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Inhibition of HIV Entry by Targeting the Envelope Transmembrane Subunit gp41

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Abstract

Background—The transmembrane subunit of the HIV envelope protein, gp41 is a vulnerable target to inhibit HIV entry. There is one fusion inhibitor T20 (brand name: Fuzeon, generic name: enfuvirtide) available by prescription. However, it has several drawbacks such as a high level of development of drug resistance, a short-half life *in vivo*, rapid renal clearance, low oral bioavailability, and it is only used as a salvage therapy. Therefore, investigators have been studying a variety of different modalities to attempt to overcome these limitations.

Methods—Comprehensive literature searches were performed on HIV gp41, inhibition mechanisms, and inhibitors. The latest structural information was collected, and multiple inhibition strategies targeting gp41 were reviewed.

Results—Many of the recent advances in inhibitors were peptide-based. Several creative modification strategies have also been performed to improve inhibitory efficacy of peptides and to overcome the drawbacks of T20 treatment. Small compounds have also been an area of intense research. There is a wide variety in development from those identified by virtual screens targeting specific regions of the protein to natural products. Finally, broadly neutralizing antibodies have also been important area of research. The inaccessible nature of the target regions for antibodies is a challenge, however, extensive efforts to develop better neutralizing antibodies are ongoing.

Conclusion—The fusogenic protein, gp41 has been extensively studied as a promising target to inhibit membrane fusion between the virus and target cells. At the same time, it is a challenging target because the vulnerable conformations of the protein are exposed only transiently. However, advances in biochemical, biophysical, structural, and immunological studies are coming together to move the field closer to an understanding of gp41 structure and function that will lead to the development of novel drugs and vaccines.

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INTRODUCTION

Structure of gp41 in the Env Complex

HIV-1 is an enveloped virus with 8-10 envelope 'spike' complexes per virion [1]. The envelope spike is made up of two protein subunits, the surface subunit, gp120, and the transmembrane subunit, gp41. These two subunits form a trimer of heterodimers that are non-covalently associated on the virus or on infected cells and are attached to the membranes *via* the transmembrane region of gp41.

The envelope complex is expressed as a precursor protein encoded by the envelope gene of HIV-1. It is expressed into the endoplasmic reticulum and transits the Golgi apparatus during which 25-30 N-linked glycans are added and trimmed [2-4].

The surface subunit, gp120, has a globular structure composed of five conserved domains (C1-C5) and five variable loops (V1-V5) [5, 6]. Gp120 has 18 cysteine residues, which form a loop structure connecting V1 to V4 by disulfide bonds [7]. These highly glycosylated variable loops shield the conserved regions of gp120 and protect the virus from antibodies. This is a protective barrier that the virus utilizes to evade the immune system, which is often referred to as the glycan shield [8].

Gp41 is divided into multiple functional domains (Fig. **1**). Beginning at the N-terminus, there is a fusion peptide, which is necessary for membrane fusion. Moving toward the C-terminus there are two helical heptad repeat (HR) regions, which are designated N-terminal heptad repeat (NHR) and C-terminal heptad repeat (CHR). These two regions are connected to a loop region that is more mobile than the helical heptad repeat regions and also contains an important disulfide bond [9-12]. The CHR is followed in sequence by a membrane proximal external region (MPER). This region has been a very promising target for drug and immunogen development as it contains epitopes that bind some of the neutralizing antibodies that have been identified such as 2F5, 4E10, Z13, and 10E8 [13-20] (see below). Next in sequence is a highly conserved transmembrane domain (TM) of 22 amino acids followed by a C-terminal cytoplasmic region (Fig. **1**).

Atomic level structures of portions of HIV gp41 larger than single domain studies were limited for many years to the ecotodomain in a six-helical bundle, hairpin-like conformation, which researchers in the field consider to be the post-fusion structure. Of these, there were several x-ray crystallographic structures made up of the core sequences of the gp41 NHR/CHR regions of the gp41 ectodomain either incubated together as individual peptides, and allowed to form the 6HB, or tethered covalently, and there was one NMR structure that included the NHR, the loop region, and the CHR [21-27]. The 6HB conformation is made up of three NHR regions, which bind together in parallel forming a three helical bundle. Three CHR regions wrap around in an antiparallel manner, each CHR coming into contact

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with two of the NHR helices due to the oblique angle of the CHR regions. This results in the disulfide-bonded loop region of gp41 forming the top of a hairpin-like structure.

In 2010, a crystal structure was reported that included sequences further toward the fusion peptide and further toward the viral membrane including the MPER [28]. While most of the structure showed a coiled-coil conformation, terminal sections near the fusion peptide and the viral membrane were not in a canonical coiled-coil, and several residues were situated so that their aromatic side chains would be oriented toward what would be the viral membrane. Interestingly, prior computational work [29] predicted the importance of peptide inhibitor-lipid interactions in what would be an MPER-like bound state.

A construct known as the BG505 SOSIP.664 gp140 trimer was crystallized in complex with a broadly neutralizing antibody (PGT122) and the structure was solved to 4.7 Å [30]. Very briefly, this is a construct that includes gp120 and terminates before the transmembrane region of gp41. There is a disulfide bond inserted between gp120 and gp41 and some of the residues from MPER have been deleted. Interesting findings include a similarity in structure between the internal three helix bundle made up of gp41 NHR and the same portion of the trimer in previous atomic level structures of the 6HB. Also, the authors note the presence of a "hole" in the electron density that they mention is consistent with that observed for the influenza and ebola fusion proteins. The 3HB section (NHR) is stated to be the location of stabilizing contacts between gp41 and gp120 in this structure.

Crystal structures were solved to 3.5 Å in 2014 in complex with two neutralizing antibodies (PGT122 and 35O22) again using the envelope complex mentioned above, BG505SOSIP. 664 [31]. The addition of the second antibody (35O22) helped researchers to obtain crystals that diffracted to the higher resolution. The higher resolution allowed the authors to detail very interesting portions of gp41 such as a 4 helix structure termed a "collar" that appears to hold the N- and C- termini of gp120 in a clasp or as the authors call it, a "tryptophan sandwich." This work also allowed for valuable modeling and detailed comparisons of glycan shield integrity and immune evasion between what the authors identify as their "pre-fusion mature closed state" structure to the previous structures widely considered to be post-fusion structures.

It has been suspected for many years that HIV Env functions by undergoing large, transient conformational changes. The underlying structural changes are propagated over long distances as compared to better-studied enzymatic function. Indeed, this is what has made the study of the conformational changes responsible for HIV entry, especially with regards to the membrane protein gp41, extremely challenging and also what helps to protect the virus from the immune system. HIV Env is also very sensitive to the addition of fluorescent proteins or tags that have been widely utilized to tease out the details of conformational changes in various other systems. Researchers are making advances, however, and single molecule FRET studies have revealed some very interesting clues to the details of the Env conformational changes [32, 33]. Observations of envelope from smFRET with labeled gp120 showed three distinct states representing dynamic prefusion conformations. The occupancy of these states was reported to shift upon addition of CD4 and the co-receptor mimic, antibody 17b. By studying the sampling of the three states and the effect of addition

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of CD4 and 17b in isolation and in combination, the authors were able to produce data that verify the two-state nature of the activation mechanism of Env. Interestingly it was also apparent that the conformational shift from a low FRET or "closed ground" state occurred less frequently with a primary HIV-1 strain (JR-FL) than with a lab-adapted strain (NL4-3). These data correlate well with the capacity these strains have for neutralization with the lab-adapted strains being much more susceptible to neutralization.

Hydrogen-deuterium exchange coupled to mass spectrometry has also been utilized to study the conformational sampling of Env [34]. Utilizing the soluble SOSIP.664 construct briefly described above and broadly neutralizing antibodies, these results also suggest that there is transient sampling of an 'open state' conformation and that the kinetics of this sampling is important to the capacity of antibodies to neutralize the virus. Both smFRET and hydrogen-deuterium exchange studies suggest that broadly neutralizing antibodies are recognizing a 'closed' or 'ground' state conformation of envelope and are functioning to neutralize the virus by stabilizing this state of envelope [33, 34].

Recently, the first x-ray crystallographic study with an envelope construct that is unliganded was solved [35]. Prior to this publication, structures were liganded with CD4 or a variety of antibodies. This report presents a combination of analyses that are illustrative of how structural knowledge and antigen studies can be combined to inform immunogen design. The authors use "antigenicity-guided conformational fixation" to search for a construct that will be fixed in a conformation that binds only to the most effective neutralizing antibodies and not to those that are poorly neutralizing. One of the most interesting findings of this study was evidence that the entry pathway may be asymmetric with an initial step in which a single CD4 molecule binds followed by the binding of additional CD4 molecules. The initial CD4 binding did not initiate the conformational changes considered the classical changes seen upon CD4-binding (*i.e.*, the formation of a bridging sheet in gp120), however, subsequent CD4 binding did produce these changes [35]. Although there is not a lot of information regarding the structure or structural changes specific to regions of gp41, the identification and isolation of an intact ground state trimer is a very important advancement to potential future structural and mechanistic studies involving gp41.

A key set of findings related to structural aspects of Env and specifically important for gp41 has come from an intricate set of biochemical experiments. Crooks *et al.* utilized enzyme digests followed by detailed gel analysis as a way to analyze for and eventually eliminate aberrantly folded or "junk" envelope [36]. These include a gp160 that is retained in the endoplasmic reticulum and not fully matured, a mature gp160 that is not fully cleaved at the furin cleavage site between gp120 and gp41, and trimeric and monomeric gp41 stumps along with variations in glycosylation. These aberrant forms of Env are hypothesized to act as decoys for the virus eliciting non-neutralizing antibodies. Tong *et al.* were able to show that enzyme digests could be utilized to produce virus-like particles bearing native Env trimers, which were only able to be recognized by neutralizing antibodies [37]. The varied population of variants of Env is an important aspect of consideration in the design of targeted inhibitors and also in the design of potential immunogens.

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HIV Entry Mechanism

HIV-1 entry is initiated by binding of gp120 to CD4 which is a primary receptor for HIV-1 infection on the target cell surface [38-42]. Association of the conserved domains of gp120 to the CD4 receptor causes rearrangements of the V1 and V2 loops, and then the V3 loop is able to bind to the co-receptor, either chemokine receptor 5, CCR5 (CD195 [43, 44]) and C-X-C chemokine receptor 4, CXCR4 (fusin or CD184 [45, 46]). Receptor binding leads to exposure of hydrophobic regions of gp41 and insertion of the fusion peptide into the host cell membrane.

Upon insertion of the fusion peptide of gp41 into the host cell membrane, the NHR and CHR regions of gp41 come together in an antiparallel fashion forming the hairpin-like six-helix bundle (6HB) conformation of gp41 which is exceptionally hyperthermostable [22]. 6HB formation is thought to be the driving force that brings the viral and cellular membranes in close proximity to facilitate fusion (reviewed in [47, 48]). Initial contact is of the outer leaflets of the viral and cellular membrane, which undergo lipid mixing followed by small pore formation. As merging of the two membranes progresses, the fusion pore enlarges and finally, the viral core enters into the cell cytoplasm [49-54]. It has not been definitively shown whether one or multiple trimers are needed for membrane fusion and this is an area of ongoing debate in the field [48, 49].

NHR- and CHR-Based Peptides

Peptide inhibitors made up of sequences from NHR or CHR (Table **1**) when added exogenously bind to form heterogeneous 6HB molecules preventing a collapse of gp41 to the stable 6HB conformation and thereby, inhibiting membrane fusion and viral entry or cell-cell fusion [55-58] (for a recent review see [59]). Various fusion inhibitors targeting pre-hairpin intermediate structures have been under development [55, 56, 58-61]. Entry inhibitor studies also have been very important in helping to shed light on how gp41 mediates membrane fusion [62-71].

Although there are NHR-based peptides such as N36, N42, and N36F10, investigators have placed a greater focus on the development of CHR-based peptides [27, 72, 73]. The primary reason is that CHR-peptide inhibitors are monomeric in solution and show a higher potency, whereas, NHR-peptides tend to aggregate in solution and exhibit lower potency [74].

The pre-hairpin intermediate structure is the target of the only fusion inhibitor in the clinic. The inhibitory peptide T20 (brand name: Fuzeon, generic name: enfuvirtide) is a peptide sequence from the CHR/MPER regions (residues 638-673) of gp41 [55, 57, 63], which was approved by the US FDA as an injectable HIV-1 fusion inhibitor in 2003. Since that time, many other peptides have shown great promise and are in continuing development (reviewed in [59, 75]).

There is a need to develop novel fusion inhibitors that have higher potency than T20, are active against T20 resistant viruses, and exhibit a higher genetic barrier to resistance. To fulfill this mission, many researchers have focused on the highly conserved hydrophobic pocket of gp41. The notion behind this is that the pocket is at the c-terminus of the NHR trimer and is bound to the pocket-binding domain (PBD) in the six helix bundle, made up of

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residues W628, W631, and I635 [22, 76, 77]. However, the T20 sequence does not include the PBD so that this inhibitor does not take advantage of the interaction between the NHR and PBD. Examples of next generation fusion peptide inhibitors that include the PBD are T1249 [78, 79], T1144 [80], TLT35- a chimera protein of T20 and T1144 [81], and 2DLT-a bivalent recombinant protein of T1144 and the CD4 D1D2 domains [82, 83].

T1249 and T1144 were designed to overcome T-20 resistance. T1249 is composed of gp41 sequences derived from HIV-1, HIV-2, and SIV and this construct exhibited anti-HIV potency to T20 resistant isolates [78, 79]. T1144 was designed to have enhanced helical structure with multiple substitutions of amino acid residues from T2410, which is a peptide that begins with Met and Thr followed by the C34 sequence [80]. This peptide was active against T20- and T1249-resistant HIV strains [80], however, rapid proteolysis of the peptide inhibitor *in vivo* and high cost of the peptide synthesis remain issues.

To solve these problems, two chimeric proteins were created. TLT35 is a chimeric protein based upon T20 and T1144, which is large enough to be produced in *E. coli*. This can be produced with lower cost than synthetic peptides. TLT35 exhibited anti-HIV activity in the low nM range against lab-adapted strains, T20 resistant strains, and primary isolates of a variety of clades [81]. There was ~10-100 fold increased antiviral potency compared to T20 and T1144 alone. TLT35 exhibited notably increased stability in PBMC culture (the half-life was increased ~18 fold over that of T20) and in the presence of proteinase K and pepsin (the half-life was increased 8-35 fold over that of T20) [81].

Another bivalent chimeric protein called 2DLT was made up of T144 and the D1D2 domains of CD4. 2DLT was also produced in *E. coli* and showed promising antiviral potency [82]. However, the mechanism by which this construct inhibits is unique compared to most peptide inhibitors. It is able to trigger pre-exposure of vulnerable gp41 regions before viral fusion occurs. The D1D3 fragment binds to gp120 of HIV, it triggers conformational changes and exposure of gp41 and then another fragment, T1144 binds the exposed gp41 region and results in rapid inactivation of cell-free virus [82]. Importantly, 2DLT resulted in synergistic effects in combination with other antiretroviral drugs [83].

Sifuvirtide (SFT) is the most thoroughly studied peptide inhibitor after T20 [84-88]. It was designed to have a unique 36 amino acid sequence different from the previously studied peptide inhibitors (C34, T20, and T-1249). It has 16 residues that are different than in C34 (underlined below in the SFT sequence: SWETWEREIENYTRQIYRILEESQEQQDRNERDLLE), 22 residues different from T20 (underlined in SFT sequence: SWETWEREIENYTRQIYRILEESQEQQDRNERDLLE), and 24 residues different from T-1249 (underlined in SFT sequence: SWETWEREIENYTRQIYRILEESQEQQDRNERDLLE) [84]. The peptide maintains the gp41 pocket binding sequence that is included in C34, but that T20 is lacking. SFT is missing, however, the lipid binding domain that is present in T20. As such, SFT shares more characteristics in common with C34 than with T20 such as a random coil conformation pattern, secondary α-helix formation in the presence of N36, inability to interact with the lipid membrane, and formation of a 6HB with the N36 peptide [84]. However, the additional residues over the base sequence of C34 that are contained in SFT seem to provide the

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capacity for SFT to form a tighter 6HB with N36 than the 6HB formed by N36 and C34 (Tm of N36/SFT = 72°C > Tm of N36/C34 = 62°C), which will provide stronger activity against NHR [84]. This peptide produced promising results both *in vitro* and in clinical studies [84, 86]. It exhibited high potency against a wide range of HIV strains including T20-resistant strains [86, 88] and showed improved clinical pharmacokinetics characteristics when compared to T20 in a clinical trial reported in 2014 [89].

Modified Peptides

CHR peptides have exhibited great promise as inhibitors, however, there are still many problems in using peptide inhibitors as therapeutic agents. Peptides have short half-lives due to degradation by cellular enzymes and rapid renal clearance. There is also limited oral bioavailability so injections are necessary and compliance becomes an issue especially when there are injection site reactions as is the case with T20. Peptides are also very expensive to produce and require specialized storage methods such as either refrigeration or reconstitution with solvent. Thus, investigators have been trying to overcome these problems by modifying peptide inhibitors with a variety of design strategies.

Covalent Peptides—Covalent inhibitors are designed specifically to bind permanently to their targets of inhibition (for a general review on covalent drugs, see [90]). Our group applied this concept to gp41 fusion peptide inhibitors. We designed covalent inhibitors to trap the vulnerable gp41 fusion intermediate permanently [71, 91] using a base C34 peptide. We tested covalent CHR peptide inhibitors made up of residues 628-661 of gp41 [22, 26, 27]. Placement of the maleimide reactive group was based upon the atomic level structures of the HIV gp41 ectodomain, which are considered to be the postfusion structure of gp41 [10, 92, 93]. The maleimide modification of the peptide inhibited gp41 mediated cell-cell fusion [71] and virus-cell fusion [91] after extensive washing by making the association between the peptide and gp41 permanent as shown by extensive washing followed by functional assays.

Albuvirtide (ABT, also known as FB006) is another C34-based covalently reactive peptide, which is chemically modified with 3-maleimidopropionic acid on the Ser^{640} residue after it had been mutated to a Lys [94]. This construct was able to form secondary α-helical structures with N36 with slightly higher thermostability (Tm of ABT/N36=56°C) than C34/N36 (Tm=54°C). It also blocked formation of the 6HB between C34 and N36 in a dose-dependent manner and inhibited gp41 mediated cell-cell fusion as well as virus entry with more anti-HIV potency than T20 [94]. However, it is not yet known which residues of NHR react with the maleimidopropionic acid group of ABT [94].

Recently, an alternative reactive group has been utilized to create a covalent linkage. A thioester was placed onto C34 in order to initiate an acyl transfer reaction. This is projected to be a better mimic of what is happening under physiological conditions when polypeptides are synthesized [95, 96]. In the process of nonribosomal peptide synthesis, a peptide bond is formed between a carboxy-thioester of the upstream substrate and a free amine on a downstream amino acid by 4’ phosphopantetheinyl cofactor (reviewed in [97]). This acyl transfer reaction is able to form a covalent bond between the modified C34 and the NHR

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resulting in high potency anti-HIV activity, which correlated to increased thermostability of 6HB when studied *in vitro* [95, 96].

The benefits of covalent peptides are not limited to improving efficacy of inhibition against gp41. This design methodology can also be used in a variety of areas such as improving drug delivery by attaching molecules to prolong the half-life of the peptides. One such strategy was utilized by attaching human serum albumin [98, 99] to the peptide inhibitor C34. Surprisingly, this did not result in a loss of potency as was expected because the conformational space available between the cellular and viral membranes was thought to be restrictive to something as big as Albumin-C34 (~MW 72 kDa). Inhibition potency was the same as the peptide alone and animal studies showed that the inhibitor efficacy was three fold greater with the albumin attachment.

Constructs that entrap the fusogenic intermediate are also promising tools for mechanistic studies of viral entry with the potential to allow for localization, kinetic, and biophysical studies of the intermediate. A covalently trapped gp41 intermediate also has the potential to elicit novel immune responses. It will be important to carefully consider the increasing amount of structural, biophysical, and immunological information regarding envelope conformational changes when designing these strategies for gp41.

D-Peptides—Investigators designed D-peptide inhibitors, which are protease-resistant to overcome the sensitivity of L-peptide inhibitors to proteolytic degradation. These were designed to increase the half-life in blood and to increase the potential for oral bioavailability. The first D-peptide, D10-p5-2K identified by mirror-image phage display inhibited both gp41-mediated cell-cell membrane fusion and viral entry [77]. However, the potency of D10-p5-2K was only in the micromolar range.

The next generation of D-peptides were discovered by modified mirror-image phage display screening. The D-peptide, PIE7, was reported to have high anti-HIV potency (picomolar range) in its trimeric version against many HIV strains [100]. However, the PIE7 trimer was only moderately potent for the neutralization-resistant clinical isolate JR-FL (IC_{50}=220 nM) compared to HXB2 (IC_{50}=250 pM) or BaL (IC_{50}=650 pM).

The third generation of D-peptide PIE12 trimer showed improved potency against diverse pseudotyped viruses (including clades A-D, circulating recombinant forms, and T20-resistant strains) and replication-competent viruses [101, 102]. Importantly, PIE12 trimer exhibited a better potency for JR-FL (about 4 times better for a pseudotyped virus and 2 times better for a replication-competent virus) than PIE7 trimer [102]. It also displayed better inhibition than T20 in all virus strains that were tested. This improved efficacy of the PIE12 trimer was attributed to a high affinity for the gp41 pocket as the Tm value of PIE12 trimer from its *in vitro* binding target IZN17 was measured to be 81°C (compared to the Tm of IZN17+PIE7 trimer = 73°C) [102].

Cholesterol-Conjugated Peptides—Another valuable modification that has been made is the covalent addition of cholesterol to a peptide inhibitor. The cholesterol group was chosen as a lipid anchor for the C34 peptide inhibitor based on the importance of its role in

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HIV fusion [103]. Cholesterol is highly enriched in the lipid membranes of HIV [104-106] and CD4 receptor is enriched in lipid rafts where cholesterol is prevalent [107]. The membranotropic nature of the cholesterol-conjugated C34 (C34-Chol, also known as DS007 or L'644) enhances the interaction between the conjugated peptide and the biological membrane where viral membrane fusion occurs [108].

Indeed, this type of modification provided C34-Chol with ~25-100 fold more potency than C34, ~50-400 fold more potency than T20, and ~15-300 fold more potency than T1249 in multiple isolates *in vitro* [103]. Further investigation of cholesterol conjugation of a C34 dimer was analyzed and this construct showed improved anti-HIV potency (IC_{50} = 210±370 pM for HIV-1_{IIIB} and IC_{50} = 10±10 pM for HIV-1_{BaL}) over C34 (IC_{50} = 31.12±36 nM for HIV-1_{IIIB} and IC_{50} = 60.14±84 nM for HIV-1_{BaL}), but it exhibited comparable potency with monomeric C34-Chol (IC_{50} = 80±70 pM for HIV-1_{IIIB} and IC_{50} = 360±330 nM for HIV-1_{BaL}) [109].

Importantly, the cholesterol modification allowed the peptide inhibitor to retain antiviral potency even after washing [103] and it was more resistant to proteinase K than T20 and C34 [110]. It also exhibited promising anti-HIV potency in *ex vivo* genital and colorectal tissue explant models [111]. Therefore, C34-Chol seems to be an attractive topical microbicide candidate as well [111].

The conjugation of cholesterol has been shown to improve the antiviral potency of HR-derived peptide inhibitors of the viral membrane fusion machinery in many other virus systems (reviewed in [112]) such as influenza [113] and Ebola virus [114]. Interestingly, this cross-virus effectiveness could make this strategy a potential rapid response alternative to combat emerging viral infections if combined with rapidly acquired sequence information from advanced bioinformatics (for more perspective on this see review [112]).

Small Molecule Inhibitors

In addition to peptides, considerable efforts to identify small molecule inhibitors of fusion have also been reported. Recent reviews include Cai and Jiang [75], Gochin and Zhou [115], and Allen and Rizzo [116]. Representative examples of small molecule inhibitors are shown in Fig. (**2**). In general, small molecules have been designed to disrupt 6HB formation by targeting a known hydrophobic pocket on the N-helical interface [76]. Jiang, Debnath and coworkers reported the first such inhibitors, ADS-J1, which was discovered in 1999 through computational docking [117]. Later experiments by the same group found low molecular weight compounds NB-2 and NB-64 using an experimental high throughput ELISA assay [118]. These compounds required a carboxylic acid for activity that is believed to interact with K574, a residue that forms a key salt bridge with the CHR during 6HB formation [119]. This salt bridge is usually recreated by later generations of compounds. Follow-up studies by the group designed several additional inhibitor series [120-122].

Developing a novel 5-helix bundle protein construct, presenting a solvent accessible N-helix groove, allowed Frey *et al.* [123] to perform a high throughput screen. Looking for ligands that could displace a fluorescent probe from the N-helix groove, the authors report several inhibitors including 5M038 and 5M041. The authors note that because the 5-helix bundle

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construct presents only one binding groove per unit instead of the three native ones, this construct represents a more stringent test of molecular activity. Stable folding and a single exposed binding site makes this construct a promising target in ongoing attempts to crystallize a small molecule inhibitor with gp41.

Knowledge-based scoring functions have been used to select active compounds from virtual screens. Holden *et al.* [124] performed a virtual screen and selected compounds using both traditional energy-based and knowledge-based scoring functions, specifically measuring the ability of a small molecule to recreate key interactions seen between the NHR and CHR. Three of the seven hits identified, including SB-C01 would not have been tested if the authors had used only traditional scoring functions. Compound SB-C09 was well ranked by both traditional and knowledge based scoring functions.

Peptidomimetic small molecules have also shown promise against gp41. Gochin *et al.* screened a library of 400 molecules using a competitive inhibition fluorescence intensity assay [125] and showed the activity for some molecules was correlated with the presence of aromatic groups at two customizable regions of the peptidomimetic scaffold (analogous to sidechains at i and i+4 of an alpha helix; e.g. O_2N-[Ala]-Nap-OH, Fig. **2**). NMR based constraints in conjunction with computational docking were used to identify binding poses for two of the compounds. The same group also had success using structure-based design to develop a series of indole inhibitors, the most active compound, 14g shows sub-micromolar activity [126].

Wang *et al.* [127] designed a series of inhibitors by covalently linking a series of small molecules based off of NB-2 and A_{12} to a truncated version of peptide C34 termed P26. Although the constructs were unable to obtain the full potency of C34 the constructs performed one-two orders of magnitude better in cell-cell fusion assays compared to P26. Ferrer *et al.* [128] employed a similar strategy to the discovery of small molecule inhibitors by covalently linking molecules from a combinatorial chemical library to a CHR peptide.

Work has not been limited to only the hydrophobic pocket. NMR-based fragment screening studies have identified an alternative pocket proximate to the hydrophobic region [129]. The authors posit that small molecule potency could be improved through a strategy of tethering binders of each pocket together. More recently, Allen *et al.* [130] used computational screening to identify inhibitors predicted to bind to theoretical pockets on the inner groove of two N helices thereby preventing N-helical trimer formation. Two molecules, which were selected based on their predicted ability to recreate interactions of the third N helix, were shown to have dose-dependent anti-fusion activity in the micromolar range. Although there is no direct experimental evidence the inhibitors act *via* the proposed NHR-trimer obstruction mechanism, companion experiments suggest the inhibitors do not work through binding to the familiar hydrophobic pocket. Follow up molecular dynamics simulations showed compound D9 was particularly structurally and energetically stable in terms of the predicted binding pose.

Work has also been done to test the ability of natural products to inhibit HIV, some of which have been found to inhibit 6HB formation. Interested readers should consult reviews by Cai

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and Jiang [75] and Teixeira *et al.* [131]. Natural products include triterpenoids [132] [133] [134], tannin [135], theaflavin and catechin derivatives [136], and Chinese medicinal herbal extracts [137].

Neutralizing Antibodies

The MPER of gp41 located between the CHR and the viral membrane is highly conserved and has long been known as an important region for the development of neutralizing antibodies [138-147]. Human antibodies, 2F5, 4E10, Z13, and 10E8 bind to MPER and neutralize a broad range of HIV-1 strains [13-20]. 2F5 and 4E10 were the first to be identified followed by Z13, which was identified from a phage display library using a peptide containing epitope sequences from both 2F5 and 4E10 [19, 148-150]. 10E8 was identified by analysis of healthy HIV-infected patient sera [17]. It was found to be targeted to MPER but to not bind phospholipids or cause auto-reactivity. These antibodies in general are known to disrupt MPER function during the membrane fusion step of the entry mechanism [151, 152]. However, MPER has also been shown to be occluded in the native structure before contact with the cellular receptors and is exposed only transiently at a relatively late stage in the entry mechanism [153-156]. MPER is widely considered to be a membrane interacting domain and inaccessibility to MPER has been one of the obstacles in developing vaccines and therapeutic intervention methods targeted to this region. Structural studies of MPER have produced diverse details depending upon the sequence, the experimental method utilized, and binding partners that were present (for a more extensive review of MPER structure and immunogenicity, see reference [138]).

Recent strategies to target Env have included more detailed studies of B cell maturation, antigen-specific single B-cell sorting, and the use of mimotopes in phage display (for a recent review, see [157]). Studies have been done with sequences from both the immunodominant loop (603-609) and from MPER [13, 143, 158, 159].

An alternative strategy was reported that utilized the isolation and cloning of switch memory B cells from peripheral blood in order to identify human monoclonal antibodies without prior information regarding target sequences [160]. This strategy revealed a broadly neutralizing and potent antibody that binds to the non-covalent interface between gp41 and gp120 [161]. This epitope was found to be cleavage dependent and structural details have been reported [162].

CONCLUSION

The trimer of noncovalently bound heterodimers that is the HIV envelope spike is responsible for specificity to the cell surface, bringing two phospholipid bilayers into close proximity, forming a hemifusion diaphragm and finally opening of a fusion pore to allow for entry of the viral genome into the cytoplasm. This process requires a complex set of large conformational changes that are most likely propagated over long distances and may involve oligomerization state changes or asymmetries yet to be delineated. Because this process is not synchronized between the very sparse number of envelope spikes that are present on the virus surface, it has been exceedingly challenging to solve structures for the transient intermediate states that are the most vulnerable to inhibition. However, due to the elegant

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use of classic biochemical techniques and advances in a combination of biophysical, structural, and antigenic studies, the field is moving closer to an understanding of the complex conformational changes that the HIV-1 envelope spike undergoes during attachment and membrane fusion. These are key advancements to identifying the optimal target to elicit effective neutralizing antibodies and model and screen for potential small molecule inhibitors. It remains to be stated, however, that studies of the details of the conformational changes, especially at the atomic level, in the case of the transmembrane subunit gp41 still lag behind those of the surface subunit gp120.

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Biography

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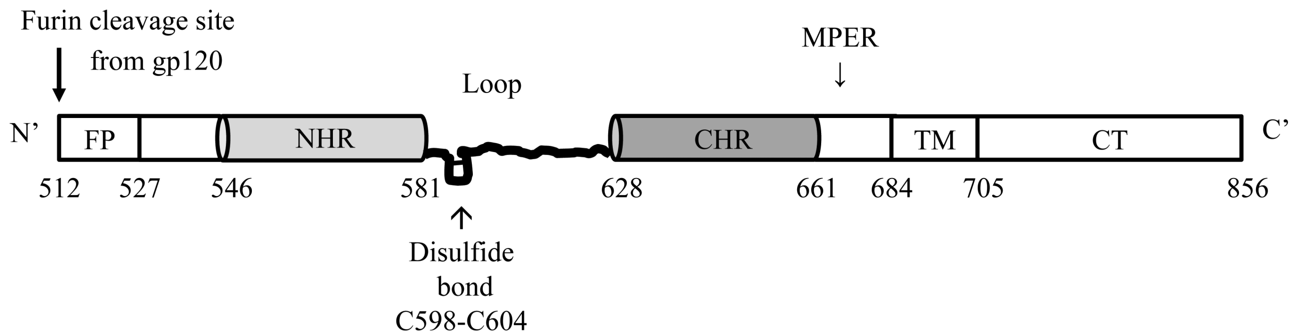

Fig. (1). The primary structure of gp41

Functional domains of gp41 from the N-terminus to C-terminus are: the fusion peptide (FP), N-terminal heptad repeat (NHR), a disulfide-bonded immunodominant loop region, C-terminal heptad repeat (CHR), a membrane proximal external region (MPER), and a transmembrane domain (TM) followed by a C-terminal cytoplasmic tail (CT). (Amino acids numbers are noted based on conventional numbering of the HIV-1 HXB2 strain).

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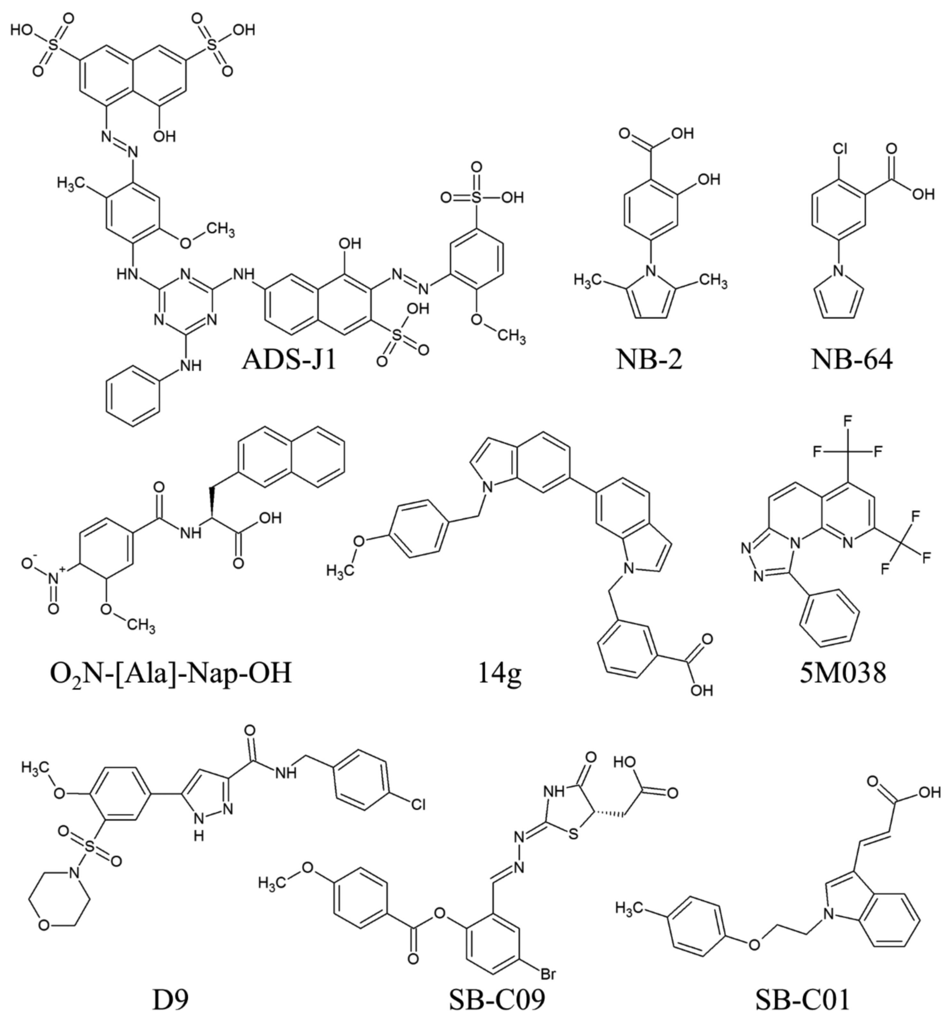

Fig. (2).

Representative small molecule inhibitors of HIV gp41. See text for references.

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Table 1

Peptide sequences targeting HIV-1 gp41.

Type	Name	Sequence	gp41 Location	Ref.
NHR Peptides	DP-107 (T21)	NNLLRAIEAQQHLLQLTVWGIKQLQARILAVERYLKDQ	553-590	[55]
	N36	SGIVQQQNNLLRAIEAQQHLLQLTVWGIKQLQARIL	546-581	[22]
	N42	STMGAASMTLTVQARQLLSGIVQQQNNLLRAIEAQQHLLQLT	528-569	[72]
	N36F10	TLTVQARQLLSGIVQQQNNLLRAIEAQQHLLQLT	536-569	[73]
CHR peptides	SJ-2176	EWDREINNYTSLIHSLIEESQNQQEKNEQEGGC	637-666	[56]
	T20	YTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWF	638-673	[55, 57]
	C34	WMEWDREINNYTSLIHSLIEESQNQQEKNEQELL	628-661	[22] [27]
	T1249	WQEWEQKITALLEQAQIQQEKNEYELQKLDKWASLWEWF	Chimera	[78, 79]
	T1144	TTWEAWDRAIAEYAARIEALLRALQEQQEKNEAALREL	Chimera	[80]
	TLT35	a chimeric peptide of T20 and T1144	Chimera	[81]
	2DLT	a chimeric peptide of T1144 and CD4 D1D2 domains	Chimera	[82, 83]
	Sifuvirtide	SWETWEREIENYTRQIYRILEESQEQQDRNERDLLE	Chimera	[84]