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Amphipathic properties of HIV-1 gp41 fusion inhibitors

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Abstract

Small molecule inhibition of HIV fusion has been an elusive goal, despite years of effort by both pharmaceutical and academic laboratories. In this review, we will discuss the amphipathic properties of both peptide and small molecule inhibitors of gp41-mediated fusion. Many of the peptides and small molecules that have been developed target a large hydrophobic pocket situated within the grooves of the coiled coil, a potential hotspot for inhibiting the trimer of hairpin formation that accompanies fusion. Peptide studies reveal molecular properties required for effective inhibition, including elongated structure and lipophilic or amphiphilic nature. The characteristics of peptides that bind in this pocket provide features that should be considered in small molecule development. Additionally, a novel site for small molecule inhibition of fusion has recently been suggested, involving residues of the loop and fusion peptide. We will review the small molecule structures that have been developed, evidence pointing to their mechanism of action and strategies towards improving their affinity. The data points to the need for a strongly amphiphilic character of the inhibitors, possibly as a means to mediate the membrane - protein interaction that occurs in gp41 in addition to the protein – protein interaction that accompanies the fusion-activating conformational transition.

Introduction

The advent of numerous antiretroviral drugs has resulted in a decline in AIDS-related deaths but has not reduced the number of people living with Human Immunodeficiency Virus Type 1 (HIV-1) infection or significantly affected the number of new infections annually. An effective vaccine is the best hope for prevention, but the foreseeable future of HIV vaccines is still unclear [1–3]. HIV-1 fusion/entry inhibitors, unlike most clinical anti-HIV drugs that act after infection occurs, not only intercept the virus before it invades the target cell, but also can be used as prophylactic agents to assemble a barrier against the initial infection. Maraviroc, originally designated as UK-427857 and approved in August 2007 [1], blocks the binding between gp120 and chemokine receptor CCR5 which HIV-1 uses as a coreceptor. Enfuvirtide, a peptide originally designated as T20 and approved in April 2003 [2], is the first fusion inhibitor used in combination therapy for the treatment of HIV-1 infection. T20 binds to gp41 to prevent the formation of an entry core for the fusion of the virus, keeping it out of the target cell. Enfuvirtide therapy costs an estimated US\$25,000 per year in the United States. Its high cost and inconvenient dosing regimen are two factors behind its use as a reserve for “salvage” therapy in patients with multi-drug resistant HIV. There has been great interest in discovering small molecule alternatives as inhibitors targeting gp41 over the past decade.

Inhibitors against gp41 have the capacity to provide universal protection, since gp41 mediates viral fusion in both cell-free and cell-associated HIV-1 transmission, independent of co-receptor subtype [4–6]. In another review in this issue, the protective effect of a compromised gp41 fusion mechanism on bystander T-cell infection is discussed. A large number of antiviral peptides have been developed against HIV fusion (for review, see [7] as well as Cai et al in this issue), but small molecule drug development has proved particularly challenging for a number of reasons. Inhibition of a 40Å long protein – protein interface requires a somewhat non-traditional approach to drug development, and attempts at computational prediction of binding have been complicated by the flexibility of the interface. Structural studies to inform inhibitor development have been lacking, due to the difficulty in handling the aggregation-prone N-heptad repeat (NHR), or in obtaining crystals with small molecules bound. Biochemical studies of drug binding to the gp41 protein must be conducted on a transient intermediate state, prior to hairpin formation, a state which is not particularly stable or soluble in solution. In another review in this issue (Cai et al) a detailed account of biochemical and biophysical studies on gp41 demonstrates the large amount of work that has been applied in this area to design appropriate forms of the protein for targeting.

Despite the challenges, there are significant advantages to small molecule inhibition of fusion, including the potential for low cost and oral bioavailability, simpler formulation, and the ability to overcome steric and kinetic limitations that apply to large peptide or protein inhibitors. A steric block protects highly antigenic regions of gp41 such as the NHR and membrane-proximal external region from access to antibodies [8]. Root and colleagues have reported on kinetic limitations associated with the limited lifetime of the susceptible gp41 intermediate, which play a role in limiting potency of protein constructs such as 5-helix and T20 [9, 10]. These authors reported that C37 and T20 binding affinity to an extended 5-helix construct, 5H-ex, was not completely correlated with inhibitory activity, implying kinetic restriction of these inhibitors. An elegant study by Kahle et al [11] contrasted between affinity-dependent and kinetically restricted inhibitory potency of gp41 intermediate state inhibitors. As a general property, NHR targeting inhibitors including C-peptides derived from the C-heptad repeat (CHR) and hydrophobic pocket binding inhibitors that have been the focus of small molecule development, are reversible inhibitors with affinity-dependent inhibitory potency irrespective of inhibitor size and chemical nature [11, 12], except for extremely tight binding inhibitors. Highly potent (pM) C-peptide mutants of C37 lost their correlation of IC_{50} to the value of K_D [11]. CHR targeting inhibitors such as 5-helix variants were kinetically restricted, with an IC_{50} against viral fusion that remained unchanged (in the nM range) despite binding affinity variations from sub-pM to nM. This points to a fundamental mechanistic difference between NHR- and CHR-targeting gp41 inhibitors, and implies that viral fusion should be more susceptible to highly potent small molecules with much faster k_{on} rates than C-peptides, provided sub-nM affinity could be achieved for the small molecules.

There are several excellent reviews on peptide and small molecule inhibitors, to which the reader should refer for more background information [7, 13–16]. This review will focus on identifying coherent molecular features potentially helpful for inhibitor design, including the amphiphilic or lipophilic features characteristic of the most potent fusion inhibitors.

Linear peptide inhibitors

Studies of linear peptides with lipophilic or amphiphilic character emulating portions of the CHR have greatly informed the field of gp41 inhibition. It is from peptide as well as structural studies [17–21] that a model for the gp41 conformational change effecting fusion has been derived (Figure 1). Briefly, a metastable state of the HIV-1 envelope complex of gp120 and gp41 (Figure 1A) undergoes a conformational change following gp120

detachment from gp41. Highly potent fusion inhibitors derived from the CHR region of gp41 [12, 22–29], provide mechanistic evidence for the transient intermediate state of gp41 that is produced, in which NHR helices of gp41 form an extended trimeric coiled coil structure and gp41 fusion peptides (FP) insert into the host cell membrane (Figure. 1B). In subsequent steps, gp41 rearranges into a 6-helix bundle (6HB) (Fig 1C, 1D). The inner coiled coil is transiently exposed as a target for C-peptide and small molecule inhibition in the prehairpin and prebundle conformations. A review on the implications of the exposed FP, fusion-peptide proximal region (FPPR) and membrane-proximal external region (MPER) for drug discovery is included in this issue.

The essential features of successful peptides include:

- 1) An amphiphilic structure in which the helix is stabilized and hydrophobic side chains project from one face of the helix. Helix stabilization takes the form of *i*, *i*+4 salt bridges, sequence modification [28, 30–32], covalent bridges, α/β -peptides [33] and covalent constraints [34, 35].
- 2) A long chain, 34 residues or more, for nanomolar potency, and an overall negative charge to counteract the positively charged NHR.
- 3) Inclusion of both a hydrophobic pocket binding domain (628-WMEWDREI-635) and a lipid binding domain (667-WASLWNWF-674) (peptide T-1249) for greatly increased potency and activity against resistant strains, compared to peptides containing only one of these regions [36, 37].

Key to the activity of T-1249 was its ability to adsorb to lipidic bilayers [38], especially to lipid-raft structures high in cholesterol, which likely localizes the peptide to the site of action. Similarly, the highly potent peptide fusion inhibitor sifuvirtide [30] was found to partition preferentially into rigid membrane surfaces where most receptors are found, thus providing a high local concentration at the fusion site [39]. In fact, only part of the activity of T20 (Table 1) is directly attributable to its binding to the NHR [9, 40], and a significant contribution to activity is due to the hydrophobic C-terminus “WNWF” interacting with the lipid membrane [41, 42] and / or with the fusion peptide [43–45].

The amphipathic structure requirement for gp41 inhibitors was further illustrated by studies which showed that the dramatically reduced activity of truncated T20 missing the lipid binding domain (peptide DP in Table 1) could be restored with a fatty acid chain (DP-C16). Various lengths of conjugated fatty acid were tested, demonstrating a correlation between length, membrane association and inhibitory potency. Similarly, attaching a cholesterol group to C34 increased its potency 17-fold [46]. Activity enhancement is believed to be due to anchoring of the C-peptides in the membrane, consequently enhancing their local concentration and providing the correct alignment for interacting with the N-terminal coiled coil [41, 42, 46]. As expected, adding cholesterol to the end of T20 did not improve its activity, because the hydrophobic C-terminus of T20 already performs the membrane interacting function [46].

Similar studies were conducted on conjugated NHR peptides [47]. Activity increased with length of the lipid group and there was a small but not very significant advantage of N-terminal vs. C-terminal lipidation. Membrane binding and mutation studies of peptides C16-N36 and N36M-C16 showed that they had increased monomeric content when alkylated compared to trimeric N36, but that antiviral activity required the ability to form trimers. Ability to associate with the CHR domain was not required. This suggested a mechanism of action targeting endogenous NHR helices, and suggested that the NHR helices are loosely associated and oriented parallel to the membrane surface prior to gp41 trimer formation.

NHR association with the lipid membrane was verified in other studies. Peptides from the NHR (FPPR, loop and pre-TM) [48, 49] are capable of interacting with membranes and changing the stability of phospholipid membranes [50]. It appears that the NHR participates in the fusion event, since composite peptides can induce fusion [51], even without the FP. Recent studies show that the 6HB is destabilized by membranes [50, 52] and that pore formation likely begins prior to hairpin formation (Figure 1C) [53]. It is the hydrophobic nature of the NHR that leads to a tendency to partition into the zwitterionic outer leaflet of eukaryotic cell membranes. This suggests that small molecules that target the NHR that are capable of partitioning into membranes could possibly antagonize the membrane – NHR interaction, not just the protein – protein interaction that occurs between the NHR and CHR [54]. Furthermore, the 6HB is highly destabilizing of negative phospholipid membrane so its role in destabilizing of the inner leaflet is likely to contribute to fusogenicity.

Another study has tested the effect on humoral immune response of a variety of lipid anchors attached to peptides in or near the MPER region (662-ELDKWASLWNWFNITNWLWYIK-683), applying the reasoning that MPER immunogens are presented in a lipid environment [55]. Lipid conjugation affected peptide secondary structure and partitioning into lipid bilayers in a site and lipid – structure dependent fashion. The authors concluded that improved peptide association with the bilayer was correlated to improved humoral response, especially for the more hydrophilic N-terminal segment 656-NEQELLELDKWASLWNGGK, much as was found for DP and C34 as described above. Lipid structure also played a role, with sterol-anchors providing the greatest adjuvant activity.

Non-specific interactions with the NHR

Surprisingly, attaching a 16-carbon fatty acid to the N-terminus of the peptide DP also improved its potency significantly (C16-DP) [42], although not quite to the level of C-terminal attachment. There is some uncertainty as to the role of this fatty acid group. One possible mechanism is that it enhances local concentration in a similar way to DP-C16, and is effective against a pre-bundle conformation in which the gp41 NHR trimer has moved parallel to the membrane surface rather than perpendicular as modeled in the prehairpin complex [47, 56] (Figure 1C). This concept is supported by the NHR studies described above. However, an alternative mechanism involves fatty acid interaction in the hydrophobic pocket. In support of the latter mechanism, we have recently reported that long chain fatty acid salts can bind to the hydrophobic pocket with sub- μ M affinity [32]. We showed that the degree of negative charge at one end of the molecule as well as the length of the carbon chain contributed proportionately to binding affinity in the pocket (Figure 2, 3). This is in agreement with the observed correlation of lipopeptide potency with length of the fatty acid chain [47]. Palmitic acid ($\text{CH}_3(\text{CH}_2)_{14}\text{COOH}$) has also been observed to bind to a hydrophobic pocket on CD4 [57, 58]. The fact that μ M pocket binding affinity can be achieved by non-specific interactions of fatty acid salts is important in small molecule design. We surmise that it is critical to include amphiphilic character which can satisfy the need for hydrophobic interactions in the grooves of the coiled coil as well as polar (negatively charged) interactions with positively charged residues. Additional interactions with lipid membrane may be key to improving potency. Furthermore, the length of the peptide – peptide interface plays a key role and is likely to be important.

Another example of non-specific but potent binding to the NHR was demonstrated by Vaillant et al [59] who showed that phosphorothioate oligodeoxynucleotides (PS-ODNs) (Figure 3) have sequence – independent activity, resulting from association with the NHR and blocking of 6HB formation. Potency is correlated to the length of the PS-ODN's and requires phosphorothioation. The authors reasoned that the non-specificity of the interaction would lead to a far lower likelihood of resistance development and could provide broad-

spectrum antiviral activity. Viral infectivity inhibition exceeded fusion inhibition to some degree (Figure 3) and the authors indicated that PS-ODN's could bind to the V3 loop of gp120 as well. They have since demonstrated the activity of amphipathic ODN's against hepatitis C entry [60, 61] and human papillomavirus [62].

Polyanionic detergents and dyes

The polyanionic character of PS-ODN's is reminiscent of many tested microbicides that were assumed to act by preventing HIV-1 entry. Many of them show fusion inhibitory activity by targeting HIV-1 gp41 and / or HIV-1 gp120 to prevent productive 6HB formation [63–66]. Their polyanionic character may cause them to interact preferentially with positively charged gp41 NHR. Preclinical and clinical trials of several detergents, including SDS [67] and cellulose acetate phthalate (CAP) [68] (Figure 3) as candidate microbicides were abandoned after they were shown to actually enhance HIV transmission because their surfactant-like properties induced epithelial damage including irritation and vaginal lesions [68, 69]. It appears that CAP might bind non-specifically to the coiled coil since it interferes with antibody binding to the hydrophobic pocket [63]. Most of its activity is considered to be due to gp120 binding.

Highly chromophoric negatively charged compounds ADS-J1 and XTT-formazan were found to inhibit gp41. ADS-J1 was the first small molecule HIV fusion/ entry inhibitor targeting gp41 identified with a molecular modeling-based virtual screen in combination with ELISA [70]. ADS-J1 inhibits HIV-1 mediated membrane fusion and HIV-1 replication with $IC_{50}=4.95\mu M$ and relatively low cytotoxicity (selectivity index: SI= 35). XTT-formazan also showed low μM activity [14]. Computer-aided molecular docking analysis suggested that ADS-J1 and XTT-formazan could be docked into the deep hydrophobic pocket of gp41 [16]. ELISA, competitive inhibition and cell fusion experiments confirmed binding [71, 72]. These molecules are not ideal lead compounds since they contain several sulfonic acid groups and an azo group that may induce tumorigenesis [16]. ADS-J1 has a molecular weight of 1089, problematic for subsequent drug development. Optimization was attempted by removing certain parts of ADS-J1 that were regarded as making no noticeable contacts with the gp41 core structure based on docking results. The resulting structures lost activity; only derivative 4 (Figure 4) with a molecular weight of 724 showed comparable anti-HIV activity to ADS-J1 in cytopathic effect (CPE) and cell fusion assays, but 10-fold reduced activity in a p24 production assay [73]. This suggests an additional mechanism of action for ADS-J1. One study has reported that it binds to gp120 [74]. Derivative 4 demonstrates amphiphilic character with hydrophobic and charged groups at opposite ends of the molecule.

Natural products

Several natural products that have been described as fusion inhibitors are polyphenolic compounds, including tannin, and various tea and medicinal herb extracts [75, 76] (Figure 5). Some of them show interaction with HIV-1 gp41 to prevent 6HB formation [75–77]. Tannin (a heterogeneous mixture of compounds) tested positive in both the 6HB assay and our hydrophobic pocket (HP) binding assay, but two 6HB inhibitors gallic acid and epigallocatechin gallate (GCG, EGCG) from green tea tested negative in the HP assay, although they may bind at an alternative site on gp41 [72]. Oleuropein (Ole) and hydroxytyrosol (HT) extracted from olive leaf were reported to inhibit gp41 fusion core formation in the nM range, with no detectable toxicity [78]. A molecular docking study indicated that Ole and HT could bind to the conserved hydrophobic pocket on the surface of the HIV-gp41 and that the catechol structure present in Ole and HT was the important moiety for binding to the pocket [79]. The exact mechanism of inhibition of HT is uncertain,

since it failed to show any activity in tests of hydrophobic pocket binding using our HP assay at concentrations up to 1.8mM (unpublished data).

Although these and other polyphenolic natural products show HIV-1-cell fusion inhibitory activity and may target different stages of the HIV-1 life cycle, their structure, including lack of homogeneity in some cases, and absence of a well-defined mechanism of action precluded their use for preclinical trials. Polyphenolic compounds bind non-specifically to proteins and peptides through the phenol groups [80]. In general, the non-specificity and / or toxicity of polyphenolic and polyanionic compounds limit their potential as HIV fusion inhibitors, without substantial modifications.

Compounds designed for hydrophobic pocket binding

The hydrophobic pocket, as described by Kim and coworkers, [12] is formed on the surface of the NHR coiled coil by residues from two parallel helices with the sequence 565-LLQLTVWGIKQLQARIL-581. Sequence conservation is high and the pocket is considered to provide approximately half of the energy required for C36 binding [9, 81, 82]. It has been intensely studied as a target for small molecule inhibition of fusion. Figure 6 depicts the hydrophobic pocket and surrounding residues. There are polar and non-polar regions of the pocket at the C- and N-terminal edges respectively, which could provide the chemical complementarity for amphipathic inhibitors. Figure 6 also depicts the variability of side chain positions in an overlay of 5 pdb structures. The effect is to subtly alter the charge distribution and make it difficult to predict with high reliability how a particular inhibitor may bind.

Cyclic peptides

Recently described cyclic D-peptides [83, 84] which built upon early studies [85] have been shown to have very potent activity against HIV fusion. Their target is the hydrophobic pocket. Individual peptides were constructed with IC₅₀ optimized to 33nM. Crystal structures confirmed hydrophobic pocket binding, with all interactions involving pocket residues, and no interactions outside of the pocket. These peptides are critical in proof of principle for inhibition targeting the hydrophobic pocket, since they demonstrate that nM inhibition is possible. This confirms earlier descriptions of the hydrophobic pocket as a hotspot for inhibiting the protein – protein interaction between the NHR and CHR [12, 18, 20]. D-peptide constructs with sub-nanomolar potency were generated by tethering two or three monomers to form a multivalent inhibitor. It was reasoned that substantially increased potency was due to the multiplicative effect on inhibition constant of targeting symmetrical binding sites around the coiled coil. The fact that a polyethylene glycol cross-linker was used as the tether may confer membrane binding potential, although the authors did not suggest or confirm this, assuming instead the multiplicative effect of an inhibitor binding in more than one pocket.

Small molecule hydrophobic pocket inhibitors

It was recognized that small molecules targeting the hydrophobic pocket typically have a negative charge, which could form an electrostatic interaction with either Lys574 or Arg579' [86, 87]. A review of the literature and our own work suggests that when the molecules are small enough to be fully contained within the cavity (about 400Da or lower), an amphiphilic structure is required, with one end of the molecule supporting the carboxylate group, and the other end of the molecule being non-polar. A subset of thioxothiazolidine – furan containing compounds screened by S. Jiang's group [88] and a subset of indole compounds developed in our lab [89] illustrate this point. These molecules were generated from initial fragment-like hits by screening and synthetic optimization (Figure 7). The small compounds,

including carboxylphenylpyrroles such as **NB-2**, **NB-64** [90] and **A12** [86], **M1** [87] and **I-1** [89, 91] are too small to be described as amphiphilic, but required carboxylate groups for activity.

Examples of the amphiphilic effect are shown in Table 2. This is by no means an exhaustive list of the compounds that have been tested [88, 92] (Zhou and Gochin, submitted)[91], but is culled from available data for which cell fusion inhibition has been reported. Compounds where R₁ or R₃ contains a carboxylate are not active, while similar molecules with non-polar R₁, R₂ and / or R₃ show low μM activity. This is perhaps a reflection of the fact that the non-polar end of the molecule is buried into the cavity. Additional carboxylates on the same side of the molecule do not necessarily abrogate activity, as in the example **NB-225** (Table 2). A similar result was obtained for peptidomimetic inhibitor **11{6,11}** (Figure 7) [72] and a benzamide series of fusion inhibitors [93] (Figure 8). Other small molecules have been described that lack an obvious negative charge, with fusion inhibition in the tens of μM [94, 95]; these are not discussed here.

Larger molecules, developed from the compounds listed in Table 2 or from other sources are shown in Figures 7 and 8. Some of them have improved binding affinity and / or cell fusion activity. These molecules are likely to occupy the pocket more fully. They include furan **12l** [96], a derivative of the thioxothiazolidine – furan series described above, indole **14g**, an indole inhibitor from our lab [91], a peptidomimetic terphenyl derivative [97] and **benzamide 11** [93] (Figure 8). A strong correlation between inhibition of 6HB and inhibition of fusion was observed for these compounds, confirming that activity involved disruption of the fusion core. Competitive inhibition studies [72] confirmed the hydrophobic pocket as the binding site for the indole **14g** and **benzamide 11**, and an NMR study of a related benzamide triacid demonstrated the binding mode, including hydrogen bonds between ligand carboxylates and Ser640' and Lys574 in the pocket, with the non-polar biaryl moiety projected into the hydrophobic cavity [93]. Interestingly, furan **12l** and other derivatives in the series [96] demonstrated very high potency against HIV-infectivity [92], more than two orders of magnitude higher than their ability to inhibit fusion [15], suggesting that these molecules have a secondary mode of action. The structure of the molecules in Figure 8 continue to demonstrate amphiphilic character with separated polar and non-polar functional groups, with the exception of the boloamphiphilic terphenyl compound **1a** that may be able to reach across the pocket and connect with both Lys574 and Arg579' [98]. This tri-functionalized 3,2',2''-terphenyl derivative is a helical peptidomimetic that projects hydrophobic side chains at the equivalent of the *i*, *i*+3, *i*+7 positions of the W-W-I motif in the CHR helix that is known to interact in the hydrophobic pocket. The carboxylates were essential for activity although data for a molecule with one carboxylate was not provided.

Small molecule gp41 inhibitors targeting the gp41 loop and fusion peptide

Early studies showed that a betulinic acid derivative **RPR103611** (Figure 9) inhibited HIV-1 replication at μM levels through inhibition of gp41 [99, 100]. Resistant strains were found to have a mutation in the loop region at I595S. Recently, a low molecular weight benzene sulfonamide **PF-68742** (Figure 9) was described with $\sim 200\text{nM}$ activity in cell fusion and antiviral assays [101]. A G514R mutation in the FP sequence rendered HIV strains resistant to **PF-68742** and reduced infectivity of the virus to 10% of WT, but did not appear to affect the ability of **PF-68742** to bind. At low levels of the compound, negative inhibition of G514R mutant virus was observed. G514R mutant virus was also 10 fold more sensitive to T20, suggesting disruption of the pre-fusion conformation of Env and overexposure of the T20 binding site. The authors found that escape viruses retaining infectivity and WT sensitivity to T20 had mutations in the disulfide loop (DSL) region (residues 599–619) consistent with a binding pocket in this region. This reinforces the role of the DSL in fusion

and suggests a functional link between the DSL and FP sequences that may involve gp120 and lipid binding.

Challenges and future directions for drug development

The relatively low activity in the sub- to low μM range for small molecules targeting the hydrophobic pocket may reflect an intrinsic inability to inhibit formation of the NHR - CHR protein – protein interface with a small molecule, or could be the result of study limitations, including scant evidence that most described inhibitors actually target this pocket, a lack of structural data defining the interaction and the quality of the chemical libraries from which inhibitors have been found. Chemical phenotypes in current libraries were generated from past classical drug discovery processes. This new class of targets involving protein-protein interactions requires new chemical entities [102]. The known inhibitors of protein-protein interactions do not show high similarity to any set of compounds developed against traditional targets. They typically do not follow the Lipinski rule of 5, having larger molecular weight and lower polarity [103]. It seems that fragment screening may be more successful than traditional HTS when applied to protein-protein interfaces [104, 105]. Several successful outcomes have been achieved by using fragment screening in inhibition of protein-protein interactions [103, 106]. In theory, fragments (150~200Da) have higher ligand efficiencies than typical compounds. In the case of gp41, no attempts have been reported. Furthermore, no accounts to date have considered the role of lipids in modulating the protein – protein interaction, as appears to occur in this case (see above).

Structural data is critical in order to move forward with ligand optimization in the pocket, especially considering the flexibility of the side chains involved in a protein – protein interaction [106, 107]. There are 170 protein data bank entries for gp41 [108], and they illustrate the variability typical of a protein - protein interface, as demonstrated in Figure 6. The base of the pocket, including main chain atoms and residues internal to the coiled coil, is invariant between structures, but side chains of surrounding residues adopt multiple conformations, likely associated with differences in structural resolution, length of the peptides used in structure determination, and the induced fit associated with a bound inhibitor or peptide. There is no crystal structure of a small molecule complex with the hydrophobic pocket of gp41. The reason maybe due to limitations of the inhibitors discovered, such as low solubility, low binding affinity or uncertainty of the binding mechanism. Direct information on binding poses has been obtained from NMR studies in three instances [87, 93, 107], which help to suggest routes for optimization.

Other reviews in this issue suggest additional targets involving other regions of gp41. For structure-based drug design, structures of gp41 MPER peptides with antibodies offer an interesting starting point for small molecule mimetics (see review by Gach et al in this issue). A high resolution structural study of gp41 containing both the FPPR and the lipid binding MPER domain, but missing the FP and loop region, indicates that a long rod-shaped coiled coil is formed in which FPPR and MPER interact [109]. The coiled coil is slightly splayed at the end with several MPER residues positioned to interact with the lipid membrane. This is considered to be the post-fusion conformation. Other studies indicated that FP and MPER regions could associate, perhaps in stabilizing the pre-fusion conformation [44] (see Huarte et al in this issue). A recent computational study of the molecular details of the interaction of T20 with gp41 containing complete pre-TM with FP in explicit DOPC membrane [110] suggested strong interactions between the C-terminal region of T-20 and both lipids and an extended N-helical coiled coil. Extension of the N-terminal coiled coil into the FPPR has also been demonstrated in peptide studies [111]. Structures of MPER peptides with cognate antibodies have revealed diverse structural features, only some of which are consistent with α -helical structure (Gach et al review in this

issue). Thus consideration of lipid interactions and conformational flexibility under different conditions and at different time points during fusion is critical in inhibitor design.

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Figure 1. Schematic illustration of the gp41 conformational rearrangement accompanying fusion Shown in dark and light gray, respectively, are the NHR and CHR domains. The fusion peptide and polar segment are shown as solid lines attaching NHR to the cell membrane, the MPER and TM domains as gray lines anchoring CHR to the virus, and the viral and host cell membranes in shades of gray. The loop region links the NHR and CHR. A. Depiction of the metastable conformation (resting state) of the gp41 – gp120 complex. The NHR and CHR domain structure is unknown; extensive glycosylation of gp120 indicated by Y. B. Transient fusion intermediate with exposed coiled coil domain, and fusion peptides inserted into the host cell membrane. Sites for small molecule binding (hydrophobic pocket and loop region) are indicated. C. Snapshot along the path of the conformational rearrangement bringing the viral and host cell membranes into close apposition for fusion. D. trimer of hairpins (six helix bundle) conformation required for fusion.

Figure 2. Affinity (K_d) of various surfactant molecules to the gp41 NHR binding pocket as a function of carbon chain length and head group ionic strength
(●)R-SO₄⁻; (■)R-SO₃⁻; (▲)R-COO⁻; ■: R is saturated hydrocarbon chain of indicated length. Error bars are standard deviation of 4 independent measurements. (Reprinted with permission from Gochin and Cai, J. Med. Chem. 52(14), 4338-44, 2009)

Figure 3. Non-specific inhibitors of gp41

Compounds are polyanionic and / or amphiphilic. Activity data is defined as follows: K_I (HP) = inhibition constant obtained in hydrophobic pocket binding assay [72]; IC_{50} (6HB) = IC_{50} obtained in ELISA assay for the 6HB [112]. EC_{50} (CCF) is the compound concentration required to inhibit cell-cell fusion by 50%. EC_{50} (p24, BaL / IIIB) is the compound concentration required to inhibit viral replication by the indicated virus subtype.

Figure 4. Fusion inhibitor ADS-J1 and a partially active derivative
See caption to Figure 3 for activity definitions.

Figure 5. Examples of natural products described as HIV fusion inhibitors

The compounds are polyphenolic. TGGP is a substructure of tannin [113]; GCG is a catechin containing compound from green tea[76]. Ole and HT were extracted from olive leaf and further work on these compounds has not been described. See caption to Figure 3 for activity definitions.

Figure 6. The hydrophobic pocket on the coiled coil of HIV-1 gp41

The pocket is formed by residues 565–581 of two NHR chains. Residues surrounding the pocket are shown explicitly for 5 PDB structures, 2R5D, 2R5B, 2KP8, 1IF3 and 3P7K, overlaid by matching NHR backbone atoms. HXB2 numbering is used. A hydrophobic patch is formed by residues Leu565', Leu568', Val570 and Trp571' and a polar patch by residues Gln575', Gln577 and Arg579'. Additional positive charge is provided by Lys574, the side chain of which does not point towards the pocket in all structures. The partially transparent surface is depicted for 3P7K.

Figure 7. Low molecular weight specific inhibitors

A. Development of a series of carboxyphenyl pyrrole inhibitors [86, 92], and B. fragmentation of a peptidomimetic inhibitor **11{6,11}** with poor solution behavior and development into indole inhibitors [89]. See caption to Figure 3 for activity definitions.

Figure 8. Larger designed inhibitors

Binding and cell fusion (CCF) data for larger molecules suggested to occupy the hydrophobic pocket more fully. Compounds are identified by a central structural feature of the scaffold. See caption to Figure 3 for activity definitions. SI = Selectivity Index = CC_{50} / EC_{50} (CCF).

Figure 9. Inhibitors targeting the loop region of gp41
See caption to Figure 3 for activity definitions.

Table 1The effect on activity of fatty-acid or cholesterol modifications of gp41 N- and C-peptides[†]

Peptide	Sequence	EC ₅₀ (nM) [*]
T20	(638)-YTSLIHS ^{LI} EE ^{SQ} NQ ^Q EKNEQELLELDK WASLWNWF -(673)	0.44 ^(a) ;
DP	YTSLIHS ^{LI} EE ^{SQ} NQ ^Q EKNEQELLE	2864 ^(a)
DP-C16	YTSLIHS ^{LI} EE ^{SQ} NQ ^Q EKNEQELLEK- C16	1.3 ^(a)
C16-DP	C16 -YTSLIHS ^{LI} EE ^{SQ} NQ ^Q EKNEQELLE	40 ^(a)
T20	(638)-YTSLIHS ^{LI} EE ^{SQ} NQ ^Q EKNEQELLELDK WASLWNWF -(673)	0.67 ^(b)
T20-chol	YTSLIHS ^{LI} EE ^{SQ} NQ ^Q EKNEQELLELDK WASLWNWF -GSGC-chol	3.7 ^(b)
C34	WMEWDREIN NYTSLIHS ^{LI} EE ^{SQ} NQ ^Q EKNEQELL	0.21 ^(b)
C34-chol	WMEWDREIN NYTSLIHS ^{LI} EE ^{SQ} NQ ^Q EKNEQELL-GSGC-chol	0.004 ^(b)
chol-C34	chol-CGSG- WMEWDREIN NYTSLIHS ^{LI} EE ^{SQ} NQ ^Q EKNEQELL	9.5 ^(b)
T20	(638)-YTSLIHS ^{LI} EE ^{SQ} NQ ^Q EKNEQELLELDK WASLWNWF -(673)	7.4 ^(c)
T1249	WQEW EQKI-----TALLEQAQIQ ^Q EKNEYELQKLDK WASLW EW F	2.15 ^(c)
N36	(546)-SGIVQQ ^Q NNLLRAIEAQQHLLQLTVWG ^{IK} QLQARIL-(581)	488 ^(d)
C16-N36	C16- SGIVQQ ^Q NNLLRAIEAQQHLLQLTVWG ^{IK} QLQARIL	72 ^(d)
N36-C16	SGIVQQ ^Q NNLLRAIEAQQHLLQLTVWG ^{IK} QLQARILK-(δNH- C16)	259 ^(d)

[†] Hydrophobic pocket binding domain bolded in dark gray, lipid binding domain bolded in gray, chol = cholesterol, C16=16 C-atom hydrocarbon chain

^{*} fusion or viral infectivity inhibition varies per study; data is provided on unmodified T20, C34 or N36 for reference

^(a) Wexler-Cohen et al, 2007 [42]

^(b) Ingallinella et al, 2009 [46]

^(c) Eggink et al, 2009 [37]

^(d) Wexler-Cohen and Shai, 2009 [47].

Table 2

Amphipathic properties of low molecular weight μM inhibitors of gp41*

Name	R ₁	R ₂	Mol. Wt.	IC ₅₀ (μM) [†]	Name	R ₁	R ₃	Mol. Wt.	IC ₅₀ (μM) [†]
NB-179	CH ₂ CH ₃	H	359.4	3.7±0.2	1	H	H	251.1	4.7±0.6
NB-180	CH ₂ COOH	H	389.4	-	2	p-OMe-benzyl	H	371.4	3.7±0.5
NB-214	CH ₂ CH ₃	2-Cl	393.9	2.2±0.08	3	H	-COCOOH	323.3	-
NB-225	CH ₂ CH=CH ₂	3-COOH	415.5	34±0.53	4	m-carboxybenzyl	H	385.4	-
NB-228	CH ₂ COOH	6-CH ₃	403.4	-	6	m-carboxyphenyl	H	371.4	-
NB-235	CH ₂ COOH	4-Cl	423.9	-	7	p-OMe-phenyl	H	357.4	1.8±0.2

* ≤424Da

[†] for cell fusion, " - " means IC₅₀ > 100 μM , References [88,91,96]