3.

Sedimentation equilibrium analytical ultracentrifugation experiment on 12 μ M C39(L4)N40 at 25°C in 10mM MES, pH 5.5 containing 50mM arginine base and 50mM glutamate. Centrifugation speeds of 25000, 30000, 35000, 40000 and 45000 are shown in red, green, blue, black and cyan, respectively. Residuals between calculated and observed data are shown.

Figure 4.

CD measurements of C39(L4)N50 in the absence (black) or presence (red) of 10mM n-dodecylphosphocholine (DPC). (A) CD spectra of C39(L4)N50 at 20°C in MES buffer (10mM MES, 50mM KCl, pH 5.5) (red and black circles), and of MES buffer with and without 10mM DPC in MES (red and black diamonds); (B) Mean residue ellipticity at 222nm over the temperature range 20 – 90°C.

Figure 5.

CD measurements of C28(L4)N50 in the absence (black) or presence (red) of 10mM n-dodecylphosphocholine (DPC). (A) CD spectra of C28(L4)N50 at 20°C in MES buffer (10mM MES, 50mM KCl, pH 5.5). (B) Mean residue ellipticity at 222nm over the temperature range 20 – 90°C. The melting curves were fit with parameters shown in Table 3 and described in the text.

Figure 6.

CD measurements of C21(L4)N50 in the absence (black) or presence (red) of 10mM n-dodecylphosphocholine (DPC). (A) CD spectra of C21(L4)N50 at 20°C in MES buffer (10mM MES, 50mM KCl, pH 5.5). (B) Mean residue ellipticity at 222nm over the temperature range 20 – 90°C. The melting curves were fit with parameters shown in Table 3 and described in the text.

Table 1

Bioactivity of reverse hairpin constructs, in nM*

	EC ₅₀ (CCF) [§]	EC ₅₀ (VCF) [†]	CC ₅₀ [‡]
C39(L4)N50	> 2000	> 2000	> 2000
C39(L4)N40	> 2000	> 2000	> 2000
C28(L4)N50	9.6 ± 1.5	9 ± 4	> 2000
C28(L4)N45	43 ± 10	409 ± 90	> 2000
C28(L4)N40	122 ± 24	> 2000	> 2000
C21(L4)N50	10 ± 3	11 ± 5	> 2000

* HXB2 Env;

[§] concentration inhibiting 50% of cell-cell fusion events,[†] concentration inhibiting 50% of viral infectivity,[‡] concentration resulting in 50% cell viability

Table 2

CD studies of reverse hairpin constructs *

	%helix [¶]		$\theta_{222} / \theta_{208}$	
	pH 7.4 [‡]	pH 5.5 [§]	pH 7.4 [‡]	pH 5.5 [§]
C39(L4)N50	97	85	1.03	1.10
C28(L4)N50	8 [‡]	81	0.70 [‡]	1.10
C21(L4)N50	87	80	1.02	1.11
C39(L4)N40	97	n.d	1.02	-
C28(L4)N45	n.d.	22 ^a	-	1.07
C28(L4)N40	9 [‡]	12	0.77 [‡]	1.50

n.d. not determined.

* measured at 20 μ M, 20°C;[‡] in PBS;[§] in 10 mM MES, 50 mM KCl, pH 5.5, except^a in 25mM Tris-acetate, pH 5.5;[‡] poor solubility at 20 μ M;[¶] Calculated assuming 100% helix at -33000 deg.cm².dmol⁻¹;

Table 3

Effect of DPC on helicity and stability of reverse hairpin constructs*

		Observed			Calculated			
	[DPC]	$\theta_{\text{MRE}} / 10^3$ [¶]	% helix	$\theta_{222} / \theta_{208}$ ^a	$T_{\text{M}} / ^\circ\text{C}$ [§]	$\theta_{\text{F}} / 10^3$ ¹	$\theta_{\text{U}} / 10^3$ ²	$H_{\text{fold}} / \text{kcal.mol}^{-1}$ ³
C39(L4)N50	0	−28.0	85	1.1	> 100	n/a	n/a	n/a
C39(L4)N50	10mM	−32.9	100	1.1	> 100	n/a	n/a	n/a
C28(L4)N50	0	−26.6	81	1.1	112 ± 3 [†]	−28.1	$-5 - 0$ [†]	−35
C28(L4)N50	10mM	−28.3	86	1.0	74	−28.1	−18.7	−37
C21(L4)N50	0	−26.3	80	1.1	97 ± 3 [†]	−26.2	$-5 - 0$ [†]	−31
C21(L4)N50	10mM	−23.7	72	1.0	45	−26.2	−17.5	−30

* measured at 20 μ M, in MES, pH 5.5;[¶] mean residue ellipticity (MRE) at 20°C in deg.cm².dmol^{−1};^a at 20°C;[§] midpoint of unfolding transition;¹ MRE of folded (20°C) state;² MRE after transition;³ free energy of unfolding; T_{M} , θ_{F} , θ_{U} and H_{fold} were determined by fitting the data to equation 3, except[†] estimated θ_{U} for 100% unfolded protein and estimated T_{M} .