Published in final edited form as:

Expert Opin Ther Targets. 2015 June ; 19(6): 765–783. doi:10.1517/14728222.2015.1010513.

HIV-1 gp120 as a therapeutic target: Navigating a moving labyrinth

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Abstract

Introduction—The HIV-1 gp120 envelope (Env) glycoprotein mediates attachment of virus to human target cells that display requisite receptors, CD4 and co-receptor, generally CCR5. Despite high affinity interactions with host receptors and proof-of-principle by the drug maraviroc that interference with CCR5 provides therapeutic benefit, no licensed drug currently targets gp120.

Areas covered—An overview of the role of gp120 in HIV-1 entry and of sites of potential gp120 vulnerability to therapeutic inhibition is presented. Viral defenses that protect these sites and turn gp120 into a moving labyrinth are discussed together with strategies for circumventing these defenses to allow therapeutic targeting of gp120 sites of vulnerability.

Expert opinion—The gp120 envelope glycoprotein interacts with host proteins through multiple interfaces and has conserved structural features at these interaction sites. In spite of this, targeting gp120 for therapeutic purposes is challenging. Env mechanisms evolved to evade the humoral immune response also shield it from potential therapeutics. Nevertheless, substantial progress has been made in understanding HIV-1 gp120 structure and its interactions with host receptors, and in developing therapeutic leads that potently neutralize diverse HIV-1 strains. Synergies between advances in understanding, needs for therapeutics against novel viral targets, and characteristics of breadth and potency for a number of gp120-targeting lead molecules bodes well for gp120 as a HIV-1 therapeutic target.

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Financial and competing interests disclosure

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

Keywords

HIV antibodies; miniprotein mimetics; natural products; receptor mimetics; small molecule inhibitors; structure-based design

1. Introduction

Entry into host cells is an essential step in HIV-1 life cycle that can be targeted by anti-HIV-1 drugs. HIV-1 entry is mediated by the viral spike (Env), which comprises three gp120 and three gp41 subunits (Figure 1A) [1]. The gp120 envelope glycoprotein, which HIV uses for attachment, undergoes receptor-driven conformational changes, first upon engaging the CD4 receptor, then on CCR5 co-receptor binding, to trigger fusion of viral and host cell membranes. Apart from being critical in entry of cell-free virus particles into host cells, interaction of gp120 with CD4 and CCR5 is also implicated in spread of virus through cell-to-cell transfer via the virological synapse [2].

Key findings highlighting entry inhibition as an effective therapeutic strategy include (1) a 32 base-pair deletion (Δ32) in the entry co-receptor CCR5 gene that confers resistance to HIV infection in populations that carry this mutant allele [3], (2) an HIV-1-positive, ART-experienced patient (Berlin, Germany) who received a hematopoietic stem cell transplant from a donor homozygous for the Δ32 CCR5 mutation, and subsequently went off antiretrovirals, has survived HIV-free without the need for antiretroviral therapy for more than 5 years [4,5], (3) favorable efficacy of drugs maraviroc [6] and enfurvirtide [7], each targeting different steps in HIV-1 entry, and (4) emerging data predicting efficacy of the gp120-targeting drug BMS-663068 in ART experienced HIV-1 positive patients [8]. Thus multiple lines of evidence indicate that blocking entry can provide therapeutic benefit to combat HIV-1 infection.

Of the two licensed entry inhibitors currently in market, maraviroc targets the HIV-1 co-receptor CCR5, and enfurvirtide targets the gp41 glycoprotein. In spite of its critical role in HIV-1 entry and exposed location on viral surface, currently no FDA-approved drug targets the HIV-1 gp120 glycoprotein. Recent advances in Env understanding, multi-target efficacy, and lead characteristics, however, have heightened interest in gp120 as a therapeutic target. First, with Env understanding, atomic-level structures of soluble trimeric HIV-1 Env, in the pre-fusion mature closed conformation, were recently published (Figure 1B) [9-11], and single-molecule analyses of functional HIV-1 Env trimers on virion surface [12] showed that this pre-fusion ground state is targeted by many broadly neutralizing gp120-directed antibodies and drugs. Second, with multi-target efficacy, treatment with combinations of antibodies that target different gp120 sites were shown to reduce viremia to undetectable levels in a subpopulation of rhesus macaques infected with a simian-HIV chimera [13]. Moreover, HIV-1 antibodies have recently been shown to work synergistically with HAART to suppress viremia [14]. Together, these results indicate that use of multiple entry inhibitors or entry inhibitors in combination with other therapeutics might be an effective therapeutic strategy [13,15]. Third, with lead characteristics, a number of recently described leads against different sites of vulnerability on gp120 have demonstrated favorable neutralization

breadth and potency (Table 1). These include (i) at the CD4-binding site, BMS-626529 that targets the pre-fusion closed conformation of HIV-1 Env [8,12], and a CD4-mimetic miniprotein named M48U1 with picomolar affinity for gp120 that has demonstrated *in vitro* efficacy including near-pan reactive neutralization [16], and *in vivo* efficacy as a microbicide [17], (ii) antibodies such as VRC01 that neutralize over 90% of circulating HIV-1, providing proof-of-principle that near pan-neutralization can be achieved despite the vaunted diversity of gp120 [18], (iii) lectins such as cyanovirin that can neutralize most strains of HIV-1 indicating that targeting gp120 evasion, in this case, its *N*-linked glycosylation, can effectively inhibit HIV-1 entry *in vitro* [19] and prevent rectal transmission of SHIV in rhesus macaques [20]. The goal of this review is to explore the potential of HIV-1 gp120 as a therapeutic target, to discuss challenges and opportunities for therapeutics development against various target sites on gp120, and to review new developments and promising leads.

2. HIV-1 gp120: a moving labyrinth

Functional constraints necessitate both a high degree of conservation of host-receptor binding sites on HIV-1 gp120, as well as exposure of these conserved sites during viral entry to enable receptor engagement. HIV-1 employs a number of evasion techniques to protect these vulnerable sites from surveillance and attack by the host immune system [21-28]. Evasion mechanisms include 1) high degree of overall sequence variability with sequence conservation limited to functional regions such as receptor binding sites, 2) rapid emergence of escape variants under selection pressure of antibodies and drugs, facilitated by low-fidelity HIV-1 replication, 3) steric occlusion of receptor sites, 4) extensive glycosylation that limits access to conserved receptor binding sites and allows the virus to masquerade as self, and 5) conformational masking that necessitates receptor-triggered conformational changes for exposure of some of the conserved receptor-binding elements. These elaborate defense mechanisms turn gp120 into a fortified, moving labyrinth [29], with recessed receptor-binding pockets and a multi-domain, cavity-ridden structure further protected by a mobile architecture involving large flexible loops and glycans, and conformational masking of gp120 itself (Figure 1). This ability of gp120 to morph shape and structure has recently been experimentally observed by single molecule fluorescence resonance energy transfer (smFRET) measurements that show HIV-1 Env trimers on virus surface as intrinsically dynamic with conformation changing in response to host receptors [12]. While the entire HIV-1 Env, including the gp120 and gp41 subunits, undergoes conformational changes during entry, much of the receptor-driven conformational transitions occur within and are driven by gp120 (Figure 1A). These conformational dynamics confound not only the human immune system, but also challenge the development of effective gp120-targeting drugs. This review focuses on the gp120 subunit of the HIV-1 Env. The next two sections summarize current knowledge of gp120 in the pre-fusion mature ground state and receptor-activated states of HIV-1.

2.1 gp120 architecture in pre-fusion, mature HIV-1 Env

Advances in structural determination of native gp120, both on intact virion [30] and within soluble trimeric mimics of the HIV-1 spike [9-11] have revealed its structure in the

conformation presented to the immune system prior to its interactions with host cellular receptors (Figure 1B). Accompanying smFRET measurements showed that the conformation captured in the 3.5 Å crystal structure of the HIV-1 Env, obtained in complex with broadly neutralizing antibodies PGT122 and 35O22, closely resembled its pre-fusion, closed conformation. Within the HIV-1 trimer, gp120 subunits form the blades of a 3-blade propeller, and are held by interactions with gp41 at one end and by interactions between its variable loops, V1V2 and V3 at the other. V1V2 and V3 loops within each monomer tightly wrap against each other, while also interacting with the V1V2 loop of an adjacent monomer, creating a “wound-up” pre-fusion ground state [11].

2.2 gp120 in receptor-activated HIV-1 Env

While atomic level structures of receptor-activated trimeric HIV-1 Env are yet to be determined, details of the interface between gp120 and the CD4 receptor were revealed by structures of monomeric gp120 bound to interactive domains of the CD4 receptor and co-receptor-mimetic antibodies [31-33] (Figure 1C and 2B). Additionally, insights into overall subunit motions in receptor-activated Env have emerged from cryo-EM studies [30,34]. The CD4-binding site is accessible on the native trimer, although stable CD4 engagement and formation of the co-receptor binding site require gp120 structural rearrangements [11,30,34,35] (Figures 1 and 2). In the absence of an atomic resolution structure of CD4/co-receptor-bound HIV-1 spike, comparison of structural features of monomeric gp120 bound to CD4 with that of gp120 within the pre-fusion closed trimer reveals regions likely to undergo conformational changes to transition the HIV-1 spike from its pre-fusion closed state to its receptor-activated conformation. Although relative positions of gp120 outer and inner domains appeared similar in both closed and CD4-activated conformations, transition to CD4-activated conformation would likely require substantial Env structural rearrangements including a) dissociation of interactions between the gp120 V1V2 and V3 loops, forcing the Env trimer to lose its tight packing and adopt an open conformation [30,34], thereby exposing immunodominant epitopes in the V3 loop [36], b) reorganization of the base of the V1V2 loop to form the bridging sheet [11,31], c) formation of the Phe43 cavity and a solvent channel adjacent to it at the CD4-binding site [31], and d) disengagement of gp120 from gp41 that accompanies structural rearrangements in gp120 resulting in the formation of the immunodominant epitope for the A32-like antibodies [16] (Figure 1).

3.0 Targeting gp120 sites of vulnerability

In the following sections we describe vulnerable sites on gp120 that emerge during HIV-1 entry, and strategies to target these sites for therapy and prevention.

3.1 CD4-binding site

The CD4-binding site on HIV-1 gp120 is a well-established site of Env vulnerability (Figure 2). Present on the surface of the native viral spike as the initial site of attachment for the CD4 receptor (Figure 2A) [35], the CD4-binding site, together with other regions on gp120, morphs upon CD4 engagement, into an altered structure that can stably engage CD4. The CD4-binding site, therefore, is an example of a region on the gp120 “moving labyrinth” that alters itself in response to an incoming ligand. Each of these presentations of the CD4-

binding site has distinct structural features and vulnerabilities that can be exploited for therapeutics development.

3.1.1 CD4-binding site on the native trimer—In the pre-fusion, closed HIV-1 Env, the initial site of CD4 attachment allows access to CD4, which binds gp120 via the membrane distal subunit of a 4-domain tandem immunoglobulin repeat, but severely restricts access of antibodies through glycan shielding and quaternary constraints [26]. Despite these barriers, the human immune system has found ways to breach viral defenses to target this site on gp120 (Figure 2A). That this site is a natural target is highlighted by the discovery that antibodies from multiple patients effectively neutralize HIV-1 by targeting the CD4-binding site (Table 1) [37]. Over the last few years, the invention of new antibody discovery and isolation techniques have led to a remarkable increase in the number of broadly neutralizing antibodies identified from HIV-1 infected patients [18,38], and have generated an arsenal that can potentially be used for therapy and prevention.

In some patients broadly neutralizing antibodies arise within a few years of HIV-1 infection in an ongoing battle between the virus and the immune system, with the immune system working to clear infection and the virus fighting to escape antibody recognition. In most cases, this battle culminates in viral escape [21,22], showing that emergence of broadly neutralizing antibodies in patients following infection is ultimately ineffective at controlling infection. The question then is - with such evidence of ineffective protection during natural infection- can antibodies be effectively used in therapeutic regimens?

3.1.1.2 Antibodies targeting the CD4-binding site on pre-fusion, closed HIV-1 spike:

Earlier studies on passive administration of antibodies had shown limited success in viremic control due to emergence of resistant viruses [39,40]. More recent studies (reviewed in [41]) in humanized mice [15], and in macaques [13,42] using cocktails of newer broadly neutralizing antibodies (bNabs) with extremely high potency and breadth, have demonstrated effective control of HIV-1 infection, suppression of viral load below detection, and delayed viral rebound upon cessation of therapy (compared to ARV drugs). The antibody-cocktail used for therapy included members of the VH1-2 germline derived VRC01-class of antibodies that potently neutralize >90% of HIV-1 isolates by targeting the CD4-binding site in the mature, closed, pre-fusion conformation of the HIV-1 spike (Table 1).

CD4 mimicry is a common mechanism adopted by antibodies to target the CD4-binding site (Figure 2C). Antibody recognition by this mechanism is achieved by copying two key CD4 interactions with gp120- i) the interaction of the CDR2 loop of CD4 via a β sheet interaction with the β 15 strand of gp120, and ii) salt bridge interaction with the highly conserved Asp368_{gp120}. Discovery of a number such CD4-mimetic antibodies [18,43,44] from different patients emphasize the generality of this mechanism of recognition. Apart from this characteristic mode of recognition by precise CD4 mimicry, other antibodies that originate from diverse germline genes and use long loops to reach into the recessed CD4-binding site can also effectively target the CD4-binding site on the native HIV-1 spike [45].

CD4-binding site antibodies such as VRC01 are among the most potent and broad, with VRC01 neutralizing greater than 90% of circulating HIV-1 isolates (Table 1) [18]. While precise mimicry of the CD4 receptor has resulted in high virus neutralization efficacy, antibodies still need to contend with their size, which is considerably larger than the single headed immunoglobulin-like CD4-receptor. Consequently, resistance mutations resulting from antibody selection pressure typically emerge at antibody contact sites that lie outside the CD4-recognition region. In the case of the VRC01-like antibodies, for example, resistance mutations have been mapped to the gp120 variably loop 5 (V5 loop) [43]. Passive immunization strategies that use antibody combinations targeting different epitopes on gp120 are, therefore, necessary to prevent rapid viral escape under selection pressure from a therapeutic antibody regimen [13,15,42,46].

3.1.1.2 Small molecules targeting the CD4-binding site on pre-fusion, closed HIV-1 spike:

Apart from naturally elicited antibodies, a class of indoleoxoacetyl piperazine compounds developed by Bristol Myers Squibbs targets the pre-fusion, closed HIV-1 envelope and potentially inhibits viral entry by preventing receptor-induced structural changes of the envelope (Figure 2B) [12,47]. Prodrug BMS-663068 that metabolizes into active drug BMS-626529 has shown *in vivo* efficacy in ART-experienced patients (Table 1) [48]. Resistance mutations against BMS-626529 typically develop within and near the Phe43 cavity at the CD4 binding site consistent with its predicted binding site. Recent structural studies on class member BMS-806 [49] should allow structure-guided design of variants with improved potency, breadth and/or tolerance for commonly occurring resistance mutations. The success of BMS-626529 demonstrates that it is possible for a small molecule to achieve high efficacy by preventing receptor binding to HIV-1 gp120, and effectively places gp120 on the roadmap for small molecule drug development.

3.1.2 CD4-binding site on receptor-activated gp120—HIV-1 gp120 alters its architecture in response to CD4-engagement, with its CD4-activated conformation characterized by formation of the bridging sheet and the Phe43 cavity at the CD4-binding site (Figure 1C and 2B) [31]. Due to energetic requirement for conformational change, molecules that target the CD4-activated conformation of gp120 were considered unlikely candidates for development of potent antivirals. This was exemplified by CD4 itself; although CD4-engagement is an essential step in viral entry, soluble CD4 fragments do not neutralize HIV-1 potently [50]. The sections below explore the utility of the CD4-activated conformation as a therapeutic target.

3.1.2.1 CD4-mimetic miniproteins and small molecules: Structural solution of gp120 in complex with a soluble CD4 fragment, and a CD4-induced (CD4i) antibody 17b, yielded the first atomic level image of the gp120 core in its CD4-triggered conformation [31] and elucidated details of the interactions between gp120 and CD4. CD4 binds a large (~800 Å²) depression on the gp120 surface by using 22 residues in the span of amino acids 25-64 of the CD4 D1 domain and centered on the CDR-2-like 36-47 loop. The critical elements in the CD4 interface include i) Phe43_{CD4}, the side chain of which protrudes and plugs the entrance of a deep cavity in gp120, ii) Arg59_{CD4}, which makes multiple contacts with gp120, and iii) strand C", which interacts with strand β15 of gp120 in a β-sheet alignment. These critical

functional elements of the CD4-binding site were transplanted first onto a structurally compatible scorpion charybdotoxin scaffold, and next onto a scorpion scyllatoxin scaffold to produce a molecule that prevented HIV-1 attachment to cells and subsequently, HIV-1 entry [51]. Using iterative feedback from structural methods- first NMR and subsequently X-ray crystallography- this inhibitor was progressively improved by optimization of the scaffold backbone, and gp120-interactive residues, including the critical residue 23 of the scaffold- a position analogous to Phe43_{CD4} [52] [53] to yield M48U1 (Figure 2B and 2C), which binds gp120 with pM affinity, neutralizes with extraordinary potency and breadth [50], and has demonstrated efficacy as a microbicide in a macaque challenge study (Table 1) [17]. X-ray crystallographic analyses on M48U1 bound to gp120 at 1.5 Å resolution revealed details of its interaction with the gp120 Phe43 cavity, and its mechanism of action. Because M48U1 reaches deep into the Phe43 cavity and interacts with residues that do not contact CD4 contact region, emergence of resistance mutations within the Phe43 cavity is a possibility and has been shown to occur *in vitro* [54]. Overall, the success of M48U1 as an entry inhibitor shows that the CD4-activated state can be a drug target.

Another class of CD4-mimetics that utilize the Phe43 cavity includes drugs that belong to the NBD/DMJ series [55,56] (Table 1). A recent review [57] covers progress in the development of this class of drugs, including the structure-guided conversion of an agonist that facilitated HIV-1 entry into CD4- cells to an antagonist that does not cause such entry.

3.2 Co-receptor binding site

The co-receptor binding site is one of the most conserved regions of gp120, and is protected from the human immune system by conformational and steric restrictions (Figures 1 and 3) [28]. Unlike the CD4-binding site, where the initial site of CD4 attachment is exposed on the viral surface in its pre-fusion closed conformation, the co-receptor binding site is not accessible in the pre-fusion, closed conformation of the HIV-1 spike, and becomes exposed only after gp120 binding to cell surface CD4 triggers conformational changes in Env (Figures 1 and 3) [27]. After CD4 binds, proximity of viral and cellular membranes restricts access of antibodies to the co-receptor binding site [28]. While full-length antibodies cannot effectively target this site, smaller antibody fragments have been shown to have better access (Figure 3) [28,58], suggesting that targeting this highly conserved region with small molecules may be a potential intervention strategy. While the structure of gp120 in complex with co-receptor has not yet been determined, structural, biochemical and virological analyses with co-receptor fragments [32,59-65] and co-receptor-mimetic antibodies [32,66-68] have provided information on gp120 interactions with its co-receptors, and these insights can be exploited for developing inhibitors that target the gp120 co-receptor binding site (Figure 3).

Similar to the CD4-binding site, receptor-mimicry is a common mechanism of inhibition at the co-receptor-binding site, both by antibodies [32] and synthetic molecules [69]. GPCR coreceptors utilize tyrosine sulfated N-terminus and extracellular loops to interact with gp120. HIV-1 gp120 harbors highly conserved tyrosine sulfate binding sites at its co-receptor interactive region, which are critical for engaging co-receptor N-terminus [32,59,60]. Naturally elicited antibodies use post-translationally modified sulfated tyrosine

residues to target this region on gp120, which includes two sulfated tyrosine binding sites, one at the base of the V3 loop centered around Arg 298, and the other around Arg 327, both highly conserved gp120 residues [32,67]. This mimicry has been further exploited to develop lead inhibitors against this site [69]. An *in silico* structure-guided, ligand-based search of chemical libraries, coupled to a rapid ELISA-based screen has yielded small molecule inhibitors that extend the natural mimicry of CCR5 N terminus shown by monoclonal antibody 412d to synthetically derived molecules (Table 1) (reviewed in [70]). Apart from small molecules, synthetically derived receptor fragments have the potential to inhibit HIV-1 entry, and in the absence of an atomic level structure, have allowed fine-mapping of co-receptor interaction with the HIV-1 envelope [60,61,65] and provided a framework for development of receptor-mimicking peptide or peptidomimetic inhibitors.

3.3 Simultaneous targeting of CD4 and co-receptor binding sites

Inhibitors that bind the highly conserved co-receptor binding site target a transient, receptor-activated conformation on the HIV-1 entry pathway, and depend on prior CD4-triggering for activity. One strategy to circumvent the need for Env triggering by cell surface CD4 is to use chimeric molecules that link soluble CD4 (or a CD4-mimetic) with a co-receptor binding site ligand (Figure 3). Such a molecule would first engage the pre-fusion, closed Env via its CD4 component, thus triggering the spike and allowing the co-receptor site directed moiety to bind. A number of inhibitors that exploit this conformational synergy between the CD4 and co-receptor binding sites have been developed [71-74]. Through tandem targeting of both receptor-binding sites on gp120 these inhibitors effect broad and potent HIV-1 neutralization.

3.4 Targeting loops and glycan: breaching viral defenses

HIV gp120 is a highly unusual glycoprotein with its solvent exposed surface almost entirely covered with N-linked glycans (Figures 1B and 4) [75]. These glycans have important functions for the virus: they are essential for correct folding and assembly, thereby contributing to viral fitness; they shield the most immunogenic epitopes on gp120; and because the glycans are of host origin, they can as decoy to the immune system. Though precise structures of each glycan can vary as a function of viral strain or gp120 sequence, as well as the cell type in which the gp120 was produced, biochemical and mass spectrometry studies have shown that the majority of glycans present on gp120 comprise high mannose types (namely Man-5 to Man-9) [76,77]. The gp120 glycan coat was long pictured as a shield covering conserved regions on HIV-1 Env, but is also potentially vulnerable, and is recognized by broadly neutralizing antibodies [75].

3.4.1 Carbohydrate-binding agents that target gp120's glycan shield

3.4.1.1 Glycan-dependent Antibodies: A substantial proportion of recently identified, bnAbs present in certain HIV-infected donors recognize glycan-dependent epitopes on HIV-1 gp120 (Figure 4) [38,78-80]. Generally speaking, these glycan-reactive antibodies can be classified as those that bind with high affinity to discrete epitopes featuring a Man₈₋₉ at position N332, exemplified by 2G12, PGT121, PGT122 and PGT128; and those whose epitope comprises glycan at position N160 and protein surface, exemplified by PG9 and

PG16. Atomic level structures of glycan-dependent antibodies with various glycosylated Env constructs, including a soluble trimer mimic [9,11,79], have provided structural definition for antibody recognition of these conserved structural motifs on the HIV-1 Env.

3.4.1.2 Lectins: Lectins are carbohydrate-binding proteins ubiquitous in Nature and abundant in plants, invertebrates and bacteria. By the late 1980's, it had been shown that select plant lectins such as concanavalin A (ConA) and wheat germ agglutinin (WGA) could inhibit HIV [81,82]. Those early studies were the first to establish that inhibition correlated with mannose-binding. In contrast, lectins with specificities toward galactose (Gal), *N*-acetyl galactosamine (GalNAc) and sialic acid (Sial), the components of complex-type glycans, were less effective or inactive. More recently targeted searches for mannose binding lectins have uncovered several that exhibit very potent (picomolar) and broad inhibition profiles toward HIV [83-85]. Several previously published reviews comprehensively described antiviral lectins [86-88]; here we focus only on those that show the highest potency and whose mechanisms of inhibition have been established through high resolution structural and biophysical studies or by generating resistant viruses.

Among the most potent anti-HIV lectins reported to date are the cyanobacterial and algal lectins cyanovirin-N (CV-N), griffithsin (GRFT), *Microcystis viridis* lectin (MVL), microvirin (MVN), *Oscillatoria agghardi* agglutinin (OAA), and scytovirin (SVN); the streptomycete-derived lectin actinohivin (AH) ; and the plant lectins banana lectin (BanLec), ConA, *Galanthus nivalis* lectin (GNA), and the amaryllis-derived lectin HHA [83,89-98] (Table 1). Although none of these proteins share amino acid sequence, or three-dimensional structural, homology, they can be grouped according to carbohydrate specificities (Table 1). ConA, BanLec and GRFT are mannose-binding lectins [84,91,98]. GNA and OAA are reported to bind the inner branching mannose structures Man α 1-3Man and Man α 1-6Man with or without the GlcNAc₂ core [99,100], while MVL requires a mixed epitope comprising GlcNAc₂ and at least two additional Man units [93,101]. CVN, MVN and SVN, on the other hand, are unique in that they only recognize and bind to terminal Man α 1-2Man units present on the branches of Man-7 to Man-9 [102-104]. As each of these small saccharide structures is present within high mannose-type glycans, these lectins can bind to gp120 and inhibit viral entry.

Affinity for the individual epitopes is a variable feature among lectins; whereas some lectins, like CVN, can bind their carbohydrate ligands with submicromolar affinity, others bind their ligands with only millimolar K_d s, as is the case for ConA. To augment affinity and confer specificity, lectins are usually oligomeric and/or contain more than one carbohydrate-binding site per protein unit allowing for multivalent interactions. Depending on the spatial arrangement of carbohydrate binding sites within the lectin as well as glycans on the target molecule or surface, multivalent binding can occur through different mechanisms. For example, when all binding sites are positioned in the same orientation allowing them to be engaged simultaneously, face-to-face or chelating type interactions occur. In contrast, when carbohydrate-binding sites are opposed and surface glycan densities are high, intermolecular cross-linking can occur and contributes to the potency of some carbohydrate-binding agents (CBAs) [105]. High resolution structures have been obtained for all of the lectins listed in Table 2, and their stoichiometries of binding to individual saccharides has been determined

by ITC or SPR. Interestingly, while multi-site lectins tend to bind gp120 with low nanomolar affinities, their effects in neutralization assays is much more variable with IC₅₀ values ranging from nanomolar to picomolar. At least two lectins, CV-N and GRFT [20,106], have shown outstanding efficacy as microbicides in SIV models. For reasons that are not yet understood, some lectins are cytotoxic to cells while others show no signs of toxicity [103,106,107].

3.4.1.3 Glycan-binding synthetic molecules and natural products: Design or discovery of small molecules that can bind carbohydrates with high affinity/avidity presents an ongoing challenge in glycobiology. To date two such molecules have been reported; they include the pradimicin family of natural products, and synthetic benzoboroxazoles. The pradimicins are actinomycete derived naphthacenquinones functionalized with a D-amino acid and hexose sugars. In the presence of Ca²⁺ the pradimicins form dimers and higher order oligomers that lead to high avidity binding to mannosides and potent inhibition of HIV-1 infection [108]. The pradimicins were originally discovered due to their potent antifungal activity and have since been shown to have *in vivo* efficacy as antifungal agents [109], Thus development of the pradimicins as HIV microbicides may be facilitated.

Kiser and co-workers took a different approach by synthesizing a series of benzoboroxazoles with the aim of designing artificial carbohydrate receptors. Boronic acids are known to form reversible covalent adducts with 1,2- and 1,3-diols, present in most sugars. By generating a stable, masked boronic acid in the form of benzoboroxazole, and incorporating these units into water soluble polymers, the Kiser team achieved low nanomolar inhibition in single round HIV infectivity assays. These formulations are currently being investigated as microbicides [110].

3.3.3 Resistance toward carbohydrate-binding therapeutics—Given their breadth and potency, and the absence of cross resistance toward other HIV drug-resistant strains, carbohydrate-binding agents (CBAs) represent promising microbicide candidates. As a prerequisite to development, several groups have been studying the effects of CBAs on HIV by selecting CBA-resistant viruses. Cumulatively those studies reveal that multiple rounds of selection must generally occur before resistant viruses develop. Further, in all cases the resistant viruses undergo mutations that delete multiple sites of *N*-linked glycosylation rather than single site mutants (Figure 4). When these mutations are viewed in the context of the gp120 trimer structure, several patterns emerge. First, loss of sites bearing complex type glycans are less common, and have only been observed for some CBAs such as the lectins AH, GNA and HHA [111,112]. Second, while culture in the presence of lectins such as GRFT, CVN or SVN that preferentially bind to Man-8 or Man-9 leads to the loss of multiple glycosylation sites, some of which e.g. the glycan at gp120 residue 339 are uniformly deleted (Figure 4) [113-116]. Interestingly, these glycan deletions overlap with residues that have been shown to bear high mannose structures, and to be the targets of glycan-dependent neutralizing antibodies such as 2G12, PGT121, PGT122, and PGT128 [114,117,118]. In addition to consistency between glycan specificity by the lectin and glycan type on gp120, the location of these sites suggests that the Man-9-binding lectins inhibit HIV through multivalent interactions with gp120 that involves this residue 332-clustered group of glycans

in particular [116,119]. With regard to therapeutic potential, it is noteworthy that viruses that are resistant to glycan-targeting antibodies remain sensitive to lectins. For example, HIV-1 strain YU2 as well as HIV-1 subtype C viruses are resistant to the antibody 2G12 due to lack of glycans at Asn positions 295 or 332 on gp120, yet these viruses are still potently inhibited by lectins such as CV-N and GRFT [119]. Additionally, the loss of multiple glycosylation sites that is needed to confer lectin resistance has the potential to expose other immunogenic epitopes, which are otherwise protected by the glycan shield [120,121]. The high genetic barrier to developing resistance to CBA, the limited cross resistance to glycan-targeting mAbs, and the observation that viruses with reduced numbers of glycans can show increased sensitivity to some gp120-directed antibodies [122] makes a number of these entities promising microbicide candidates, emphasizing the importance of gp120 as a therapeutic target.

3.5 Expert Opinion

HIV-1 gp120 is a complex target for inhibitor development with elaborate and intricate defenses built to combat constant surveillance and attack by the human immune system. Therefore, despite its critical role in HIV-1 entry, it has not been a preferred therapeutic target. Unlike enzyme targets, disrupting gp120 function involves interfering with protein-protein interactions that usually have larger and more complex interfaces than enzyme-substrate pairs. Despite these challenges, the quest for an HIV vaccine, with the HIV-1 Env at its center, has yielded a large volume of structural knowledge of gp120, as well as its interactions with host receptors and antibodies. This wealth of knowledge has spurred development of inhibitors, which currently are at different stages of development ranging from lead molecules with μM potency to receptor mimetics capable of broad and potent neutralization. ART is continuously evolving with new drugs being sought to address various issues with existing regimens including toxicity and treatment failure. New therapeutics against gp120 would most immediately benefit patients who have exhausted current antiretroviral regimens due to emergence of resistant viruses.

One area that is expected to see rapid development is passive therapy with broadly neutralizing antibodies. The large number of antibodies targeting various sites of vulnerability on gp120 provides a valuable arsenal for multi-“drug” therapy, which has proven critical in ARV to prevent emergence of resistant viruses. Antibodies have a number of unique advantages including low toxicity, which may benefit treatment of specific populations such as infants. Apart from the advantage of longer serum half-life, full-length antibodies also have the unique advantage of Fc-mediated functions, which have been shown to have an effect on viral control through non-neutralizing protective mechanisms. Significant drawbacks toward development of preventive and therapeutic regimens using antibodies involve their route of administration through injections, which can limit adherence, and the high cost of manufacturing compared to synthetic small molecules. Enhancing potency of antibodies can reduce the amount of antibody that needs to be administered thereby reducing cost, and strategic efforts to improve the breadth and potency of existing broadly neutralizing antibodies through structure based design have been pursued [123-125]. Despite some difficulties, passive therapy with antibodies holds immediate promise, especially for treatment of select populations, such as infants where doses are

expected to be lower. Finally, emerging gene therapy approaches using adeno-associated vectors can, in the future, make treatments involving antibody delivery more effective and less expensive [126,127].

Precise atomic-level definition of gp120 interactions with host molecules, such as receptors and antibodies, has opened doors to rational, structure-guided development of inhibitors that target the conserved, functional regions of gp120. The symbiosis between structural analyses, rational design and chemical synthesis that combine the precision of structure-guided design and the manipulative power of synthetic chemistry have great potential to deliver a diverse arsenal of HIV-1 entry inhibitors. Notwithstanding the challenges inherent in disrupting large protein-protein interactions with smaller chemical moieties, this strategy has a unique advantage of being unencumbered by the biological repertoire and can use synthetic compounds to reach where natural molecules cannot [128]. Any antiretroviral therapy has to take into account the latent viral reservoir since the ultimate goal of therapy is to purge the reservoirs and effect a cure, thus enabling patients to end life-long therapy. Emerging ideas in this area include targeted cytotoxicity and coupling inhibitors and targeting molecules with immunotoxins and cytolytic agents [129].

In conclusion, HIV-1 gp120, notwithstanding its complexities and the challenges, is an emerging therapeutic target, with a rich arsenal of therapeutic leads that can enrich the current ART repertoire. Two recent technological advances that will likely impact the development of inhibitors targeting HIV-1 gp120 are i) the atomic level definition of the pre-fusion closed state of HIV-1 Env and availability of a robust crystallization lattice for structural determination [11], and ii) an ability to visualize the gp120 moving labyrinth by smFRET [12]. While not all the activated states revealed by smFRET analyses of the HIV-1 Env have been resolved structurally, the definition of the pre-fusion, closed, ground state, which is targeted by a number of broadly neutralizing antibodies and inhibitors [12] is clear. Apart from this closed conformation of HIV-1 Env, receptor-activated Env conformations can also be accessed and targeted by broad and potent inhibitors [50,72]. Taken together, the convergence of structural and mechanistic knowledge, emerging synergy between vaccine and cure research fields, and the availability of several potent lead inhibitors targeting different binding sites and conformations of HIV-1 gp120 bode well for the development of gp120-targeting therapeutics in the next few years.

Acknowledgments

The authors were supported by the AIDS Targeted Antiviral Program, Office of the Director, NIH, Intramural Research program, NIDDK, Intramural Research Program, Vaccine Research Center, NIAID, NIH.

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Figure 1. Conformational dynamics of HIV-1 entry

(A) Schematic representation of HIV-1 entry pathway. Mature HIV-1 Env prior to receptor activation adopts a closed conformation resistant to neutralization by antibodies. On engaging CD4 receptor the trimer adopts an open conformation with the gp120 subunits undergoing rigid body motion, along with reorganization at the trimer association domain, made up by the variable loops V1V2 and V3, and at the gp120-gp41 interface. The trimer then interacts with co-receptor and undergoes additional conformational changes to effect viral and host cell membrane fusion. (B) Atomic level structure of pre-fusion closed HIV-1 BG505 SOSIP.664 trimer (PDB ID 4TVP). Panels from left to right show: gp120 in surface representation, colored by residue conservation with gp41 in blue cartoon, and glycans in pale green surface representation; a 90° rotated view looking down the viral membrane distal trimer apex with gp120 outer domain colored red, inner domain grey, V3 loop orange and V1V2 olive; HIV-1 Env with glycans removed to allow visualization of the underlying protein with gp120 protomers colored grey, pink and cyan, respectively; and gp120 subunits colored by domain. (C) Atomic level structure of monomeric HIV-1 YU2 gp120 in complex with CD4 and CD4-induced co-receptor mimetic antibody 412d. Left panel shows gp120 colored by domains, with outer domain colored red, inner domain grey, bridging sheet magenta and V3 loop orange. CD4 is shown in yellow and the antibody heavy and light chains in green and dark pink, respectively. Middle panel shows a 90° rotated view of gp120 colored by domains as described above. Right panel depicts gp120 cavities and pockets, which are shown in blue surface representation, with the gp120 polypeptide shown as white cartoon. CD4 is shown in yellow ribbon representation with its Phe 43 residue shown in stick representation.

Figure 2. Targeting the CD4-binding site of HIV-1 gp120

The initial site of CD4 engagement on HIV-1 Env is, by functional necessity, highly conserved and accessible on the viral surface. Naturally occurring antibodies target this site to neutralize virus. Synthetic molecules have been developed that target the CD4 binding site on gp120. Atomic resolution structures of CD4-binding site directed ligands reveal unifying mechanisms of recognition. **(A) (Left)** Model of IgG VRC-PG04 binding to the CD4-binding site. The model was constructed by superposing coordinates of b12 IgG (1HZH) on VRC-PG04 Fab in the BG505 SOSIP.664-VRC-PG04 complex (PDB ID: 3J5M). **(Right)** Model of HIV-1 trimeric spike bound to CD4 receptor. The model was constructed by superposing the gp120 outer domain from PDB ID 1RGK to the outer domain in PDB 4TVP (EM structure of BG505 SOSIP.664 bound to VRC-PG04), followed by superposition of 4-domain CD4 (PDB ID 1WIP). **(B)** Synthetic molecules that target the CD4 binding site to neutralize HIV-1. HIV-1 gp120 is shown in surface representation with its domains colored as in Figure 1C middle panel, and with the CD4-binding footprint overlaid in yellow. The left panel shows residues implicated in BMS-806 binding by mutagenesis studies in lime green. The predicted binding site is circled and the chemical structure of class member BMS-626529 is shown. In the gp120-M48U1 complex (PDB ID: 4JZZ) (middle) M48U1 is shown in teal cartoon representation with the U1 moiety that inserts into the gp120 Phe43 cavity shown in stick representation. In the gp120-DMJ-I-228 complex (PDB ID: 4DKQ) (right) DMJ-I-228 is shown in cyan stick representation. **(C)** Mechanism of CD4-binding site recognition. Molecular mechanisms of recognition conserved among diverse inhibitors include recognition of Asp 368 of gp120 via a salt bridge interaction, and utilization of the gp120 Phe43 cavity.

Vulnerability at the HIV-1 co-receptor binding site					
Co-receptor binding-site directed ligand	CD4i antibody	CD4i antibody fragments	CCR5 Nt peptides and mimetics	ECL2-derived peptides	CD4-CD4i chimeras
Schematic					
Recognition of pre-fusion, closed conformation	X CD4i site occluded	X CD4i site occluded	X CD4i site occluded	?	✓
Recognition of CD4-activated, open conformation	X Steric restrictions	✓	✓	✓	✓
Examples	17b, 48d, 412d, X5 [31,32,33,36]	Fab: 17b, X5 VHH: JM4, JM5 [28,58]	CCR5 Nt peptide (2 – 18) and peptidomimetics tyrosine sulfate mimetics [59,60,62,63,69]	ECL2 peptide fragments [61,64]	CD4-linker-17b Fab eCD4lg 11-Fc (71 – 74)

Figure 3. Targeting the co-receptor binding site of HIV-1 gp120

The highly conserved co-receptor binding site is occluded in the mature, pre-fusion, closed HIV-1 Env trimer. Hence this state cannot be targeted by co-receptor binding-site directed ligands that need CD4-activation to bind the HIV-1 spike. Once the spike engages cell surface CD4, the space between the viral and cellular membranes do not allow access to full-length human antibodies. Smaller antibody fragments such as Fab and VHH can, however, access the CD4-induced epitope. Peptides and small molecules that mimic the CCR5 N terminus (Nt) bind to a CD4i binding surface, although they have been shown to be active without CD4 induction with enhancement of activity in presence of CD4, suggesting that these molecules may be able to induce the CD4i state. Peptides derived from the second extracellular loop (ECL2) of CCR5, however, can work on both states and are not CD4-induced, suggesting that some regions on the co-receptor binding site may be accessible on the mature, closed, pre-fusion spike. Synthetic chimeras that include CD4 (or a CD4-mimetic) and a CD4-induced ligand within the same molecule can target the closed spike, with the CD4 component of the ligand first binding to induce the CD4i conformation, thus allowing the CD4i component to bind. This figure shows schematics of various molecules that bind to the gp120 co-receptor binding-site, and indicate whether each can target the pre-fusion, closed and the CD4-activated states of the viral spike. A () indicates that the target conformation can be accessed by the inhibitor molecule indicated, and (X) indicates that the molecule cannot target the conformational state.

Figure 4. Targeting the variable loops and glycans of HIV-1 gp120

(Left) Antigen binding fragment of antibody PGT122 bound to an epitope on gp120 that combines glycans and elements of the variable loops. (PDB ID 4TVP). **(Right)** Chart showing glycan type and observed glycan deletions as a function of gp120 amino acid sequence. Lectins for which resistance profiles have been determined are shown at the beginning and end of rows. Complex and high-mannose type glycans are shown at their respective sites and are depicted as green and red branches. Red or green vertical bars indicate loss of that glycosylation site when treated with the corresponding lectin.

Table 1

HIV-1 gp120 directed entry inhibitors: Classification, structural determination, antiviral and therapeutic efficacy.

Target	Inhibitor type	Molecules	Binding-site determination	Antiviral activity		Clinical trials
				<i>In vitro</i> efficacy	<i>In vivo</i> efficacy	
CD4-binding site	Antibodies	VRC01-like antibodies (VRC01, 12A21, 3BNC117, etc.), HJ16, b12	Crystal structures, EM structures, mutagenesis and resistance mutation [35,43,130,131]	VRC01-like antibodies neutralize > 90% of circulating HIV-1 isolates [43,44]	VRC01 protects macaques against SHIV mucosal challenge [132]. A cocktail of bnAbs including 3BNC117 reduces plasma viral loads to undetectable levels in monkeys infected with a pathogenic SHIV [133].	
				M48U1 neutralizes 90.4 % of a 180-isolate representative panel of circulating HIV-1 isolates with geometric IC50 mean of 0.13 µg/ml [50], and inhibits HIV-1(Ba-L) in human mucosal explants of cervical and colorectal tissues[18].	Used as microbicide, M48U1 blocked vaginal transmission of SHIV(162P3) in 5 out of 6 cynomolgus macaques [17].	
		Peptides and miniproteins M48U1, M48, M47, M48U7, M48U12, etc.	Crystal structures, mutagenesis, resistance mutation [17,50,52,53,134]			
Small molecules		NBD-class, DMJ-class	Crystal structures, mutagenesis, [56,135-137]	DMJ-II-121 neutralizes 83% of a 42-isolate panel of Clade B and Clade C viruses with geometric IC50 mean of 2.3 ± 0.05 µM. [56]	N.D.	
		BMS-class (BMS-806, BMS-626529)	mutagenesis, resistance mutations [8,48,138]	BMS-626529 neutralizes R5-tropic viruses with EC50 of 0.4-1.7 nM. X4-tropic viruses with EC50 between 0.7 and 16.2 nM, and dual tropic virus 89.6	Oral administration of BMS-663068 for 8 days resulted in substantial declines in plasma HIV-1 RNA levels. [8]	
						PhaseIIb trial to investigate safety, efficacy and dose-response in ART experienced HIV-1 subjects [8]

Target	Inhibitor type	Molecules	Binding-site determination	Antiviral activity		Clinical trials
				<i>In vitro</i> efficacy	<i>In vivo</i> efficacy	
Co-receptor binding site	Antibody fragments and nanobodies	m36, 17b scFv	Crystal structures, mutagenesis [28,31,33,67,139-144]	Yes [28]	N.D.	
	Peptides Small molecules	CCR5 Nt and ECL-derived peptides	NMR data, mutagenesis [61,62,65]	Yes [59,61,62,65]	N.D.	
		Tyrosine sulfate mimetic molecules	Competition assay, CD4-induction, mutagenesis [69]	Yes [69]	N.D.	
Variable loops and glycan shield	Antibodies	PG9, PG16, 2G12, PGT122, 10-1074, CAP 256, VRC26.01	Crystal structures, mutagenesis, resistance mutation [79,80,145]	10-1074 and PGT121 inhibit cell-free and cell-to-cell transmission[146]	PG9 protects macaques against SHIV mucosal challenge [132].	
	CBAs: Lectins	CVN, GRFT Benzoboroxazoles	Crystal structures, mutagenesis, resistance mutation [19,92,102,107,113,115]	Yes	Used as a microbicide blocks SHIV transmission in rectal challenge [20]	Preclinical toxicity [20]
	CBAs: Synthetic			Yes		Preclinical toxicity

Table 2

Biochemical and virological properties of lectins.

Lectin	Oligomeric state	# Sites per monomer	Total valency	Carbohydrate specificity	EC ₅₀ , IC ₅₀ (nM)	K _d , gp120 (nM)	References
ConA	<i>Concanavalin A</i>	tetramer	1	4	Man	98	[114]
OAA	<i>O. agardhii</i> agglutinin	monomer	2	2	Man	45	[95,147]
MVL	<i>M. viridis</i> lectin	homodimer	2	4	Man ₃ GlcNAc ₂	30	[93]
BanLec	Banana lectin	homodimer	2	4	Man	20	[148,149]
GNA	<i>G. nivalis</i> lectin	tetramer	3	12	α-1,3 Man; α-1,6 Man	7	[99,112]
HHA	<i>Hippeastrum</i> hybrid	tetramer	3	12	α-1,3 Man; α-1,6 Man	6	[150,151]
AH	Actinohivin	monomer	3	3	α-1,2 Man	12	[152,153]
MVN	Microvirin	monomer	1	1	α-1,2 Man	12	[103,107]
SVN	Scytovirin	monomer	2	2	α-1,2 Man	0.3	[119]
CV-N	Cyanovirin-N	monomer	2	2	α-1,2 Man	0.1	[114,119,154]
GRFT	Griffithsin	homodimer	3	6	Man, Man-9	0.04	[106,119,155]
						0.12 ^a	
						8.2 (10:1) ^b	

^a K_Ds measured by SPR, data fit to a 1:1 binding model

^b K_Ds determined by ITC, with stoichiometries of binding in parantheses.