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Peptides and Peptidomimetics as Tools to Probe Protein-Protein Interactions – Disruption of HIV-1 gp41 Fusion Core and Fusion Inhibitor Design

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1. Introduction

Protein-protein interactions play important roles in many critical processes in the life sciences, such as signal transduction, lipid membrane fusion, receptor recognition, *etc.*, and many of them are important targets for drug development and design (Wilson 2009; Tavassoli 2011). Unlike an enzyme-substrate interaction which usually has a deep binding pocket in the protein for substrate binding, protein-protein interactions usually involve a large interacting interface; as a result, it is a big challenge for small molecule drugs to efficiently competitively occupy the interface and disrupt protein-protein interactions that modulate these life processes. Proteins are natural ligands that can modulate protein-protein interactions; however, they are not ideal therapeutic agents because of their expensive production costs and the fact that they are not able to be administered orally. In protein-protein interactions, energy is not always equally distributed throughout the binding interface; a couple of focused areas may account for the main protein-protein interaction energy, called a hot spot, which can be the target for a small molecule protein-protein interaction inhibitor (PPII).

Peptides, with suitable molecular size, provide a bridge between protein and small molecule drugs. Similar to proteins, many peptides are natural ligands that modulate protein-protein interactions in important life processes; they are used as drug leads and/or modified to increase potency and selectivity. Compared with small molecules, peptides are more efficient PPIIs due to their relatively large size, and can be useful tools to probe protein-protein interactions for PPII design.

HIV-1 gp41 mediated virus-cell membrane fusion is critical for HIV-1 infection and *in vivo* propagation (Eckert & Kim 2001; Caffrey 2011), and the mechanism is shared by many other viruses using a class 1 fusion protein as membrane fusion machinery, including some life threatening pathogens such as influenza virus, respiratory syncytial virus (RSV), Ebola virus, and severe acute respiratory syndrome (SARS) virus (Harrison 2008). A critical step in HIV-1 infection is a protein-protein interaction between the gp41 N- and C-terminal heptad repeats (NHR and CHR), that form a coiled-coil six-helical bundle (6-HB), providing energy for virus-cell membrane fusion (Fig. 1). Peptides derived from CHR or NHR can interact

with their counterparts in gp41 to prevent fusogenic 6-HB formation and inhibit HIV-1-cell membrane fusion, thus preventing HIV-1 infection and replication. T20 (Fuzeon, enfuvirtide), a 36-mer peptide from HIV-1 gp41 CHR, was approved by the USA FDA in 2003 as the first fusion inhibitor for salvage therapy in HIV/AIDS patients unresponsive to common antiretroviral therapy. Its application has been limited by i) the high cost of peptide synthesis, ii) rapid *in vivo* proteolysis, and iii) poor efficacy against emerging T20-resistant strains. These drawbacks have called for a new generation of fusion inhibitors with improved antiviral and pharmacokinetic profiles.

In this chapter, we will focus on the development of HIV-1 fusion inhibitors, concentrating on C-peptide fusion inhibitors and their peptidomimetics, which have been used as probes and tools to elucidate gp41 NHR-CHR interactions for future fusion inhibitor design and improve, and in the long run, the development of small molecule inhibitors that can disrupt this important protein-protein interaction.

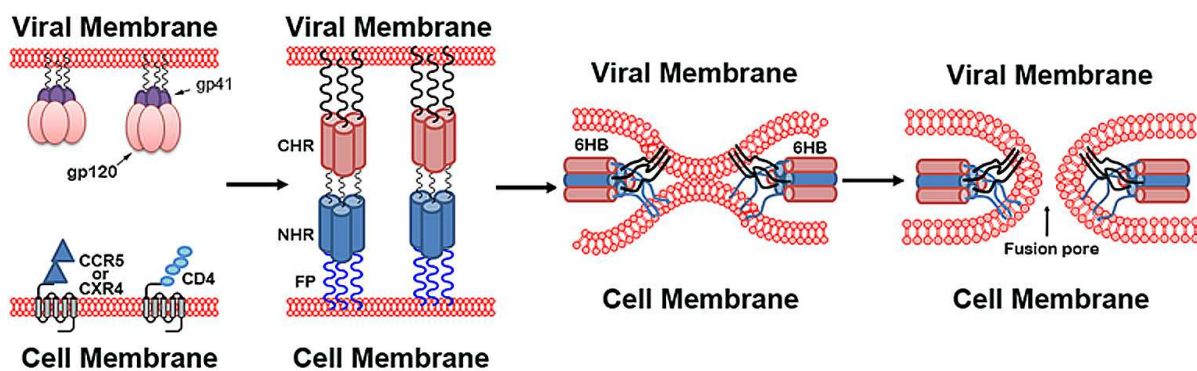


Fig. 1. HIV-1 gp41 mediated virus-cell membrane fusion.

2. Peptides as a model to build the HIV-1 gp41 fusion core

HIV-1 uses an envelope protein (ENV) mediated virus-cell membrane fusion to enter host cells for infection (Eckert & Kim 2001). HIV-1 ENV is composed of noncovalently associated gp120/gp41 trimers that form spikes and decorate the viral surface, in which the metastable transmembrane subunit gp41 is sequestered by the cell surface subunit gp120. During HIV-1 infection, gp120 first interacts with the T-cell receptor CD4, ensuring the viruses approach the target cells; then, the coreceptor binding sites in gp120 are sequentially exposed and gp120-coreceptor (CCR5 or CXR4) binding follows (Fig. 1). The resulting dissociation of the gp120-gp41 complex and the release of the unstable gp41 subunit trigger virus-cell membrane fusion. First, gp41 inserts into the target cell membrane using its fusion peptide, resulting in a pre-hairpin intermediate (PHI) in which its C-terminus anchors to the viral membrane and its N-terminus inserts into the host cell membrane, bridging the viral and cellular membranes (Fig. 1). The gp41 PHI automatically undergoes structure rearrangement with its NHR and CHR folding towards each other to form the fusogenic 6-HB. The energetic 6-HB formation drives the juxtaposition of the viral and cellular membrane, and finally results in virus-cell membrane fusion (Fig. 1). Agents that target the presumed gp41 PHI to prevent fusogenic 6-HB formation can terminate the virus-cell membrane fusion processes and be used as fusion inhibitors for antiretroviral therapy (Cai & Jiang 2010).

The discovery of potent anti-HIV peptides from HIV-1 gp41 NHR and CHR sequences suggests that gp41 is a target for fusion inhibitors (Wild et al. 1992; Jiang et al. 1993; Wild et al. 1994); these exogenous HIV-1 gp41 peptides interact with their counterparts in the gp41 6-HB, forming an unproductive complex that prevents gp41 fusion core formation. During the membrane fusion process, HIV-1 gp41 progressively undergoes a conformational change, and the gp41 PHI target exists for only a couple of minutes and then rapidly folds into a 6-HB; therefore, gp41 and its ectodomain are not suitable targets for a fusion inhibitor. Efforts to obtain a whole structure of the gp41 ectodomain also have been unsuccessful. So, the identification of a stable target in the PHI or gp41 fusion core is necessary for understanding the mechanism of gp41 mediated virus-cell membrane fusion for fusion inhibitor design and development.

The HIV-1 gp41 fusogenic 6-HB core has been constructed using synthesized peptides from the related gp41 wild-type sequences. Typical resolved crystal structures of the 6-HB fusogenic core include the N36/C34 complex (Chan et al. 1997), the IQNgp41/C43 complex (Weissenhorn et al. 1997), and the N34(L6)C28 trimer (Tan et al. 1997). These crystal structures provide atomic resolution of the interactions between NHR and CHR, verifying that NHR and CHR can be both a target and ligand from which a pharmacophore model can be deduced for fusion inhibitor design and optimization.

The crystal structures show that a parallel coiled-coil trimerized NHR forms the interior core, which is antiparallel packed with three CHR helices, to form a 6-HB (Fig. 2a,2b) (Chan et al. 1997). In the NHR interior core, the N-peptide uses its amino acid residues at the *a* and *d* positions of the heptads for self trimerization to stabilize the core; while the *e* and *g* residues of two adjacent helices form three hydrophobic grooves along the whole NHR trimer, which serve as targets that interact with the *a* and *d* residues of the C-peptides. Each groove contains a particularly deep cavity: Val-570, Lys-574, and Gln-577 from the left N36 (gp41₅₄₆₋₅₈₁) helix form the left side; Leu-568, Trp-571, and Gly-572 from the right N36 helix form the right side; and Thr-569, Ile-573, and Leu-576 form the floor, resulting in a pocket of ~16 Å long, 7 Å wide, and 5–6 Å deep (Fig. 2d). With the exception of Ile-573, all of the residues forming the cavity are identical between HIV-1 and SIV. The NHR deep pocket accommodates three hydrophobic residues from the abutting C34 (gp41₆₂₈₋₆₆₁) helix: Ile-635, Trp-631, and Trp-628 constitute a WWI motif (Fig. 2c). The interaction between the NHR pocket and the WWI motif is predominately hydrophobic. A salt bridge between Lys-574 of NHR and Asp-632 of CHR immediately to the left of the cavity is also important for the NHR-CHR interaction (Chan et al. 1997). In addition to be the main binding sites for the C-peptide, the deep NHR pocket is also an attractive target for small molecule fusion inhibitors. Besides the deep pocket, the rest of the groove along the NHR helices also makes extensive contact with CHR, providing additional energy to stabilize the 6-HB. The N36/C34 complex shows striking structural similarity to the low-pH-induced conformation of the influenza HA2 subunit (TBHA2) and the TM subunit of Mo-MLV, both of which have been proposed to be in a fusogenic conformation, suggesting a common mechanism of virus-cell membrane fusion among enveloped viruses (Chan et al. 1997).

During 6-HB formation, NHR and CHR are mutual target and ligand, so either can be the target for fusion inhibitor design. In a 6-HB, NHRs form a trimerized interior core that contains three grooves, and each with a deep pocket, which is more like a target, especially for small molecule fusion inhibitors. An electrostatic potential map of the N36 coiled-coil

trimer shows that its surface is largely uncharged; and the grooves that are the sites for C34 interaction are aligned with predominantly hydrophobic residues that would be expected to lead to aggregation upon exposure to solvent. In contrast, the N36/C34 complex shows a much more highly charged surface due to acidic residues on the outside of the C34 helices, resulting in greater solubility of the heterodimeric complex (Chan et al. 1997). As a result, N-peptides are prone to aggregate in the absence of C-peptides under physiological conditions. This also accounts for a much weaker inhibitory potency for N-peptides compared to C-peptides, since they must form a stable discrete trimerized inner core to efficiently interact with the CHR. Thus, construction of a stable and soluble discrete trimerized gp41 NHR core as a target is important for fusion inhibitor design and development.

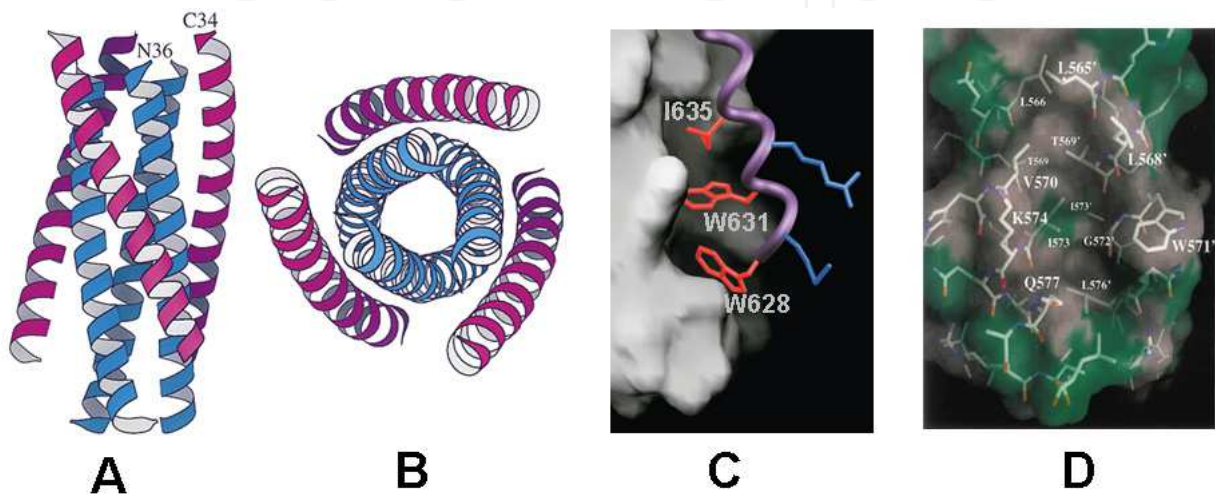


Fig. 2. Crystal structures of the HIV-1 gp41 fusion core. (A) 6-HB structure of the gp41 N36/C34 fusion core; (B) the top to bottom view of the N36/C34 6-HB structure (Chan et al. 1997); (C) the deep pocket in the NHR groove interacts with the WWI motif of CHR (Chan et al. 1998); (D) the NHR deep pocket.

The key for constructing an efficient NHR target is to promote trimerization of N-peptides without changing their native binding sites and conformation. Addition of physicochemical restraints in N-peptides has been shown to be an efficient way to construct a stable and discrete NHR trimer. Typical NHR constructs include: IQN17 (3) and IZN17 (4) (Eckert & Kim 2001), 5-helix (Root et al. 2001; Frey et al. 2006), and Env2.0 (5) and Env5.0 (6) (Cai & Gochin 2007; Cai et al. 2009). These stable NHR-trimers can be efficient targets for fusion inhibitor discovery and development. Through forming discrete and stable trimers, they are also highly potent HIV-1 fusion inhibitors by themselves. The sequences of the N-peptide targets are shown in Fig. 3.

IQN17/IZN17 (Fig. 4): A trimeric coiled-coil GCN4 isoleucine zipper was used to construct the first HIV-1 gp41 fusion core for an x-ray crystallographic study (Weissenhorn et al. 1997). IQN17 was constructed by fusing a modified GCN4-pIQI peptide sequence to the 17-mer N-peptide gp41₅₆₅₋₅₈₁ (N17) that comprises the gp41 hydrophobic pocket (Eckert et al. 1999). The resulting peptide, IQN17, is a fully helical discrete trimer in solution, as determined by circular dichroism (CD) and sedimentation equilibrium experiments. The crystal structure of the IQN17/D10-p1 complex, a cyclic D-peptide fusion inhibitor, showed that the overall architecture of the HIV-1 gp41 hydrophobic pocket in the complex is almost

identical to that in the wild-type HIV-1 gp41 N36/C34 structure, with a C_{α} root mean square deviation (rmsd) of 0.65 Å. In follow-up studies, a new version, IZN17, was designed using the same strategy. IZN17 is more thermally stable than IQN17, with a $T_m > 100$ °C, compared with ~100 °C for IQN17; the enhancement of thermal stability was further confirmed by measuring the T_m in 2 M guanidine chloride, with a T_m of 66 °C and 74 °C for IQN17 and QZN17, respectively. IZN17 is also more soluble than IQN17 under physiological conditions (Eckert & Kim 2001). Both IQN17 and IZN17 were used as targets in a mirror-image phage display experiment to identify D-peptide fusion inhibitors (Eckert et al. 1999; Welch et al. 2007; Welch et al. 2010).

DP107	(1)	NNLLRAIEAQQHLLQLTVWGIKQLQARILAVEERYLKDQ
N36	(2)	SGIVQQQNNLLRAIEAQQHLLQLTVWGIKQLQARIL
IQN17	(3)	RMKQIEDKIEEIESKQKKIENEIARIKLLQLTVWGIKQLQARIL
IZN17	(4)	IKKEIEAIKKEQEAIKKKIEAIEKLLQLTVWGIKQLQARIL
Env2.0	(5)	Bpy-GQAVEAQQHLLQLTVWGIKQLQARILAVEKK
Env5.0	(6)	Bpy-GQAVSGIVQQQNNLLRAIEAQQHLLQLTVWGIKQLQARILAVEKK

Fig. 3. NHR target sequences. The sequences and groups responsible for physicochemical constraint are shown in grey.

5-Helix (Fig. 4): 5-Helix was designed using the 6-HB as a motif (Root et al. 2001). In 5-helix, five of the six helices that make up the 6-HB core structure are connected by short peptide linkers. The 5-helix protein lacks a third C-peptide helix, and this vacancy is expected to create a high-affinity binding site for the gp41 CHR. Under physiological conditions, 5-helix is soluble and a well folded protein that adopts >95% helical content, as expected from the design, and is extremely stable. In addition, denaturation was not observed, even at 96 °C or in 8 M guanidine chloride. 5-Helix interacts strongly and specifically with C-peptides, inducing a helical conformation in the bound C-peptide as judged by CD. 5-Helix was successfully used as the target in a fluorescence polarization assay to identify small molecule fusion inhibitors (Frey et al. 2006).

Env2.0/Env5.0 (Fig. 4): A trivalent coordination metal complex was used to fortify the gp41 NHR trimer (Gochin et al. 2003). 5-Carboxy-2,2'-bipyridine (BPY) was attached to an N-peptide that contains a deep pocket. Addition of a metal ion such as Fe^{2+} or Ni^{2+} resulted in the formation of a tris-BPY metal complex, which stabilizes the coiled-coil structure. The resulting magenta $Fe^{2+}(BPY)_3$ complex solution was due to a Fe^{2+} -BPY charge transfer band at 545 nm and confirmed Fe^{2+} -BPY binding. The apo-Env2.0 displayed 40% α -helical structure that increased to 89% upon the addition of Fe^{2+} ions, as measured by CD. The integrity of the binding grooves in $Fe^{2+}(Env2.0)_3$ was confirmed by its efficient binding with a matched C-peptide, as shown by CD and NMR (Gochin et al. 2006). The 545 nm absorbance agrees well with the emission maxima of the fluorophores fluorescein and Lucifer yellow. Fluorescence quenching by fluorescence resonance energy transfer (FRET) should occur if the fluorophore is brought close to the Fe^{2+} -BPY center. This enables direct determination of binding by using a fluorophore labeled C-peptide as the probe. Compounds which are able to bind to the NHR target and displace the probe can be measured with a competitive inhibition assay by following the recovery of probe fluorescence intensity (Cai & Gochin 2007). The BPY-metal complex FRET strategy is

generally applicable to different interacting peptide pairs, as long as the two peptide sequences are matched. This has been confirmed by the development of a longer gp41 N-peptide/C-peptide pair, Env5.0 and CP5; the peptide pair showed nanomolar binding affinity and can be used to screen more potent fusion inhibitors. Env5.0 contains the whole groove, and it has been used to identify ligands that interact with the range of the groove outside of the deep pocket by designing suitable probes (Cai et al. 2009). In addition, Env2.0 has been successfully used as a target for a screening assay to identify small molecule fusion inhibitors (Cai & Gochin 2007; Zhou et al. 2010).

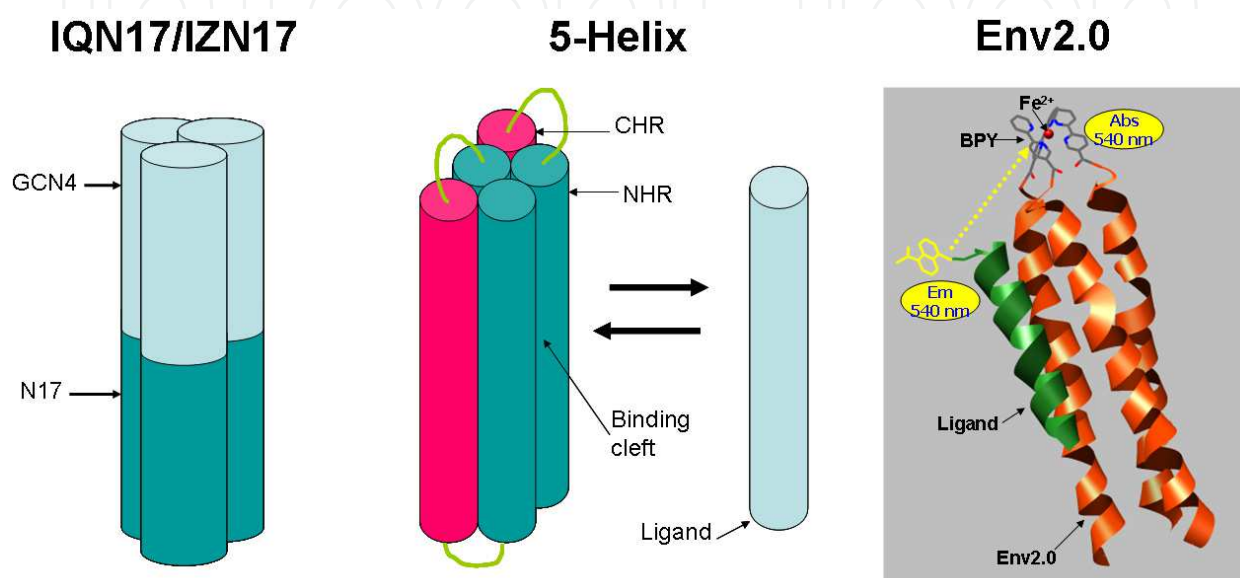


Fig. 4. Designed soluble and discrete NHR target.

In summary, peptides have been used to construct the HIV-1 gp41 fusion core, which is a 6-HB. Crystal structure analysis showed that the gp41 NHR trimer forms an interior core, which contains three hydrophobic grooves as the binding site for C-peptide. A deep pocket in the groove is a hot spot for the NHR-CHR interaction, and can be a target for small molecule fusion inhibitors. The NHR target can be constructed by adding physicochemical constraints in the N-peptides to promote the formation of a soluble and discrete NHR trimer, which can be used for screening HIV-1 fusion inhibitors targeting gp41 NHR or the deep pocket.

3. Peptide fusion inhibitors target the gp41 NHR core

Peptides, especially C-peptides (sequence see Fig. 5), can efficiently block the gp41 NHR-CHR interaction to inhibit HIV-cell membrane fusion and infection. They act in a dominant-negative manner by binding to the transiently exposed coiled-coil N-peptide region in the PHI (Eckert & Kim 2001). The wild-type C-peptide sequences have been shown to have low nanomolar IC_{50} values for HIV-1 ENV mediated membrane fusion and viral infection. Peptide engineering has been employed on wild-type C-peptide sequences to obtain structure activity relationship (SAR) data for the peptide fusion inhibitors, resulting in peptides with an improved anti-HIV profile and a better understanding of the mechanism of gp41 mediated virus-cell membrane fusion (Otaka et al. 2002; Dwyer et al. 2007). The insight gained from these works was finally tested by the artificial design of peptide fusion inhibitors with few sequence homologies to natural peptides or protein sequences (Qi et al. 2008; Shi et al. 2008).

3.1 Peptides from the wild-type gp41 sequence

The first highly potent HIV-1 fusion inhibitors were independently discovered by two groups in the early 1990s, including SJ-2176 (gp41₆₃₀₋₆₅₉) (Jiang et al. 1993; Jiang et al. 1993) and DP178 (gp41₆₃₈₋₆₇₃, later named T20) (Wild et al. 1994), which were both derived from the gp41 CHR wild-type sequence. Due to their stronger anti-HIV activity compared with N-peptides, most of the exploited fusion inhibitors were C-peptides, among them, T20 (7) and C34 (gp41₆₂₈₋₆₆₁, 8) were extensively studied. C-peptide fusion inhibitors are usually unstructured in solution by themselves, and form α -helical structures in a 6-HB after interaction with NHR.

T20, originally named DP178, was developed into the first HIV-1 fusion inhibitor with the brand name Enfuvirtide (Lazzarin et al. 2003; Walmsley et al. 2003; Su et al. 2004). It has low nanomolar antiretroviral activity. Under physiological conditions, it is unstructured and cannot form a stable 6-HB with N-peptide; however, it is highly soluble, making it a good drug candidate. Its mechanism of action has been controversial until now, since it cannot form a 6-HB with N-peptide, which is an established interaction model of HIV-1 peptide fusion inhibitors that has been supported by x-ray crystallography (Chan et al. 1997). T20 does not contain the WWI motif necessary to bind with the primary NHR deep pocket. This may account for its relatively weak binding with NHR and the resulting loss of activity against emerging drug resistant HIV-1 isolates. The 8-residue C-terminus of T20 contains three Trp residues and is highly hydrophobic, which enables T20 to bind with the lipid membrane; thus, this 8-residue motif is called the lipid binding domain (LBD). The hydrophobic residues in the LBD are critical for T20 to maintain high anti-HIV activity, although the LBD elicits no anti-HIV activity by itself. It seems that T20 may interact with both the gp41 NHR groove and the lipid membrane to interfere with 6-HB formation, thus inhibiting HIV-1 infection (Liu et al. 2005; Liu et al. 2007).

C34 forms a stable 6-HB with NHR, thus preventing productive 6-HB formation, a mechanism well supported by x-ray crystallography (Chan et al. 1997). It also displays stronger antiretroviral activity than T20, while its poor solubility under physiological conditions hinders it as a promising drug candidate (Otaka et al. 2002). Like all other wild-type gp41 C-peptides, C34 is unstructured under physiological conditions, while it adopts a nearly full α -helical structure when interacting with N-peptide to form a 6-HB. C34 contains the WWI motif, so it can interact with the primary binding pocket in NHR to form a stable complex with N-peptides. N-PAGE has shown that C34 can form a stable 6-HB in the presence of N36 or N46; and thermal denaturation has shown that the N36/C34 complex displays typical two-state denaturation behavior with a T_m value of ~ 61 °C (Pan et al. 2009). In a 6-HB, C34 uses residues *a* and *d* to interact with NHR. The *a* and *d* residues in the N-terminal half of C34 are uniformly hydrophobic and elicit a predominantly hydrophobic interaction with residues *e* and *g* in NHR and bury these residues in the 6-HB; and the *a* and *d* residues in the C-terminal half of C34 form a hydrophilic layer spanning four α -helical turns, which is assumed to match the similar hydrophilic layer in the related NHR sequence. Thus, C34 is widely used as a tool to study the mechanism of HIV fusion inhibitors, as well as the lead or template for next generation fusion inhibitor design, and will be discussed in Section 3.2.

CP32 (gp41₆₂₁₋₆₅₂, 14) is another identified highly potent wild-type gp41 C-peptide fusion inhibitor that targets NHR sequences other than T20 and C34 (He et al. 2008). It contains a 7-residue motif upstream of the C34 sequence. Interestingly, the CP32 sequence matches the

T21 sequence, the first identified peptide HIV-1 fusion inhibitor from gp41 under the name DP107 (Wild et al. 1992); the match is expected based on the N36/C34 complex and the anti-parallel interactions between gp41 NHR and CHR. CP32 contains the WWI motif, so it can interact with the NHR deep pocket to form a stable 6-HB. The CP32/T21 complex is ~100% α -helical with a T_m of 82 °C, which is more stable than the N36/C34 complex. The discrete 6-HB conformation of the CP32/T21 complex was supported by N-PAGE, size exclusion chromatography, as well as analytical ultracentrifugation. CP32 showed an IC_{50} value of 4.2 nM against HIV-1 ENV mediated cell-cell fusion and an IC_{50} value of 4.6 nM against HIV-1_{NL4-3wt} infection of MT-2 cells. Although it has a similar potency as C34 against wild-type HIV-1 isolates, CP32 is ~20-fold and >500-fold more potent than C34 and T20, respectively, against the drug-resistant HIV-1_{NL4-3-V38SE/N42S} isolate, possibly due to the fact that it targets a different sequence in gp41 NHR.

There are also longer C-peptides fusion inhibitors, such as C43 and C52, which include both partial or complete sequences of C34 and T20; however, none of these longer peptides have improved anti-HIV potency compared with T20 and C34 (Deng et al. 2007). In the PHI, a long groove may expand throughout the gp41 NHR and beyond, and it may be targeted by its CHR counterpart. The C-terminal half of the gp41 ectodomain may make contact with the N-terminal half at a certain time during fusion processes, so the C-peptide sequence may expand to the whole C-terminal half of the gp41 ectodomain and interact with the PHI to inhibit gp41 mediated virus-cell membrane fusion. The energy along the gp41 NHR-CHR interface is not evenly distributed; the WWI motif and the LBD serve as hot spots in the gp41 NHR-CHR interaction. A highly potent C-peptide fusion inhibitor must contain at least the WWI motif or the LBD; in addition, a suitable length of total peptide sequence is required to provide additional interactions in the NHR groove to stabilize the C-peptide-NHR interaction. Though they form stable α -helical structures in the 6-HB, C-peptides and N-peptides from the wild-type gp41 sequence are usually unstructured in solution; thus, they are prone to proteolysis. The viral strains resistant to T20 also required the development of a highly potent fusion inhibitor to overcome drug resistance. The use of protein/peptide engineering to improve the physicochemical properties of the wild-type C-peptide sequence and to increase the stability of the C-peptide-NHR complex is discussed below.

3.2 Engineered peptides

New generations of peptide fusion inhibitors have been developed by engineering C34-related sequences in order to increase the *in vivo* stability and NHR binding affinity, and to overcome T20 resistance. It is well accepted that increasing the helicity of the peptide fusion inhibitor will increase its antiretroviral potency by increasing its binding affinity with NHR and the *in vivo* stability (Otaka et al. 2002). In a 6-HB, C-peptides interact with NHR with their *a* and *d* residues, which are considered to be critical for molecular recognition between CHR and NHR; while amino acid residues at the *b*, *c*, *f*, and *g* positions are exposed to solution and are not considered to be critical for the gp41 NHR and CHR interaction (Chan et al. 1997). However, the solvent exposed residues have a global effect on the solubility, stability, and other physicochemical properties of the C-peptides, so they affect the *in vivo* activity and the druggability of peptide fusion inhibitors. Salt bridges and helical enhancers have been engineered by replacing the solvent exposed residues with the desired residues in order to get more potent HIV-1 fusion inhibitors.

T1249 (**13**) was developed by Trimeris as a second generation peptide HIV-1 fusion inhibitor after T20 (Miralles et al. 2003; Eggink et al. 2008; Pan et al. 2009). It was designed to include both hot spots, the WWI motif of C34 and the LBD of T20. To keep the peptides a suitable length, the seven residues following the WWI motif were considered to be not critical for the NHR interaction and were deleted; thus, the WQEWEQKI motif remained. It also contained the conserved amino acid residues from SIV and HIV-2 that are essential for fighting contains the T20 resistant virus. In addition, alanine substitutions and salt bridges were added to increase the α -helicity, resulting in a 39-mer highly mutated peptide based on the wild-type HIV-1 gp41 sequence. T1249 showed enhanced antiretroviral activity against the T20 resistant virus, and ~50% α -helicity compared to the unstructured character of the wild-type gp41 peptide. It entered into phase II clinical trials, but it was terminated due to side effects (Miralles et al. 2003).

T1144 (**9**) and T2635 (**31**) are third generation peptide fusion inhibitors developed by Trimeris (Pan et al. 2011). They fully exploited the strategy used in the development of T1249, however, they are based on the gp41₆₂₆₋₆₆₃ sequence (Dwyer et al. 2007). T1144 and T2635 showed strong activity against both native and highly T20 resistant HIV-1 strains. They form stable α -helices in solution with a helical content of 97% and 75% for T1144 and T2635, respectively. In addition, they both form a very stable 6-HB with NHR under physiological conditions. Analytical ultracentrifugation showed that these highly helical peptides form trimers in solution, which may make them more resistant to proteolysis and increase their *in vivo* stability. Ultra stable C-peptides from the same CHR sequence were also obtained, which showed ~100% helical content in solution and formed ultra stable 6-HBs with N-peptide with T_m values >100 °C, even in 8 M urea solutions. However, these peptides showed very weak antiretroviral activity. This indicated that it required a suitable degree of stability and α -helical content for the C-peptide to efficiently inhibit 6-HB formation to stop the HIV-1-cell fusion process.

Sifuvirtide (SFT, **12**) was developed by FusoGen and was based on the C34-related sequence gp41₆₂₇₋₆₆₂ (He et al. 2008; Liu et al. 2011). It was derived from the HIV-1 subtype E sequence and was engineered to mutate the exposed residues to salt bridges to increase the helical content and solubility. Like C34, Sifuvirtide is featureless under physiological conditions, while it forms a nearly full α -helical 6-HB with N36, with a T_m of 72 °C, 10 °C higher than that of N36/C34. As expected from its sequence origin, Sifuvirtide does not interact with the lipid membrane. Sifuvirtide showed low nanomolar inhibitory activity against HIV-1 ENV mediated cell-cell fusion and HIV-1 infection, including T20-resistant HIV-1 isolates. It showed an *in vivo* half-life of 20 h in a single dose administration in 12 healthy volunteers, which is much more stable than T20 and suitable for a once daily administration. It has finished phase IIb clinical trials in China and has shown promising antiretroviral profiles against both T20 resistant and T20 sensitive HIV-1 strains (Wang et al. 2009). The same strategy was applied to CP32 and resulted in CP32M with an improved anti-HIV profile (He et al. 2008).

SC35EK (**10**), also based on C34, was developed by Fujii's group. Most of the *b*, *c*, *f*, and *g* residues were substituted with glutamic acid and lysine residues in order to form EE-KK double salt bridges to fortify the α -helical structure (Otaka et al. 2002). SC35EK showed a little bit more potency than C34 in a multinuclear activation of galactosidase indicator (MAGI) assay (IC_{50} from 0.68 to 0.39 nM), while the salt bridge greatly enhanced its

solubility and made it a suitable drug candidate. Its structure is still largely random in solution, while the T_m of its 6-HB formed with N36 increased from 57 °C to 77 °C, which is 20 °C higher than C34. SC35EK was further shortened to SC29EK (**11**), with similar potency (Naito et al. 2009). The same strategy was applied to T20, the resulting T20EK (**16**) showed eight times more potency than T20 and can efficiently inhibit T20 resistant HIV-1 strains (Oishi et al. 2008).

In summary, the C-peptide fusion inhibitor could be engineered to improve the anti-HIV profile. Exposed residues in the 6-HB were substituted to build salt-bridges to significantly stabilize the C-peptide-NHR complex. This type of substitution can improve the solubility of the peptide fusion inhibitor to improve its druggability. The substitution also improved the pharmacokinetic profile, resulting in a longer *in vivo* half life. The helicity of isolated C-peptides were greatly increased by replacing the *a* and *d* residues in the hydrophilic layer, resulting in thermally stable C-peptide fusion inhibitors with high α -helical content; they formed an extremely stable complex with NHR. Some of these structured C-peptides showed high anti-HIV potency, especially against highly drug-resistant HIV-1 isolates; while too thermally stable C-peptides of this type caused abolishment of their inhibitory activities.

T20	(7)	Y T S L I H S L I E E S Q N Q Q E K N E Q E L L E L D K W A S L W N W F
C34	(8)	W M E W D R E I N N Y T S L I H S L I E E S Q N Q Q E K N E Q E L L
T1144	(9)	T T W E A W D R A I A E Y A A R I E A L L R A L Q E Q Q E K N E A A L R E L
SC35EK	(10)	W E E W D K K I E E Y T K K I E E L I K K S E E Q Q K K N E E E L K K
SC29KE	(11)	W E E W D K K I E E Y T K K I E E L I K K S E E Q Q K K N
Sifuvirtide	(12)	S W E T W E R E I E N Y T R Q I Y R I L E E S Q E Q Q D R N E R D L L E
T1249	(13)	W Q E W E Q K I T A L L E Q A Q I Q Q E K N E Y E L Q K L D K W A S L W E W F
CP32	(14)	Q I W N N M T W M E W D R E I N N Y T S L I H S L L E E S Q N Q
CP32M	(15)	V E W N E M T W M E W E R E I E N Y T K L I Y K I L E E S Q E Q
T20EK	(16)	Y T S L I E E L I K K S E E Q Q K K N E E E L K K L E E W A K K W N W F

Fig. 5. C-peptide fusion inhibitors

3.3 Artificially designed peptides

Artificial design was employed to design unknown peptide sequences with few homologies to natural peptide sequences (Qi et al. 2008; Shi et al. 2008). Based on the crystal structures of the HIV-1 gp41 fusion core, C-peptide uses its hydrophobic *a* and *d* residues to interact with the NHR. An EEYTKKI heptad unit (HR) was designed, with the heptad repeat 'bcdefga', as the building block. The *d* and *a* positions in the HR were hydrophobic Tyr and Ile residues, respectively, which were expected to form a hydrophobic face to interact with the hydrophobic NHR grooves. The residues at the *b* and *c* positions in the HR were negatively charged Glu, which were expected to form an intrahelical salt bridge with positively charged Lys at the *f* and *g* positions to stabilize the helical structure; these highly polar residues also form a highly hydrophilic face that increases the solubility of the peptides.

A 35-mer 5HR (17) (Fig. 6), which contains five copies of the HR, based on the length of most highly potent HIV-1 fusion inhibitors, was used as a template to build peptides to disrupt the HIV-1 gp41 NHR-CHR interaction. The interaction between 5HR and N46 (gp41₅₃₆₋₅₈₁) was modeled by using a PyMOL program based on the crystal structure of the N36/C34 6-HB, and compared with that of C34. The binding between the residues of 5HR and N46 was less complementary than that of the residues between C34 and N46. 5HR showed weak anti-HIV-1 activity ($IC_{50} = 156 \pm 8 \mu\text{g/mL}$), as measured by a dye transfer HIV-1-mediated cell-cell fusion assay. The WWI motif and LBD were used to replace the HR unit at the N- or C-terminus of 5HR, respectively, or both, based on the SAR of the C-peptide fusion inhibitors, resulting in PBD-4HR (18), 4HR-LBD (19), and PBD-3HR-LBD (20). Inserting a LBD or WWI motif in the 5HR sequences resulted in 2-fold and 6-fold increased potency, with an IC_{50} value of 74 ± 4 and $26 \pm 0.4 \mu\text{g/mL}$ for 4HR-LBD and PBD-4HR, respectively. The increasing potency was synergistic and PBD-3HR-LBD had an IC_{50} value of $4.8 \pm 0.3 \mu\text{g/mL}$, a striking 33-fold increase over 5HR. As expected from the design, peptides containing PBD, e.g. PBD-4HR and PBD-3HR-LBD, could form a stable 6-HB with the N-peptide N46 and effectively blocked gp41 core formation, as measured by CD spectroscopy and N-PAGE; peptides containing the LBD, including 4HRLBD and PBD-3HR-LBD, were bound tightly to lipid vehicles, with an association constant of 6.80×10^4 and $1.27 \times 10^5 \text{ M}^{-1}$, respectively, as determined by isothermal titration calorimetry (ITC). These results suggest that the HR sequence can be efficiently docked into the NHR groove and act as a structural domain; and the interaction can be greatly increased by including the WWI motif and LBD in the sequence. Thus, 4HR-LBD, PBD-4HR, and PBD-3HR-LBD are artificial fusion inhibitors that mimic T20, C34, and T1249 – the three typical highly potent HIV-1 fusion inhibitors target different sites of gp41 NHR, respectively.

The anti-HIV-1 activities of 4HR-LBD and PBD-4HR are lower than those of T20 and C34, which may be due to less sequence complementarity between the artificially designed HR and HIV-1 gp41 NHR. The resulting less tight binding suggests that a specific interaction should be uncovered and be addressed for the design of highly potent fusion inhibitors targeting specific viruses.

5HR	(17)	EEYTKKIEEYTKKIEEYTKKIEEYTKKIEEYTKKI
4HR-LBD	(18)	EEYTKKIEEYTKKIEEYTKKIEEYTKKIWASLWNWF
PBD-4HR	(19)	WMEWDREIEEYTKKIEEYTKKIEEYTKKIEEYTKKI
PBD-4HR-LBD	(20)	WMEWDREIEEYTKKIEEYTKKIEEYTKKIWASLWNWF

Fig. 6. Artificially designed peptide fusion inhibitors

In summary, C-peptide fusion inhibitors interact with gp41 NHR to prevent fusogenic 6-HB formation, and thus terminal HIV-1 ENV mediated virus-cell membrane fusion. A WWI motif in C-peptide that interacts with the NHR deep pocket is critical to the C-peptide-NHR interaction, and an extended interaction between C-peptide and the rest of the groove in the NHR trimer provides additional energy to stabilize the 6-HB. Artificial peptide design, based on the knowledge learned from SAR studies of the C-peptides, provides an alternative for peptide fusion inhibitor design; it also provides a stringent test for the knowledge gained and sets a new starting point for fully understanding the fundamentals

of virus-cell membrane fusion in order to guide future fusion inhibitor design against HIV and other viruses with class I fusion proteins.

4. Peptidomimetics as probes and inhibitors to study the gp41 NHR-CHR interaction

Several SAR studies of highly potent peptide fusion inhibitors have provided an efficient way to disrupt the HIV-1 gp41 NHR-CHR interaction for anti-HIV therapy; they have also deepened our understanding of the gp41 NHR-CHR interaction. Peptide drugs have their intrinsic weaknesses, however, such as high-cost, and unsuitability for oral administration due to *in vivo* proteolysis. Peptidomimetics that use unnatural building blocks may overcome the *in vivo* instability of peptide drugs, leading to orally bioavailable drugs. Peptidomimetics are more like small molecules than peptide drugs, so highly potent peptidomimetic fusion inhibitor studies can be useful for guiding small molecule fusion inhibitor design. Peptidomimetic fusion inhibitors that target gp41 NHR, including D-peptides, foldamers, and covalently linked restrained α -helical peptides (sequences or structures see Fig. 7), are discussed in this section.

4.1 D-peptides

As enantiomers of natural L-peptides, D-peptides are not degraded by proteases and have the potential for oral bioavailability. D-Peptides that target a specific protein or peptide target can be discovered by mirror-image phage display (Eckert et al. 1999). The target is synthesized chemically with D-amino acids, resulting in a product that is the mirror image of the natural L-amino acid form, which is used to screen phage that expresses a peptide library of phage coat proteins, to select phage clones with L-peptide sequences that specifically bind to the D-target. The mirror images of the phage-expressed L-peptide sequences are chemically synthesized with D-amino acids. By symmetry, these D-peptides should bind to the natural L-amino acid target.

Cyclic D-peptide HIV-1 fusion inhibitors targeting IQN17 have been identified by mirror-image phage display (Eckert et al. 1999). The phage-expressed peptide library contained ten random amino acid residues flanked by either a cysteine or a serine on both sides. Of the 12 identified IQN17-specific phage clones, nine were pocket specific binders, and eight contained the consensus sequence CXXXXXEWXWLC. The corresponding D-peptides were synthesized and were oxidized to form disulfide bonds. Lysines were added to improve the solubility. An intramolecular disulfide bond was critical for pocket binding and viral inhibition by these D-peptides, since cysteines were selected from an initial phage library containing either Cys or Ser at these positions. Replacing the Cys with Ala in the most potent derivative D10-p5-2K (**22**, IC₅₀ of 3.6 μ M) caused complete loss of inhibitory activity in a gp41 mediated cell/cell fusion assay.

A IQN17/D10-p1 (**21**) co-crystal was obtained and resolved to 1.5 Å resolution by x-ray crystallography. Structural superposition showed that the overall architecture of the gp41 NHR deep pocket in the IQN17/D10-p1 complex is almost identical to that in the wild-type N36/C34 structure (Chan et al. 1997), with a C _{α} rmsd of 0.65 Å. D10-p1 forms a circular structure and binds only to the gp41 region of IQN17. Ala-2 to Ala-5 and Ala-11 to Ala-16 form short left-handed α -helices, and the middle region is unstructured. The overall

positions of the D10-p1 and C34 helices closely overlap, but most of the side chains are significantly different, corresponding to the opposite handedness of the inhibitors. Of the 16 residues in D10-p1, only six interact directly with the gp41 pocket of IQN17, including Trp-10, Trp-12, and Leu-13 in the conserved EWXWL sequence, and Gly-1, Ala-2, and Ala-16 in the invariant original flanking phage sequence. The side chains of Trp-10, Trp-12, Leu-13, and Ala-16 are deeply buried in the hydrophobic pocket of IQN17. A hydrogen bond is formed between a pocket residue Gln-577 and Trp-12 in D10-p1. The packing difference between the Trp-12 and Leu-13 side chains in D10-p1 and Trp-631 and Ile-635 in C34 results in slight changes in the shape of the pocket. Overall, however, the hydrophobic pocket maintains its integrity between the N36/C34 and IQN17/D10-p1 structures. NHR chemical shift differences showed that, for all of the identified D-peptides, Trp-10, Trp-12, and Leu-13 are buried in the IQN17 pocket, validating the pocket as a target for drug development.

In follow-up work, the consensus residues in the sequence (CX₅EWXWLC) reported above were fixed so that a constrained library was constructed in which the other six positions were randomized (Welch et al. 2007). The mirror-image phase display using IQN17 as the target identified, incidentally, the potent 8-mer D-peptide 2K-PIE1 (**23**) in the 10-mer template phage library. The x-ray crystal structure showed that 2K-PIE1 interacts in a similar manner as D10-p1 to IQN17, and 2K-PIE1 forms a more compact structure with IQN17. So, a comprehensive 1.5×10^8 member 8-mer phage library of the form CX₄WXWLC (3.4×10^7 possible sequences) was generated, and was screened using IZN17 as the target (Eckert & Kim 2001). The resulting PIE7 (**24**) was the most potent inhibitor ($IC_{50} = 620$ nM) and is 15-fold more potent than the best first-generation D-peptide (D10-p5). Comparison of the crystal structures of 2K-PIE1 and PIE7 complexed with IQN17 reveals several interesting differences. First, an intramolecular polar contact between the hydroxyl of D-Ser7 and the carbonyl of D-Gly3 in 2K-PIE1 is lost in PIE7 but is replaced with a new interaction between the side chain carboxylate of D-Asp6 and the amide of D-Gly3. Second, new hydrophobic interactions are created in PIE7 between the ring carbons of D-Tyr7 and the pocket residue Trp-571. Third, the carbonyl of D-Lys2 of PIE7, although somewhat flexible in orientation, forms a direct hydrogen bond with the ϵ nitrogen of Trp-571 in some of the structures. Fourth, in some of the structures the hydroxyl of D-Tyr7 in PIE7 forms a new water-mediated hydrogen bond with the pocket residue Gln-575, and this interaction cannot be formed in the 2K-PIE1 structure. Dimerized or trimerized PIE7 was constructed via PEG cross-linkers. The resulting (PIE7)₂ and (PIE7)₃ have IC_{50} values of 1.9 nM and 250 pM against HXB2, respectively. In contrast, PIE7 inhibits both JRFL, a primary R5-tropic strain, ($IC_{50} = 24$ μ M) and BaL ($IC_{50} = 2.2$ μ M) entry, although ~40- and 4-fold less potently than HXB2 entry, respectively; the PIE7 trimer is a moderately potent inhibitor of this strain ($IC_{50} = 220$ nM) and an extremely potent inhibitor against BaL ($IC_{50} = 650$ pM).

Structure-guided phage display was used to optimize the flanking residues for further improvement of PIE7 (Welch et al. 2010). The crystal structure shows significant contacts between the presumed inert flanking residues (Gly-Ala on the N-terminus and Ala-Ala on the C-terminus) and the NHR deep pocket. A new phage library was designed using XXCDYPEWQWLCXX as the template. PIE12 (**25**) was identified as the most potent (40-fold more potent than PIE7 against the JRFL strain). The x-ray crystal structure showed similarity between PIE12/IZN17 and PIE7/IZN17 structures with a RMSD of 0.6 to 1.2 Å on all C $_{\alpha}$ atoms. In PIE12/IZN17, new N-terminal flank residues (His1 and Pro2) form favorable ring

stacking interactions with the pocket (IQN17-Trp571), the substitution of Leu for Ala in the C-terminal flank sequence causes it to be buried an additional 50 Å into the hydrophobic surface area of the pocket, and the new interactions with the flanking sequence do not perturb the pocket-binding structure of the core PIE7 residues. These differences may account for the improved activity of PIE12 over PIE7. CD thermal denaturation showed that the PIE12-trimer forms the more stable complex with IQN17 with a T_m of 81 °C in 2 M Guanidine chloride (Gua.HCl), 8 °C higher than that of the PIE7-trimer complex. The anti-HIV-1 breadths of the PIE7-trimer, PIE12-trimer, and PIE12 were tested by a pseudovirion assay against a panel of 23 pseudotyped viruses representing clades A to D, several CRFs, and enfuvirtide-resistant strains. Both PIE7 and PIE12-trimers potently inhibited all strains tested, though PIE12-trimer was generally a superior inhibitor.

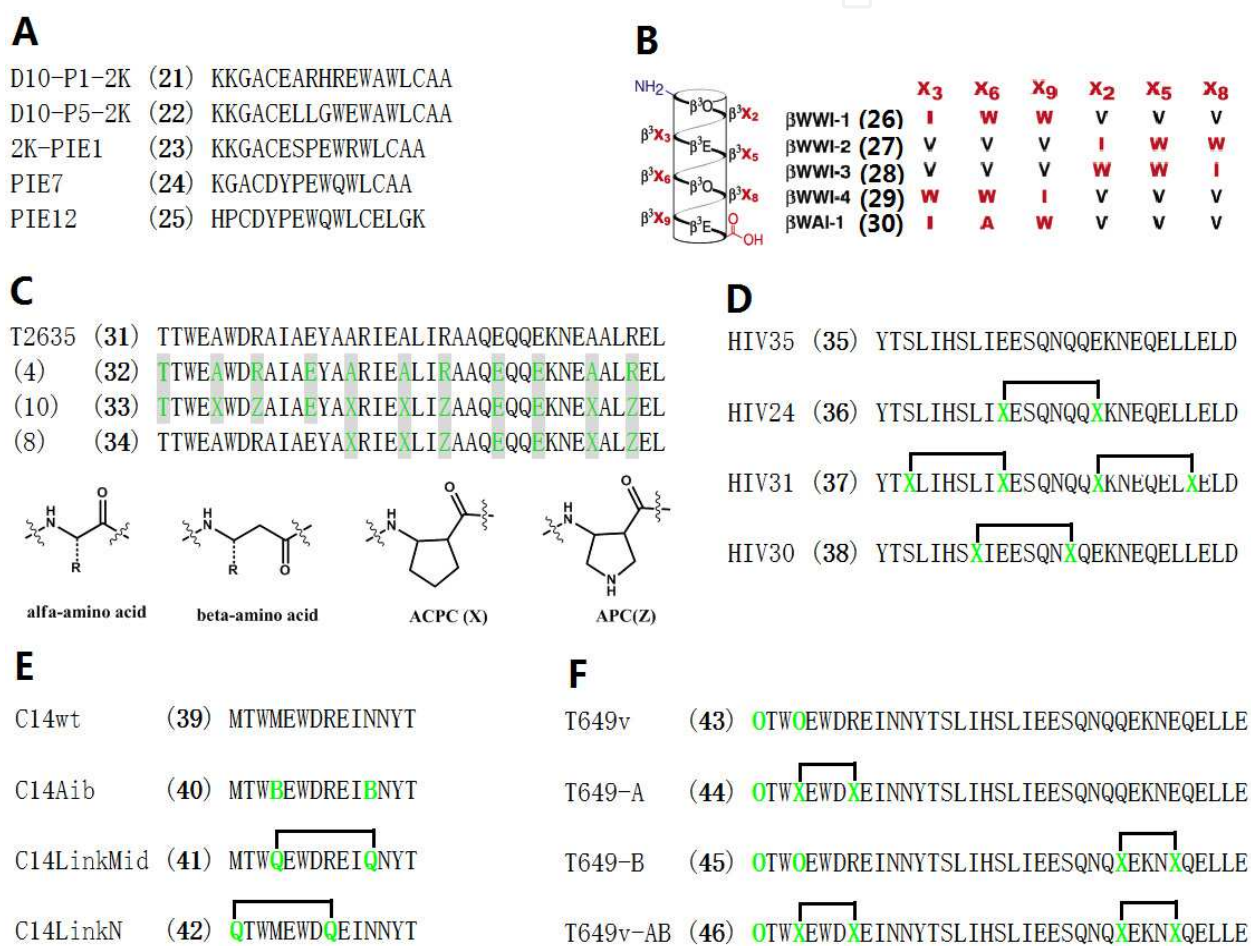


Fig. 7. Peptidomimetics used to disrupt the HIV-1 gp41 NHR-CHR interaction. (A) D-peptides; (B) β -foldamers; (C) α/β -foldamers; (D)/(E) linked peptides; (F) stapled peptides.

Viral passage studies were conducted to select for resistant strains. A strain bearing E560K/V570I mutations, which conferred a 400-fold resistance to PIE7-dimer, was selected with 20 weeks of propagation. These mutations dramatically weaken the binding of D-peptides to the gp41 pocket but not the C-peptide inhibitor C37. Despite this loss of affinity, the escape mutations had a minimal effect on the potencies of PIE12-dimer and PIE12-trimer. PIE12-dimer and PIE12-trimer resistant virus were identified after 40 and 65 weeks of propagation, respectively, using a much slower escalation strategy; only a Q577R single

substitution was identified. Interestingly, this substitution is present in nearly all group O isolates but is rare among group M isolates. Examination of the PIE12 crystal structure shows that Q577 makes hydrogen bonds with Glu7 and Trp10 in PIE12, which may explain the disruptive effects of this mutation.

4.2 Foldamers

A foldamer is a discrete chain molecule or oligomer that adopts a secondary structure stabilized by noncovalent interactions. Foldamers use unnatural building blocks instead of natural amino acids or nucleotides; as a result, they are more resistant to enzymatic degradation and show enhanced *in vivo* stability. They can mimic the ability of proteins, nucleic acids, and polysaccharides to fold into well-defined conformations, such as helices and β -sheets.

Short β^3 -foldamers have been designed that mimic the WWI motif of C-peptide fusion inhibitors (Stephens et al. 2005). A β -amino acid contains an additional methylene unit between the amine and carboxylic acid (Fig. 7c), and the amide bonds in β -peptides can resist *in vivo* proteolysis. A set of β^3 -decapeptides, β -WWI-1-4 (**26-29**), in which the WWI motif is presented on one face of a short 1,4-helix (Fig. 7b), were designed. Each β -peptide was fluorescently labeled at the N-terminus and was used in direct fluorescence polarization experiments to determine its binding affinity to IZN17. β -WWI-1-4-Flu bound IZN17 well, with equilibrium affinities of 0.75 ± 0.1 , 1.0 ± 0.3 , 2.4 ± 0.7 , and 1.5 ± 0.4 μ M, respectively. A WWI-1 analog β -WAI-1-Flu (**30**), containing Ala in place of the central Trp of the WWI motif, bound IZN17 with lower affinity ($K_d > 20$ μ M), suggesting the WWI motif is critical for pocket binding. The binding affinities are consistent with the cell-cell fusion assay results; β -WWI-1-4 inhibited cell-cell fusion with EC_{50} values of 27 ± 2.5 , 15 ± 1.6 , 13 ± 1.9 , and 5.3 ± 0.5 μ M, respectively, whereas β -WAI-1 was inactive under the same conditions.

In a follow-up study (Bautista et al. 2009), the second Trp in β -WWI-4 (**29**) was replaced with unnatural residues to probe steric and electronic effects on the NHR deep pocket binding. Most of the new β -peptides (EC_{50} 8.2–19 μ M) are more potent than β -WWI-1 (**26**) ($EC_{50} = 56$ μ M) at promoting the survival of HIV-infected cells. However, high cytotoxicities, with a selective index (CC_{50}/EC_{50}) < 10 , render these short β -peptides unsuitable as drug leads.

An α/β foldamer with partial β -amino acid replacement was used to modify a highly potent C-peptide fusion inhibitor to increase its *in vivo* stability (Horne et al. 2009). A two-stage design strategy was employed to modify T2635 (**31**), a highly potent third generation peptide fusion inhibitor (Dwyer et al. 2007). A fluorescent polarization binding assay using 5-helix as the target, a cell-cell fusion assay, and a protease K assay were used to assess the peptide and designed foldamer. In the first stage, one amino acid residue in each α -helix turn at the same position was replaced by a β -amino acid. The optimized α/β foldamer (**32**), containing systematic β -amino acid substitutions at positions *c* and *f*, showed weak binding affinity ($K_i = 3800$ nM) and cell-cell fusion inhibitory activity ($IC_{50} = 390$ nM), compared with those of T2635 ($K_i < 0.2$ nM and $IC_{50} = 9$ nM). However, **32** showed a 20-fold increase in half-life (14 min) in the protease K assay, compared with that for T2635. The β -amino acid, with one additional methylene unit, may make the backbone of the α/β foldamer too flexible to adapt a suitable conformation and results in the loss of activity. Accordingly, in the second stage of design, β -amino acids at certain positions were replaced with cyclic β -

amino acids to restore the rigidity of the backbone. The resulting α/β foldamer (**33**) had an IC_{50} value of 5 nM in the cell-cell fusion assay, similar to that of T2635; its binding affinity to 5-helix was 9 nM, similar to its cell-cell fusion inhibitory potency, despite its significantly weaker binding affinity to 5-helix than T2635. The α/β foldamer (**33**) showed a half-life of 200 min in the protease K assay, a 280-fold increase from T2635. The anti-HIV-1 infection activity of the α/β foldamer was also similar to T2635, as measured by HIV-1 infection of TZM-bl (JC53BL) cells using both R5 and X4 HIV-1 strains.

X-ray crystallography was used to characterize the structures of N36/T2635 and N36/**33** complexes. The N36/T2635 6-HB structure is almost identical to that of the wild-type N36/C34 6-HB, with a rmsd of 0.73 Å for the C_{α} atoms. However, the N36/**33** complex showed large structure distortion in the N-terminus (4.2 Å C_{α} rmsd for residues 2–15); the side chains of Trp³ and Trp⁵ were not resolved in electron density, suggesting a high degree of disorder, indicating that the N-terminal segment of **33** does not engage the NHR binding pocket in the complex. However, removal of the first ten residues of **33**, where the WWI motif is located, causes the loss of binding to 5-helix ($K_i > 10 \mu\text{M}$), indicating that the N-terminal segment of **33** is essential for high-affinity 5-helix binding. The N36/**34** complex, maintaining the intact WWI motif in the foldamer sequence (**8**), was crystallized and resolved to 2.8 Å resolution. Relative to **33**, **34** tracks much more closely with T2635, with a 1.4 Å C_{α} rmsd for residues 2–33 between the two structures. The side chains of the WWI motif in the N-terminal segment of **34** show the expected packing into the binding pocket on the NHR core trimer. The above results suggest that the lack of direct contact between the N-terminal portion of **33** and the NHR trimer in the N36/**33** complex may be an artifact of crystal packing.

4.3 Covalent-linked constrained peptides

Helical structure is critical for C-peptide fusion inhibitors to make proper contacts with the NHR binding sites to elicit potent inhibition. Constraining methods that add structural constraints into the peptide sequence by covalently cross-linking amino acid residues at suitable positions can promote the formation of the α -helical conformation, even in short peptides. The covalent linker can be a longer linker between the i and $i + 7$ residues, or a short linker called a stapler between the i and $i + 4$ residues.

The first selected gp41 C-peptide was truncated T20 that lacks the LBD sequence, called HIV35 (gp41₆₃₈₋₆₆₅, **35**) (Judice et al. 1997). A covalent cross-linker between the i and $i + 7$ residues of the polypeptide chain locks the intervening residues into a α -helical conformation. Residues at adjacent f positions on the opposite face of the helix were selected for cross-linking to enforce the residues at positions a and d to adopt a suitable conformation for target binding. Analogs of HIV35 were prepared containing either one, HIV24 (**36**), or two, HIV31 (**37**), tethers to impart increasing helicity. A control peptide, HIV30 (**38**), was prepared in which a tether was introduced between successive d residues to stabilize the helicity while blocking potential binding interactions across the a - d face. HIV24 and HIV30 were partially α -helical as measured by CD. By contrast, the doubly constrained analog HIV31 was mostly α -helical. HIV35 showed very weak inhibitory activity against HIV-1 in primary infectivity assays by using peripheral blood mononuclear cells with the virus JRCSF, a nonsyncytium-inducing strain, and BZ167, a syncytium-inducing HIV-1 strain. Single restrained HIV24 is more potent than HIV35, partially restoring the inhibitory

activity of T20. Doubly constrained HIV31 shows dramatically higher potency, and its activity was comparable with T20 in both HIV-1 infection assays.

In another report, a 14-residue C-peptide C14 (gp41₆₂₆₋₆₃₉, **39**) was selected (Sia et al. 2002). A cell-cell fusion assay was used to evaluate the biological activity of the peptides. Two strategies were employed, substitution with 2-aminoisobutyric acid (Aib) or a diaminoalkane crosslinker, to stabilize the helical conformation of C14. Six peptides were designed and produced, C14linkmid (**41**) was the most potent inhibitor against syncytia formation ($IC_{50} = 35 \mu M$), followed by C14Aib (**40**) ($IC_{50} = 144 \mu M$). C14linkmid and C14Aib bind to IQN17 with a K_d of 1.2 μM , respectively, as measured by ITC. The efficacy of the cross-linker on the inhibitory activities depends on its position in the peptide sequence, N-terminal cross-linked C14linkN does not inhibit cell-cell fusion, whereas the middle cross-linked C14linkmid inhibits cell-cell fusion at micromolar concentrations. The cell-cell fusion inhibitory activities of the peptides generally correlated with their NHR binding affinities, although the cell-cell fusion activities were consistently ~10-fold less potent than the K_d of NHR binding. Additional factors, other than binding affinity to the target, may be necessary for blocking viral entry. The crystal structure of the C14linkmid/IQN17 complex showed that C14linkmid binds to the gp41 hydrophobic pocket in essentially the same conformation as the pocket-binding region of C34, demonstrating that the crosslink imparts no detectable distortion on the backbone of the C14 peptide in the bound conformation.

Chemical staples have been used to fortify peptides to overcome the proteolytic shortcomings of highly potent peptide HIV fusion inhibitors as therapeutics. As an example, chemical staples were inserted at the N- or C-termini of T649v (**43**) by substituting (*S*)-2-(((9H-fluoren-9-yl)methoxy)carbonylamino)-2-methyl-hept-6-enoic acid at select (*i* and *i* + 4) positions, followed by ruthenium-catalyzed olefin metathesis (Bird et al. 2010). Sites for unnatural amino acid insertion were carefully selected to avoid disruption of the critical hydrophobic interface between NHR and CHR helices as delineated by the crystal structure of N36/C34. Three stapled peptides were designed by inserting single or double staples at selected positions. The activities of related peptides were measured using a luciferase-based HIV-1 infectivity assay, using viruses derived from HXBc2 and the neutralization-resistant primary R5 isolate, YU2. All of the peptides showed low nanomolar IC_{50} values against HXBc2 strains, suggesting that chemical modification in the stapled peptides does not disrupt its NHR interaction. Moreover, all of the stapled peptides showed higher inhibitory activities against drug resistant HIV-1 isolates, such as YU2 and the HIV-1 HXBc2 virus bearing the T20-resistant V38A/N42T or V38E/N42S double mutations in gp41 NHR, with a rank order of SAH-gp41₆₂₆₋₆₆₂ (**46**) > **44** > **45** > T649v > enfuvirtide. SAH-gp41₆₂₆₋₆₆₂ (**46**) displayed medium to low nanomolar IC_{50} values for all of the viruses tested, including T20 and the T649v-resistant YU2 isolate.

The pharmacokinetic properties of **44** were evaluated in a mouse model (Bird et al. 2010). The total body clearance of **44** (1.0 mL/min/kg) was 10-fold more slow than that of the unmodified T649v peptide (9.5 mL/min/kg). A proteolysis assay using both chymotrypsin and pepsin suggested that the striking protease resistance of stapled peptides is conferred by a combination of (1) decreased rate of proteolysis due to induction of α -helical structure and (2) complete blockage of peptidase cleavage at sites localized within or immediately adjacent to the (*i*, *i* + 4)-crosslinked segment. In addition, a pilot study was undertaken to compare the oral absorption of T649v and **44** using a mouse model. Measurable

concentrations of the full-length peptide were found in plasma samples from all **44** treated animals after oral dosing, and the concentration was dose dependent; no T649v was detected in plasma under the same conditions. The hydrocarbon double-stapling confers striking protease resistance of the peptide fusion inhibitor, which translates into markedly improved pharmacokinetic properties, including oral absorption, thus unlocking the therapeutic potential of natural bioactive polypeptides.

In summary, highly potent peptidomimetic HIV-1 fusion inhibitors have been discovered based on peptide fusion inhibitors, including: D-peptide fusion inhibitors discovered by mirror-image phage display using a D-amino acid form of the HIV-1 gp41 target; foldamers constructed from highly potent C-peptide fusion inhibitors by proper substitution of selected residues with β -amino acid residues; and structurally constrained peptides by covalently linking two residues at the same positions in a helical turn to promote α -helical structure formation. More like small molecule drugs, these peptidomimetics are potentially orally bioavailable and also provide clues for small molecule fusion inhibitor design.

5. Small molecule helix mimetics

The ultimate goal for drug development is small molecule drugs; it is also the main challenge in PPII development. The NHR deep pocket is a hot spot for the NHR-CHR interaction; it has an internal volume of roughly 400 \AA^3 , and could be filled by a molecule with a molecular weight of approximately 500 Da, raising the possibility that it could be targeted by small molecule drugs (Chan et al. 1997). Several groups have identified small molecules that show low micromolar inhibitory potency against HIV-1 ENV mediated cell-cell fusion and virus infection (Debnath et al. 1999; Frey et al. 2006; Cai & Gochin 2007; Zhou et al. 2010), however no direct evidence supports that these small molecule fusion inhibitors bind to the deep pocket (Gochin & Cai 2009; Cai & Jiang 2010). Therefore, providing direct structural evidence that a small molecule can bind to the NHR deep pocket, so that a small molecule pharmacophore model can be deduced, is highly desired for small molecule HIV fusion inhibitor design and development.

5.1 Small molecule-peptide conjugates

To identify small molecule ligands that specifically bind to the gp41 NHR deep pocket, Harrison's group has synthesized a biased peptide conjugate library (Ferrer et al. 1999). It contained ~60,000 compounds and used three small molecule building blocks to replace the WWI motif in C-peptide and links to the same peptide sequence. The library was synthesized and screened against 5-helix (Weissenhorn et al. 1997) using an on-bead affinity-based assay. A small molecule moiety was identified, which sequentially contained cyclopentyl propionic acid- ϵ -glutamic acid-p-(N-carboxyethyl) aminomethyl benzoic acid (Fig. 8) (**47**). The moiety alone had no activity based on an HIV-1 ENV mediated cell-cell fusion assay. However, when conjugated to a 30-mer C-peptide C30 (gp41₆₃₆₋₆₆₅) without the PBD sequence, the resulting conjugate peptide showed an IC_{50} value of $0.3 \mu\text{M}$, which was 20-fold increase compared with the IC_{50} value of $7 \mu\text{M}$ for C30. The conjugated peptide still had a much lower potency than a 38-mer (gp41₆₂₈₋₆₆₅) C-peptide containing the PBD that showed an IC_{50} value of 3 nM. The conjugated peptide could form a stable complex with N-peptide, as shown by size exclusion chromatography and native N-PAGE. This indicated that the small molecule moiety could partially mimic

the WWI motif of C-peptide to occupy the deep binding pocket of the NHR, while structure modification is needed to optimize the binding.

Crystallography was used to characterize the interaction between the conjugated peptide and gp41 NHR (Zhou et al. 2000). The full length of the non-peptide moiety is visible in electron density maps, but unexpectedly in two orientations, each with about 50% occupancy. The two binding modes share the same aminobenzoic acid position (F1) but diverge at the two more distal building blocks. Also, the electron density for the amino acid at the connection to the non-peptide moiety is poor, suggesting disorder in the peptide linkage to the non-peptide moiety.

5.2 Small molecules

A small molecule α -helical mimetic based on a substituted terphenyl scaffold was designed to inhibit the assembly of the 6-HB core (Ernst et al. 2002). Tris-functionalized 3,2',2''-terphenyl derivatives can serve as effective mimics of the surface functionality projected along one face of an α -helix. Compound 1a (**48**) was designed to mimic the side chains of an $i, i+4, i+7$ hydrophobic surface in an α -helix, using the branched alkyl substituents isobutyl and isopropyl (to avoid complications from chirality in a sec-butyl group) to mimic the side chains of the most prevalent Leu and Ile in the a and d positions of a 3-4 heptad repeat. Terminal carboxylate groups were also added to mimic the anionic character of the C-peptide and to improve the aqueous solubility. The ability of 1a (**48**) to disrupt the gp41 core was studied by CD spectroscopy, using a N36/C34 6-HB model ($T_m = 66^\circ\text{C}$). Titration of 1a into a 10 μM solution of N36/C34 resulted in a decrease of the CD signal at 222 and 208 nm, which corresponds to a reduction in the helicity of the 6-HB. A plot of θ_{222} versus inhibitor concentration shows saturation at approximately three equivalents of 1a. The CD spectrum with excess 1a was similar to the theoretical addition of the individual N36 and C34 spectra at the same concentration; and the thermal denaturation curve of the gp41 core in the presence of 50 μM 1a shows a significant 18 $^\circ\text{C}$ drop in the T_m value and closely resembles the melting transition of N36 alone at the same concentration. These data suggest that the 6-HB structure is completely disrupted by helix mimetic 1a. Both the hydrophobic and electrostatic features of 1a are important for its ability to disrupt the bundle. Analogs lacking the key alkyl side chains or carboxylic groups have little effect on the CD spectrum of the protein, even at high concentrations. Mimetic 1a effectively disrupts N36/C34 complexation with an IC_{50} value of $13.18 \pm 2.54 \mu\text{g}\cdot\text{mL}^{-1}$, as measured by an ELISA assay using NC-1 (Jiang et al. 1998). Compound 1a inhibits HIV-1 mediated cell-to-cell fusion with an IC_{50} value of $15.70 \pm 1.30 \mu\text{g}\cdot\text{mL}^{-1}$, using a dye-transfer cell fusion assay. In comparison, analogs lacking hydrophobic side chains or carboxylic groups had no inhibitory activity and proved to be cytotoxic at similar concentrations. Compound 1b (**49**), with larger hydrophobic groups than 1a, showed marginally enhanced activity than 1a.

Cai and Gochin identified a set of small molecule fusion inhibitors from a peptidomimetic library using a fluorescent biochemistry assay using Env2.0 as the target (Cai & Gochin 2007). Compounds **54** [3,5] and **55** [6,11] showed K_i values of 1.51 ± 0.16 and $1.34 \pm 0.19 \mu\text{M}$, respectively, in a competitive binding assay using Env2.0 as the target; and an IC_{50} value of $\sim 8 \mu\text{M}$ in an HIV-1 gp41 mediated cell-cell fusion assay using a CCR5/CXR4 dual dependent target cell line (JI et al. 2006). These compounds contain two units that are covalently linked by an amide bond, and each unit contains two aromatic rings that may

bind into the gp41 NHR hydrophobic pocket. A carboxyl group provides electrostatic interaction with K574 in the binding pocket and is critical for the activity of these small molecules; methylation of the carboxyl group resulted in loss of activities of the compounds in both the biochemical assay and cell-cell fusion assay. Three-unit compounds are prone to form aggregates under the assay conditions used and showed no activity, while single-unit compounds, such as M1 (56), display submillimolar inhibitory activity (Cai et al. 2009). Compound 1 (57), based on M1, was developed, which displayed an IC_{50} value of 4.5 ± 0.5 and 3.2 ± 0.5 μM in a fluorescence biochemical assay and a cell-cell fusion assay, respectively (Zhou et al. 2010). Compound 1 (57) showed very low cytotoxicity ($IC_{50} > 500$ μM); with a relatively small size, it is a promising lead for fusion inhibitor design.

Others have reported well-characterized small molecule fusion inhibitors targeting gp41, including SDS-J1 (50) (Debnath et al. 1999), NB64 (51), NB2 (52) (Jiang et al. 2004), and 4M041 (53) (Frey et al. 2006). These fusion inhibitors were selected from an active compound library by visual screening, then identified by high-throughput screening, and finally verified by a cell-cell fusion assay or HIV-1 infection assay. They usually showed low micromolar IC_{50} values for fusion inhibition; however, the following work to optimize the structures to obtain more potent fusion inhibitors were less fruitful, resulting in the identification of more small molecules with similar activity (Jiang et al. 2011). Also, their exact binding model with the gp41 NHR deep pocket still needs to be verified.

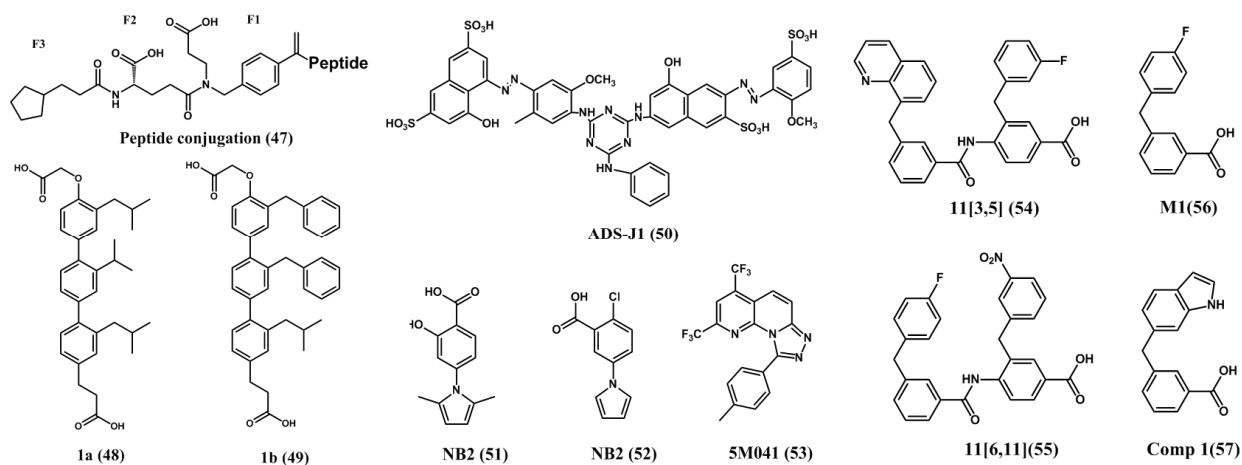


Fig. 8. Small molecule fusion inhibitors.

6. Conclusion

Peptides and peptidomimetics are efficient tools to study the HIV-1 gp41 NHR-CHR interaction, a key protein-protein interaction for HIV-1 gp41 mediated virus-cell membrane fusion, which enables HIV-1 enters and ultimately infects host cells. Peptides derived from wild-type HIV-1 gp41 sequences have been used to model the HIV-1 gp41 fusogenic core, a 6-HB formed by the NHR trimer as the inner core, and anti-parallel bind with three CHRs. Crystallographic structure analysis of the 6-HB has uncovered structure details for the gp41 NHR-CHR interaction. A deep pocket in the surface of NHR is a hot spot for the NHR-CHR interaction and a potential target for small molecule fusion inhibitors. N-peptides can be efficient targets for screening fusion inhibitors targeting the gp41 deep pocket by adding structural modulators to promote the trimeration of N-peptide.

Natural C-peptides can efficiently inhibit the gp41 NHR-CHR interaction by interacting with their counterpart in the gp41 6-HB; therefore, they can be used as fusion inhibitors against HIV-1 ENV mediated virus-cell fusion. They use residues at the *a* and *d* positions in heptad registration to bind the NHR hydrophobic grooves. The WWI motif of C-peptide provides a critical interaction with the NHR deep pocket, and an additional interaction between the C-peptide and NHR groove is required for a highly potent peptide fusion inhibitor of 30–40 residues. The *b*, *c*, *f*, and *g* residues in the C-peptide that form the predominantly hydrophilic surface in the 6-HB can be modified for increasing the secondary structure and solubility of the C-peptide, in order to increase its anti-HIV potency. The knowledge gained has been tested by artificial design of highly potent peptide fusion inhibitors with few similarities from known peptide sequences.

Peptidomimetics using unnatural building blocks have been successfully employed to mimic the molecular structures involved in the gp41 NHR-CHR interaction, resulting in highly potent HIV-1 fusion inhibitors with extraordinary *in vivo* stability to overcome the weakness of peptide drugs with potential oral administration possibilities. The achievements of the high potency peptidomimetic fusion inhibitors can also be used to guide small molecule fusion inhibitor design to disrupt this important protein-protein interaction.

In summary, HIV-1 fusion inhibitor development provides a model for using peptides as tools to probe protein-protein interactions for small molecule PPII design and development. The methods and results described in this chapter not only provide clues for future HIV-1 fusion inhibitor design, but also can be used for other viruses using a familiar virus-cell membrane fusion mechanism, as well as to guide other PPII design and development.

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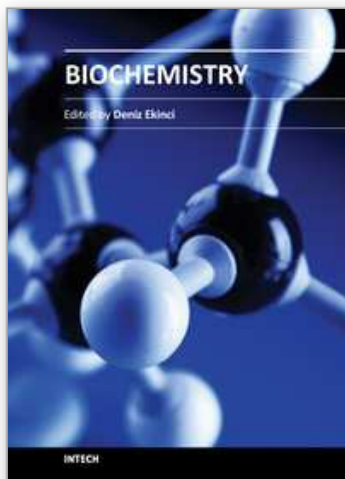
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Over the recent years, biochemistry has become responsible for explaining living processes such that many scientists in the life sciences from agronomy to medicine are engaged in biochemical research. This book contains an overview focusing on the research area of proteins, enzymes, cellular mechanisms and chemical compounds used in relevant approaches. The book deals with basic issues and some of the recent developments in biochemistry. Particular emphasis is devoted to both theoretical and experimental aspect of modern biochemistry. The primary target audience for the book includes students, researchers, biologists, chemists, chemical engineers and professionals who are interested in biochemistry, molecular biology and associated areas. The book is written by international scientists with expertise in protein biochemistry, enzymology, molecular biology and genetics many of which are active in biochemical and biomedical research. We hope that the book will enhance the knowledge of scientists in the complexities of some biochemical approaches; it will stimulate both professionals and students to dedicate part of their future research in understanding relevant mechanisms and applications of biochemistry.

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