Molecular Therapy
Nucleic Acids
Review

HIV Entry and Its Inhibition by Bifunctional Antiviral Proteins

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HIV entry is a highly specific and time-sensitive process that can be divided into receptor binding, coreceptor binding, and membrane fusion. Bifunctional antiviral proteins (bAVPs) exploit the multi-step nature of the HIV entry process by binding to two different extracellular targets. They are generated by expressing a fusion protein containing two entry inhibitors with a flexible linker. The resulting fusion proteins exhibit exceptional neutralization potency and broad cross-clade inhibition. In this review, we summarize the HIV entry process and provide an overview of the design, antiviral potency, and methods of delivery of bAVPs. Additionally, we discuss the advantages and limitations of bAVPs for HIV prevention and treatment.

Introduction

Passive infusion of broadly neutralizing antibodies (bnAbs) is extensively being investigated for HIV treatment and prevention. Advantages of bnAbs include their long half-life *in vivo*, effectiveness against virus isolates that are resistant to antiretroviral drug therapy (ART), and potential to mediate the destruction of HIV-infected cells.[1] However, the high specificity and potency with which antibodies bind to their target may be a disadvantage against the highly variable HIV envelope glycoproteins (Env). Indeed, clinical trials based on the infusion of single antibodies have shown that pre-existing as well as emerging resistance are obstacles to bnAb-based approaches.[2,3] Similar to ART and pre-exposure drug prophylaxis (PrEP), a combination of bnAbs will likely be necessary for therapy and prophylaxis.

Bifunctional antiviral proteins (bAVPs) consist of two protein-based entry inhibitors that target different steps of the HIV entry process. They exploit the multi-step nature of HIV entry to overcome the limitations of single inhibitors and exhibit exceptional antiviral effects. In this review, we summarize the HIV entry process and provide an overview of the design and antiviral potency of the currently identified bAVPs. Additionally, we discuss different methods of delivering bAVPs for HIV prevention or treatment as well as the advantages and limitations of bAVPs. It is of note that intracellularly acting antiviral proteins, such as intrakines and intrabodies,[4–6] or transmembrane proteins, such as membrane-anchored fusion inhibitors and interferon-induced transmembrane proteins,[7,8] will not be covered in this review, as they can only be delivered and expressed in the target cells. Bispecific T-cell-engaging antibodies are also covered elsewhere.[9]

HIV Entry into Target Cells

HIV entry represents the first step in the virus life cycle and is an attractive target for controlling HIV replication. To enter host cells, HIV Env needs to interact with CD4, which serves as the primary receptor, and CCR5 or CXCR4, which serve as the major coreceptors. The following section provides an overview of the structure of the HIV Env, the receptor, coreceptors, and the HIV entry cascade.

HIV Envelope Glycoproteins

Mature HIV particles have a spherical structure and measure approximately 100–140 nm in diameter.[10,11] The virus membrane is embedded with viral envelope glycoprotein (gp) complexes and membrane proteins of host cell origin.[12] Cryoelectron microscopy has revealed that virions contain 7–14 spikes on average.[11,13,14] Each spike is generally comprised of three surface HIV gp120 subunits that are non-covalently bound to three transmembrane HIV gp41 subunits.[15] In addition to functional trimers, the envelope can also contain non-functional gp120/gp41 monomers as well as gp41 stumps that lack the gp120 subunits.[16] The surface gp120 contains five highly variable regions (V1–V5) and five conserved regions (C1–C5).[17] A schematic representation of gp120 and gp41 is shown in Figure 1A. The extracellular region of gp41 contains the fusion peptide (FP), the fusion peptide proximal region (PR), the N-terminal heptad repeat-1 (HR1), a loop, the C-terminal heptad repeat-2 (HR2), and the membrane proximal external region (MPER). The extracellular domains of gp41 are followed by the transmembrane domain (TMD) and the cytoplasmic tail (CT). Recently, cryoelectron microscopy has revealed the structure of the gp120/gp41 heterotrimer in its native conformation (Figure 1B).[15,18] The variable regions of gp120 form exposed loops that cover the surface of the trimeric complex. Although some of these regions are highly immunogenic, their variable nature eventually leads to the evasion of antibody-mediated responses that are directed against them.[19] Especially the V1 and V2 loops at the apex of the trimer shield more conserved epitopes from being accessed by antibodies.[20] The HR1 of gp41 forms helices at the center of the trimeric complex, and the HR2 assumes a helical conformation at the base close to the MPER.[15,18] The variable and

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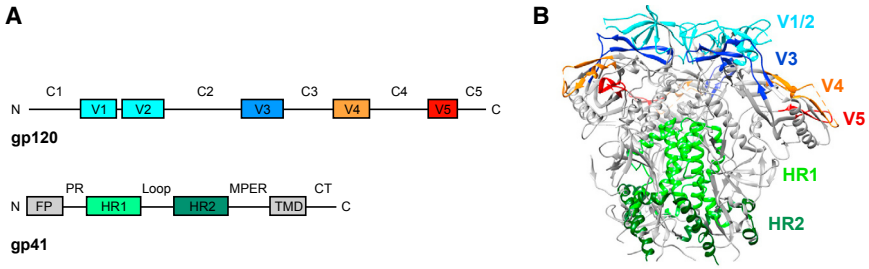

Figure 1. HIV Env Structure

(A) Schematic representation of gp120 and gp41. (B) Crystal structure of native HIV Env. The positions of V1–V5 of gp120 as well as of HR1 and HR2 of gp41 are indicated. The image was generated with the software Chimera by using the coordinates from PDB: 5FUU.[18]

conserved regions of gp120 contain 24 glycosylation sites.[21,22] The glycans represent 50% of the molecular mass of gp120 and act as an additional shield that masks conserved epitopes.[23]

The Receptor and Coreceptors

In order to enter host cells, HIV Env g120 needs to interact with a primary receptor, CD4, and a coreceptor, usually CCR5 or CXCR4. CD4 is a glycoprotein that is mainly expressed on the surface of T cell subsets and monocytes.[24] CD4 interacts with the major histocompatibility complex class II (MHC II) and plays an important role in T cell development and function.[25] The extracellular region of CD4 is comprised of four immunoglobulin (Ig)-like domains (D1–D4).[26] The gp120-binding region is contained in D1, which is located at the N terminus.[27] The CD4-binding pocket of gp120 was mapped to the base of the V1, V2, and V5 loops as well as the C2, C3, and C4 regions.[28,29]

The CXCR4 and CCR5 chemokine receptors are members of the G-protein-coupled receptor (GPR) superfamily and contain seven transmembrane domains. CXCR4 is ubiquitously expressed on all blood cells[30] and interacts with the CXC chemokine stromal cell-derived factor-1 (SDF-1),[31] which functions as a chemoattractant and regulates hematopoiesis.[32,33] CXCR4 is essential during embryonic development and plays an important role in the tissue recruitment of immune cells in adults.[30] In contrast, CCR5 seems to be dispensable in humans. Individuals born with a naturally occurring mutation in the *CCR5* gene (*CCR5Δ32*) are apparently healthy and show an increased resistance to HIV infection.[34] CCR5 is mainly expressed on $CD4^+$ T cells and macrophages but can also be found on a variety of other cell types, including microglia, astrocytes, and neurons.[35] Natural ligands for CCR5 include macrophage inflammatory protein-1α (MIP-1α), MIP-1β, and regulated upon activation normal T cell expressed and secreted (RANTES).[36] CCR5 is involved in recruiting immune cells to the sites of inflammation,[37] and it has been suggested that individuals with the *CCR5Δ32* gene are more susceptible to some infectious diseases, such as infections with the West Nile virus.[38] HIV strains that utilize CCR5 as a coreceptor are referred to as R5 HIV, and CXCR4-utilizing strains are referred to as X4 HIV.[39] Dual-tropic HIV strains are referred to as R5X4 HIV.[39] R5 HIV is predominantly involved in transmission of the virus.[40] However, HIV switches coreceptor usage from CCR5 to CXCR4 in about 50% of the patients in later stages of the disease.[41] Although CCR5 and CXCR4 are the most commonly used coreceptors,[42] some HIV isolates can utilize other GPRs as coreceptors. Examples of alternative coreceptors include the apelin receptor, CCR1, CCR2b, CCR3, CCR8, CCR9, chemokine-like receptor 1, CXCR3, CXCR5, CXCR6, CXCR7, formyl peptide receptor-like 1, GPR1, and GPR15.[43,44] Although the significance of alternative coreceptor usage is still under investigation, it was suggested that coreceptors other than CCR5 and CXCR4 could play a role in HIV dissemination and pathogenesis.[44,45] Coreceptor binding and specificity are dependent upon the V3 loop of gp120[46,47] and additional conserved regions in proximity of the stem of the V3 loop.[48–50] Binding of the chemokine receptor to gp120 is largely dependent on the tyrosine-sulfated N-terminal region and the extracellular loop 2 but may also be influenced by additional residues present in other extracellular loops.[51]

HIV Entry Cascade

HIV entry is a highly sequential and time-sensitive process that can be divided into receptor binding, coreceptor binding, and membrane fusion (Figure 2). Receptor and coreceptor binding is mediated by gp120, and membrane fusion is mediated by gp41. The first step in the viral entry process is binding of gp120 to CD4. CD4 binding induces extensive conformational changes in HIV Env that cause the trimeric complex to assume an open or activated state.[52] The V1 and V2 regions and the CD4-binding sites move away from the center of the trimer, resulting in the exposure of the V3 loop and of the central gp41 stalk.[53] Interaction of the CD4-bound trimer with either coreceptor induces additional conformational changes that prime gp41 for membrane fusion. Specifically, gp41 forms a pre-hairpin intermediate, in which the HR1 and HR2 form extended helices and the FP is inserted into the host cell membrane.[54] Interaction of HR1 with HR2 causes gp41 to fold back on itself, which results in the formation of a six-helix bundle.[55] This conformational change is thought to bring the viral and cellular membranes together, causing the lipids from the cellular and viral membranes to mix. Lipid mixing results in the formation of a fusion pore and release of the virion contents into the cytoplasm.[56] It is of note that membrane fusion can occur directly at the plasma membrane and in endosomes upon receptor-mediated internalization of the virus particles.[57] Furthermore, HIV Env expressed on the surface of infected cells can also interact with CD4 on uninfected HIV target cells, which in turn can lead to the fusion of the cellular membranes or the formation of virological synapses and cell-cell transmission.[58]

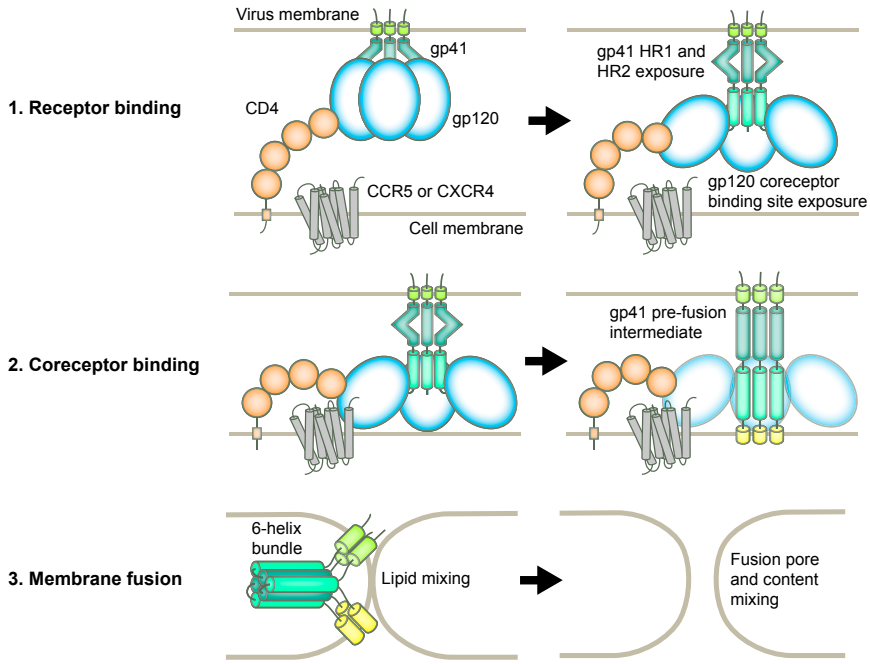

Figure 2. The HIV Entry Cascade

A schematic representation of the HIV entry process is shown. Receptor binding induces conformational changes in gp120 that result in the exposure of the coreceptor-binding site on gp120 and the HR1 (light green) and HR2 (dark green) of gp41. Coreceptor binding induces additional changes that result in the release of the FP of gp41 (yellow) and cause the HR1 and HR2 of gp41 to assume an extended conformation (pre-fusion intermediate). Insertion of the FP into the host cell membrane initiates the formation of the 6-helix bundle and lipid mixing between the viral and cellular membranes, leading to the formation of a fusion pore and content mixing.

Protein-Based HIV Entry Inhibitors

Entry inhibitors interfere with the first step in the HIV replication cycle and can prevent cells from becoming infected. Small-molecule inhibitors, peptides, and proteins have been described against each step of HIV entry. This section focuses on peptides and proteins that interfere with individual steps of HIV entry by targeting viral or cellular proteins.

Soluble Receptors Targeting HIV Envelope Glycoproteins

Soluble CD4 (sCD4) represents the first HIV entry inhibitor that has been developed. It is a truncated version of the CD4 receptor that contains the gp120-binding site but lacks the transmembrane domain. sCD4 did not interact with MHC II and exhibited excellent antiviral activity against X4 HIV_{IIIB}.[59–61] Based on the *in vitro* data, a clinical trial examining the benefits of administering recombinant sCD4 was conducted. Although daily injections of up to 30 mg sCD4 for four weeks were safe, only modest reductions in viral load (23% reduction at the highest dose in comparison to baseline) were observed despite reaching serum concentrations of sCD4 that were sufficient to inhibit X4 HIV_{IIIB} *in vitro* (~100 ng sCD4 per mL blood).[62] Follow-up studies showed that significantly higher concentrations of sCD4 were required for inhibition of HIV strains newly isolated from patients.[63] Based upon these findings, the effects of a single infusion of up to 10 mg sCD4 per kg patient weight were examined.[64] Serum sCD4 concentrations reached 30–200 μg/mL, and all patients showed a dose-dependent reduction of viral load. Remarkably, three out of four patients in the highest dose group became aviremic.[64] In a follow-up trial based on the twice-daily administration of up to 10 mg sCD4 per kg patient weight, two out of three patients in the highest dose group became and remained aviremic during a four-week treatment period.[65]

To improve the antiviral effect and stability, sCD4 was covalently linked to the constant region of the IgG1 heavy chain (sCD4-IgG1).[66] sCD4-IgG1 lacked the first domain of the constant heavy chain, but contained the hinge region and the crystallizable fragment (Fc) region, and formed dimers. sCD4 was also used to replace the V1 and V2 regions of the IgG2 light and heavy chains to generate a heterodimer containing four copies of sCD4 (sCD4-IgG2 or PRO542).[67] Electron microscopy revealed that sCD4-IgG1 in complex with two gp120 molecules assumed a Y-shaped structure, and the PRO542-g120 complex consisted of four gp120 molecules arrayed around a central PRO542 molecule.[68] Based on this result, it was proposed that PRO542 can crosslink multiple HIV Env trimers.[68] sCD4-IgG1 and PRO542 showed improved antiviral activity and stability in the serum,[66,67] but they failed to show a dose-dependent inhibition in patients, which was attributed to potential interactions between the Fc regions of these inhibitors and the Fc receptors present on human immune cells.[69,70]

The gp120-binding site is located in D1 of CD4, which is unstable when expressed on its own. Screening a large library of D1 mutants has recently revealed two stable 100-residue D1 proteins (mD1.1 and mD1.2).[71] The antiviral effect of mD1.1 was comparable to that of sCD4 when tested against a panel of 13 HIV isolates.[71] In a follow-up study, an mD1.2 mutant with increased stability and solubility has been identified.[72] Interestingly, this mutant showed a 10-fold increased neutralization activity against two R5 HIV strains when compared to sCD4. One of the greatest advantages of sCD4 is that it is identical to CD4. Therefore, HIV cannot escape binding to sCD4 without losing affinity for CD4 on target cells. It remains to be seen to what extent resistance to these modified sCD4 variants develops.

Antibodies Targeting HIV Envelope Glycoproteins

The CD4-binding site is one of the few conserved epitopes that is accessible in native HIV Env. In contrast to sCD4, monoclonal antibodies (mAbs) targeting the CD4-binding site do not induce conformational changes in HIV Env. The mAb b12 was one of the

first well-characterized CD4-binding site antibodies with broad specificity.[73] Since the discovery of b12, the development of more sensitive techniques has resulted in the isolation of novel antibodies with improved antiviral activity.[74] Two of the most promising antibodies are 3BNC117 and VRC01, which neutralize 78% and 84% of circulating HIV isolates, respectively, at a concentration of 50 μg/mL (compile, analyze, and tally neutralizing antibody panels [CATNAP]).[75–77] The effect of a single infusion of 3BNC117 or VRC01 (up to 30 mg/kg patient weight) was recently examined in clinical trials.[2,3] Although injection of either antibody resulted in a drastic reduction of plasma viral load (0.8–2.5 $\log_{10}$), emergence of resistant isolates was observed.

The mAb 2G12 binds to a distinct gp120 epitope that is formed by multiple glycans.[78] Recently, mAbs that can target three additional glycan epitopes have been identified. These epitopes are formed by the N_{332} glycan and the V3 loop, the N_{160} glycan and V1 and V2 loops, or the N-linked glycans near the interface between gp120 and gp41.[79] Examples of mAbs that can recognize these epitopes are PGT121, PG9, or 35O22, respectively. Although newly identified glycan-targeting mAbs are potent inhibitors of HIV entry,[79] their neutralization breadth seems to be lower than that of mAbs targeting the CD4-binding site. For example, mAb PGT121 targets the N_{332} glycan and neutralizes 61% of circulating HIV isolates at a concentration of 50 μg/mL (CATNAP).[77] In comparison, the mAb VRC01 targets the CD4-binding site and neutralizes 84% of circulating isolates at the same concentration.[77] Similar to CD4-binding site mAbs, mutations that confer resistance to glycan-targeting mAbs have been identified.[80,81]

Binding of sCD4 to gp120 results in the exposure of several conserved discontinuous epitopes on the surface of gp120.[28,82] The human mAb 17b targets a conserved CD4-induced epitope on gp120 that partially overlaps with the coreceptor-binding site.[28,46] Binding of 17b to this epitope prevents interaction of gp120 with CCR5 and CXCR4. Antibodies targeting CD4-induced epitopes are readily formed in individuals infected with HIV and can mediate antibody-dependent cellular cytotoxicity (ADCC).[83] However, their antiviral activity is limited because their target sites only become available when the viral and cellular membranes are in close proximity, and at this point, steric hindrance restricts antibody access. Therefore, antibodies targeting the coreceptor-binding site are generally not considered as bnAbs.

HIV gp41 MPER-targeting antibodies bind to a fusion-intermediate of gp41 and prevent conformational changes in gp41 that are necessary for fusion.[84] 2F5, 4E10, and 10E8 are among the best-characterized MPER-targeting mAbs.[85–87] 4E10 and 10E8 exhibit exceptional neutralization breadth and neutralize $\geq$90% of circulating HIV isolates; 10E8 is ~10-fold more potent at inhibiting HIV entry than 4E10.[77] The epitopes of anti-MPER mAbs were shown to include membrane lipids in addition to the gp41 MPER.[88–90] For example, 4E10 has been shown to bind to several phospholipids, cardiolipin, sulfatide, galactosyl ceramide, glucosyl ceramide, and lipid A.[91] Therefore, their unspecific binding to membrane lipids is a concern in the clinical development of these antibodies.[92]

The field of bnAb research for HIV treatment and prevention is developing rapidly, and many new bnAbs within the above-mentioned classes have been described in recent years. These novel bnAbs were either isolated from patients or were engineered by rational design. There is a series of exceptional reviews that describe newly identified bnAbs and pre-clinical research in non-human primates in more detail.[1,93–97] bnAbs are of special interest for HIV cure research due to their ability to mediate the destruction of infected cells via ADCC. When latently infected cells become active, they start to express HIV Env. The bnAbs can bind HIV Env present on the surface of infected cells. In turn, cells that express the Fc receptor CD16 on their surface, mainly natural killer cells, recognize infected cells that are coated with antibodies and mediate ADCC. Therefore, bnAbs have the potential to reduce the viral reservoir in patients. Reduction of the viral reservoir can be accelerated when bnAbs are used in combination latency-reversing agents.[98] However, the ability to bind to cell surface HIV Env and induce ADCC varies considerably between individual bnAbs and does not necessarily correlate with their ability to neutralize cell-free HIV.[99–101] Further research will be necessary to find optimal combinations of bnAbs that effectively induce ADCC.

Lectins Targeting HIV Envelope Glycoproteins

Lectins are proteins that bind complex carbohydrate structures. Examples of lectins with anti-HIV activity are cyanovirin-N (CV-N), griffithsin (GRFT), and banana lectin (BanLec). CV-N was isolated from the cyanobacterium *Nostoc ellipsosporum* and GRFT from the red algae *Griffithsia*, and BanLec was isolated from *Musa acuminate*.[102–104] Although the exact mechanism of action is unknown, the lectins bind to high mannose glycans on gp120 and interfere with the binding of gp120 to CD4 on the target cells.[103–105] GRFT is a highly potent inhibitor of HIV entry, inhibiting a broad range of HIV isolates at picomolar concentrations.[103] However, loss of glycosylation sites on gp120 and rearrangement of glycans in gp120 can lead to resistance to CV-N and GRFT.[106] Nevertheless, their use for HIV prophylaxis as topical microbicides is currently under investigation.[107] Both CV-N and GRFT were shown to prevent infection on cervical explants and virus dissemination by tissue-emigrating cells.[108,109] It is of note that prolonged exposure to CV-N resulted in activation of peripheral blood mononucleocytes and an increase in inflammatory cytokines, but these effects were not evident upon shorter incubation times.[109] More recently, a BanLec variant was engineered by introducing a mutation within the sugar-binding site to eliminate its mitogenicity while retaining its antiviral activity.[110]

Peptides Targeting HIV Envelope Glycoproteins

The complementary determining region 2 (CDR2)-like domain of the D1 of CD4 contains residues critical for interaction with gp120 but is unstable when expressed on its own. CD4-mimicking peptides were designed by inserting critical residues from the CDR2-like region of CD4 into the CDR2-like region of stable but inactive scorpion toxin derivatives.[111] Residues of the CD4 mimetic peptides were further

optimized based on the crystal structure of the CD4-gp120-mAb 17b complex.[112] In comparison to sCD4, the gp120 binding and antiviral activity of the resulting CD4 mimetic CD4M33 (27 amino acids) was ~3- to 5-fold lower.[112]

CCR5mim is a tyrosine-sulfated CCR5-mimetic peptide (15 amino acids) derived from the variable region of the sCD4-induced epitope-targeting mAb E51.[113] CCR5mim fused to IgG1 (CCR5mim-Ig) inhibits both R5 and X4 HIV entry at micromolar concentrations.[113] In comparison to CCR5-mimetic peptides derived from CCR5 and fused to IgG1, CCR5mim-Ig inhibited HIV entry 20 times better but was significantly inferior to the previously mentioned sCD4-Ig, which inhibited HIV entry at nanomolar concentrations.[113] Potential interactions with natural CCR5 ligands were not examined.

Fusion inhibitors (FIs) are short peptides based on the HR2 of gp41. FIs bind to the HR1 of gp41 and prevent the formation of the six-helix bundle that is necessary for the fusion of viral and cellular membranes.[55,114] FIs inhibit both CCR5-tropic and CXCR4-tropic HIV isolates at nanomolar concentrations. FI_{T20} (also known as T20, DP-178, Fuzeon, and enfuvirtide; amino acids 643–678 of HIV_{LAI} gp160) is the first fusion inhibitor that has been approved for clinical use.[115] Due to poor absorption in the gastrointestinal tract and sensitivity to proteases, an oral formulation is not available and FI_{T20} must be injected.[116] It is used to treat patients who fail ART regimen. Another disadvantage of FI_{T20} is the emergence of escape mutants in patients.[117] FI_{T-1249} is a second-generation fusion inhibitor consisting of 39 amino acids derived from the consensus HR2 sequences of HIV-1, HIV-2, and simian immunodeficiency virus.[118] Although FI_{T-1249} showed improved antiviral activity and was effective against FI_{T20}-resistant strains, clinical development was halted due to the drug formulation difficulties.[119] Third generation fusion inhibitors, such as FI_{T2635}, were designed to have an enhanced helical structure and self-associate into stable oligomers.[120] Although mutations that confer resistance to the second-generation FIs have been described, multiple mutations that cause a severe loss of fitness are necessary to confer resistance to third generation FIs.[121–123] FI_{T2635} is currently tested in clinical trials in China.[124,125] Lipid-modified peptides (lipopeptides) are peptides modified with fatty acid, cholesterol, or sphingolipids. Lipopeptide fusion inhibitors associate with the cellular membrane and can possess improved antiviral activity and *in vivo* stability.[126,127] One of such inhibitors, LP-11, demonstrated greatly improved activity over FI_{T20} in inhibiting diverse subtypes of HIV isolates, with mean half-maximal inhibitory concentrations (IC_{50}s) of 0.83 nM and 29.45 nM, respectively.[128,129]

Antibodies Targeting CD4 or CCR5/CXCR4

The CD4 receptor plays a central role in MHC-II-mediated immune functions. Although many CD4-targeting mAbs with anti-HIV activity have been identified, most mAbs that inhibit gp120-CD4 interaction also interfere with the binding of CD4 to MHC II.[130] Ibalizumab is a humanized anti-CD4 mAb that binds to the D2 of CD4.[131] It inhibits HIV entry at a post-attachment step and does not interfere with MHC II binding.[131] Weekly infusions (10 mg/kg patient weight) or infusions every two weeks (25 mg/kg patient weight) of ibalizumab reduced viral load (0.5 to 1.7 log_{10}) in 20 out of 22 patients after 1 or 2 weeks, but the viral load started to increase again after 2 or 3 weeks of treatment.[132] Viral load eventually returned to the pre-trial levels despite repeated infusions of ibalizumab, and the emergence of resistant isolates was documented.[132]

PRO 140 is a humanized IgG4 mAb that is based on the murine anti-CCR5 mAb PA14[133] and is currently the only coreceptor-targeting mAb in clinical development. PRO 140 binds to an epitope formed by the N terminus and the second extracellular loop of surface CCR5 without inducing CCR5 signaling.[133] PRO 140 prevents binding of the V3 loop of gp120 to CCR5. PRO 140 is effective against primary HIV isolates, including those resistant to small-molecule CCR5 inhibitors (e.g., maraviroc).[134] Infusion of PRO 140 (324 mg/patient weekly or every 2 weeks) significantly reduced viral load (>1 log_{10} mean reduction after 22 days) and increased $CD4^+$ T cell count in patients harboring R5 HIV.[135] The long-term efficacy and safety of PRO 140 is under investigation in phase 2b and 3 clinical trials (NCT02483078). There is no candidate CXCR4-targeting antibody for the inhibition of HIV entry under development.

Chemokines Targeting CCR5

The chemokines MIP-1α, MIP-1β, and RANTES are natural ligands of CCR5. The three chemokines were identified as the major HIV-suppressive factors released by $CD8^+$ T cells.[136] They mainly interfere with HIV entry by inducing the internalization of CCR5.[137] Chemokine analogs have been investigated as potential entry inhibitors. For example, chemical modification of the amino terminus of RANTES resulted in RANTES analogs with more potent antiviral activity, such as PSC-RANTES.[138] PSC-RANTES is in development as a topical microbicide and has been shown to inhibit vaginal HIV transmission in a pre-clinical macaque model utilizing simian immunodeficiency virus/HIV hybrid strains.[138,139] However, the clinical use of chemokines and their analogs is limited because they also mediate acute and chronic inflammation.[140] 5P12-RANTES and 5P14-RANTES are RANTES analogs identified by the screening of phage display libraries.[141] In contrast to RANTES and previous analogs, 5P12-RANTES and 5P14-RANTES do not induce CCR5 cell signaling, and only 5P14-RANTES causes CCR5 internalization.[141] Both analogs are effective against CCR5-tropic HIV strains at picomolar concentrations, and efforts are underway to develop 5P12-RANTES as a topical microbicide to prevent vaginal and rectal transmission of HIV.[142,143]

Bifunctional Antiviral Proteins Targeting HIV Entry

Due to the highly variable nature of HIV Env, single AVPs are of limited use for HIV treatment and prevention. Similar to ART and PrEP, a combination of AVPs would likely be necessary for therapy and prophylaxis. To overcome this limitation, bAVPs are designed that simultaneously bind two different targets. bAVPs are fusion proteins containing two AVP domains connected with a flexible linker sequence (Figure 3). A summary of bAVPs and their antiviral potency is provided in Table 1.

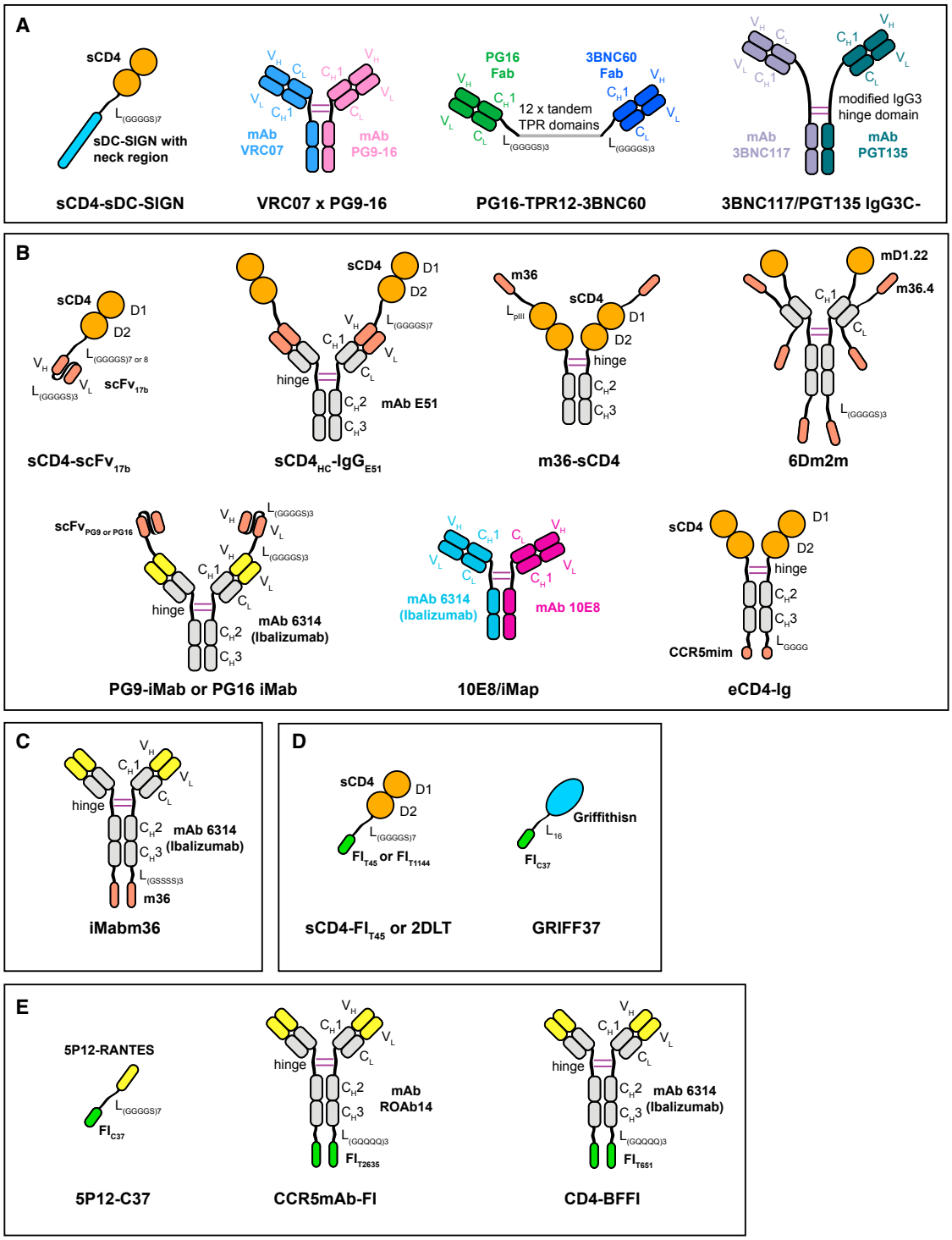

Figure 3. Design of bAVPs

(A) bAVPs targeting receptor binding. (B) bAVPs targeting receptor binding and coreceptor binding. (C) bAVP targeting coreceptor binding. (D) bAVPs targeting receptor and membrane fusion. (E) bAVPs targeting coreceptor binding and membrane fusion. C_H, antibody heavy chain constant domain; C_L, antibody light chain constant domain; D1 and D2, CD4 domain 1 and 2; L, linker sequence; L_{16}, linker sequence consisting of 16 G and S residues in random order; L_{pIII}, linker sequence derived from bacteriophage protein pIII; V_H, antibody heavy chain variable domain; V_L, antibody light chain variable domain.

Table 1. Overview of bAVPs

Entry Steps	Targets	bAVP	IC_{50} µg/mL	IC_{50} nM	No. of Isolates	Ref
Receptor binding	CD4bs	sCD4-sDC-SIGN	0.30	4.70	3	146
	gp120 glycans	VRC07 × PG9-16	0.06	0.40	206	148
		PG16-TPR12-3BNC60	0.03	0.21	12	151
		3BNC117/PGT135 IgG3C-	0.04	0.27	119	149
Coreceptor binding	CD4 CoRbs	iMabm36	NA	NA	118	153
Receptor binding Coreceptor binding	CD4bs	sCD4-scFv$_{17b}$	1.01	19.87	47	154,155
	CoRbs	sCD4$_{HC}$-IgG$_{E51}$	0.72	2.88	33	157
		m36-sCD4	0.42	3.20	14	156
		eCD4-Ig	0.05	0.39	24	158
		6Dm2m	0.07	0.32	12	72
	gp120 glycans	PG9-iMab	<0.01	0.02	118	160
	CD4	PG16-iMab	<0.01	0.01	118	160
	MPER CD4	10E8/iMab	<0.01	0.01	118	161
Receptor binding Membrane fusion	CD4bs	sCD4-FI$_{T45}$	0.10	3.52	16	164,165
	gp41 HR1	2DLT	1.43	40.83	5[a]	166,167
	gp120 glycans gp41 HR1	Griff37	<0.01	0.08	4[b]	168
Coreceptor binding Membrane fusion	CCR5	5P12-C37	<0.01	0.02	6	169
	gp41 HR1	CCR5mAb-FI	0.05	0.26	5[b]	170
	CD4 gp41 HR1	CD4-BFFI	4.63	27.23	20	173

The targeted entry steps and molecular targets of different bAVPs are shown. The mean (geometric) IC_{50}s and number of isolates that were tested in single-round infection assays are indicated for each bAVP. CD4bs, CD4-binding site; CoRbs, coreceptor-binding site; Ref, references.
[a]Virion inactivation was tested by washing out the inhibitor prior to infecting the target cells.
[b]Assays were performed using primary peripheral blood lymphocytes.

Bifunctional Antiviral Proteins Targeting Receptor Binding

Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) is a C-type lectin receptor expressed on the surface of dendritic cells that binds to high mannose glycans on gp120. DC-SIGN-bound HIV remains infectious. Dendritic cells can capture virions, transport them to lymphoid tissues, and allow infection of $CD4^+$ T cells.[144] DC-SIGN consists of an N-terminal cytoplasmic domain, a transmembrane domain, a neck region involved in tetramerization of the receptor, and a carbohydrate recognition domain (CDR) at the C terminus. Soluble DC-SIGN (sDC-SIGN) consists of the CDR and prevents the interaction of HIV gp120 with DC-SIGN but increases the exposure of the CD4-binding site on gp120 and enhances infection of T cells.[145] Based on the ability of sDC-SIGN to increase binding of sCD4, a series of sCD4-sDC-SIGN fusion proteins was created.[146] Improved antiviral effects in comparison to sCD4 were only observed for fusion proteins with six to seven repeats of linkers containing four glycines and one serine (GGGGS) that also contained the neck region of DC-SIGN (Figure 3A). Interestingly, changing the serine at position 60 of the D1 of sCD4 to cysteine (S60C) was previously shown to enhance the affinity of sCD4 to gp120 by enabling the formation of a disulfide bond between the S60C of sCD4 and the cysteine at position 126 of gp120.[147] Introducing the S60C mutation into sCD4-DC-SIGN with the neck region and a seven-repeat linker increased the antiviral potency against two additional HIV isolates by 10-fold in comparison to the fusion protein without the mutation.[146] sCD4-DC-SIGN may be used to develop a microbicide to prevent sexual transmission of HIV. However, further characterization of the fusion protein, e.g., stability and toxicity, would be necessary in more clinically relevant models.

In another study, bispecific antibodies were designed that resembled normal mAbs in their structure.[148] One arm of these bispecific antibodies was derived from one HIV-Env-targeting bnAb, and the other arm was derived from another one. The bispecific antibodies are generated by combining the CrossMap technology, which involves

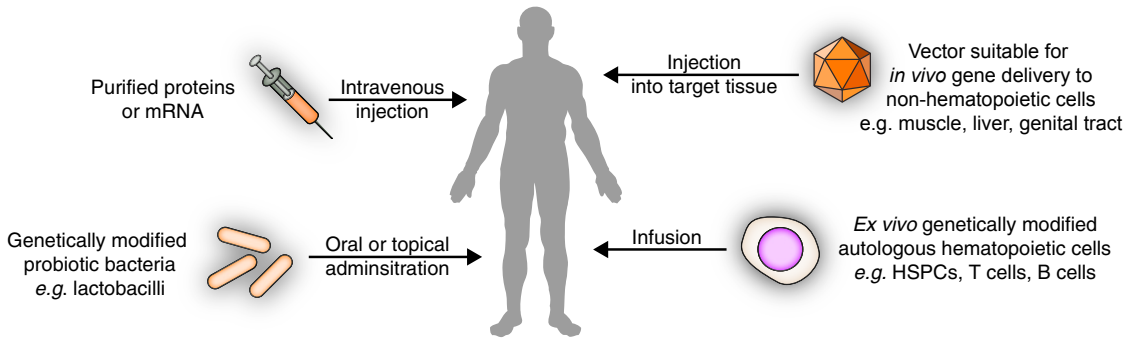

Figure 4. Delivery of bAVPs

bAVPs can be directly injected into patients. Alternatively, several genetic strategies may be used to deliver genes encoding bAVPs. Virus-derived vectors, such as AAV vectors, have been used to deliver genes to non-hematopoietic cells by direct injection into the target site. Probiotic bacteria of the genital tract can be engineered to secrete bAVPs for prevention. Autologous hematopoietic cells can be modified to secrete bAVPs with viral and non-viral vectors.

C_H1 and C_L domain swapping, with the knob-into-hole technology, which introduces a point mutation in the C_H3 domain for heavy chain heterodimerization.[149] Out of the tested combinations, VRC07 × PG9-16 (Figure 4A) was the best inhibitor and showed greater neutralization potency and breadth in comparison to the individual antibodies, but not in comparison to a combination of the two parental antibodies. Nevertheless, VRC07 × PG9-16 had a similar half-life than the parental antibodies when administered to macaques. However, further *in vivo* testing of the different bAVPs would be necessary to compare their serum half-lives.

In a different study, bispecific antibodies were found to be less effective in comparison to their parental antibodies. IgG antibodies have the potential to crosslink two different HIV Env spikes or to bind with both arms to the same HIV Env spike. However, the low density of HIV Env spikes and therewith the long distance between two different HIV Env spikes limit the ability of IgGs to crosslink two Env spikes. The structure of the individual Env spikes also impedes binding of both antibody arms to the same Env spike.[150] Among the human IgG subclasses, IgG3 encompasses an exceptionally long and flexible hinge domain. In order to allow bispecific antibodies to engage one Env spike with both arms, variants based on IgG3 were designed.[149] Of the tested constructs, 3BNC117/PGT135 with an engineered IgG3 hinge domain (3BNC117/PGT135 IgG3C-) (all cysteines were replaced with serines with the exception of the two cysteine residues close to C_H2) was the best inhibitor and over 10-fold more potent than the corresponding bispecific construct based on IgG1. Importantly, 3BNC117/PGT135 IgG3C- mediated significantly stronger inhibition of HIV replication than a mixture of the parental antibodies in humanized mice with established HIV infection.

In order to measure the distance between two antibody arms that allows binding to the same Env spike, two antibody fragments were linked using variable-length double-stranded DNA.[151] 40-bp and 50-bp double-stranded DNA (dsDNA) bridges were optimal and corresponded to 136 Å and 170 Å, respectively. The dsDNA bridge was replaced with a rigid protein-based linker of similar length consisting of 12 domains of a tetratricopeptide-repeat (TPR) protein and used to attach a PG16-based antibody fragment to a 3BNC60-based fragment (PG16-TPR12-3BNC60). The resulting fusion protein was over 33-fold more potent than the individual antibody fragments. However, due to the lack of the Fc region, PG16-TPR12-3BNC60 likely has a lower half-life than the parental antibodies and cannot induce ADCC.

Bifunctional Antiviral Proteins Targeting Coreceptor Binding

Despite binding to CD4, the mAb ibalizumab does not interfere with the binding of gp120 to CD4. Instead, ibalizumab interferes with coreceptor engagement of CD4-bound virions. Resistance to ibalizumab is readily acquired by mutations in HIV Env that allow coreceptor engagement despite binding of ibalizumab to CD4. In order to overcome this limitation, the C terminus of each ibalizumab heavy chain was linked using a four-repeat GGGGS linker to a stable antibody fragment that binds to a conserved CD4-induced epitope of gp120, m36.[152,153] The resulting fusion protein, iMabm36, inhibited HIV entry better than the individual moieties either alone or in combination. Out of a panel of 118 HIV strains, 83% were neutralized by iMabm36 at an IC_{50} of less than 0.1 μg/mL compared to 75% for ibalizumab alone. Detailed IC_{50} values for each strain and inhibitor were not provided.

Bifunctional Antiviral Proteins Targeting Receptor and Coreceptor Binding

Like CD4, sCD4 has the unique ability to induce conformational changes in HIV Env and expose the conserved coreceptor-binding site.[28,82] To exploit this sCD4-induced conformational change, sCD4 has been fused with a series of entry inhibitors targeting the coreceptor-binding site of gp120 (Figure 3B). sCD4 was combined with the single-chain variable fragment (scFv) of mAb 17b ($scFv_{17b}$) with a seven-repeat flexible GGGGS linker.[154] The resulting sCD4-$scFv_{17b}$ fusion protein was a more potent inhibitor of R5 HIV_{BaL} entry than sCD4, mAb 17b, or combinations thereof. sCD4-$scFv_{17b}$ with an eight-repeat GGGGS linker (sCD4-40-$scFv_{17b}$) did not increase the potency of the original sCD4-$scFv_{17b}$ with a seven-repeat linker.

However, when tested against a panel of 47 HIV isolates from different clades, sCD4-40-scFv$_{17b}$ mediated more potent and broader inhibition of HIV entry than the neutralizing mAbs b12, 2G12, 2F5, or 4F10.[155]

Screening a phage library of diversified human antibody heavy-chain variable domains resulted in the identification of m36, the aforementioned stable antibody fragment that binds to a conserved CD4-induced epitope of gp120.[152] Fusing m36 to sCD4 with a 27-residue linker derived from the bacteriophage protein pIII increased the potency of m36 against a panel of 14 HIV isolates.[156] The strongest inhibition was observed when m36 was fused to sCD4-IgG1.[156] In a follow-up study, the optimized one domain sCD4 variant mD1.22 and m36.4 were fused to the human IgG1 constant regions in different combination.[72] Out of the tested constructs, an octavalent construct, 6Dm2m (Figure 4), was the most potent inhibitor, inhibiting 13 primary HIV isolates at picomolar concentrations.

In another study, sCD4 was fused to modified versions of the mAb E51, which also targets the coreceptor-binding site.[157] sCD4 was either fused to the scFv$_{E51}$ or to the heavy chain, light chain, or both chains of full-length mAb E51 by using a seven- or nine-repeat GGGGS linker.[157] Of the tested fusion proteins, sCD4 fused to the heavy chain of mAb E51 by using a nine-repeat linker (sCD4$_{HC}$-IgG$_{E51}$) was the most effective inhibitor when tested against a panel of 33 HIV isolates. The peptide CCR5mim1 was derived from mAb E51 and inhibits HIV entry in the absence of sCD4, but the antiviral effect is enhanced when sCD4 is present. Therefore, sCD4-IgG1 was fused to CCR5mim1 with a short linker consisting of four G residues.[158] The resulting fusion protein eCD4-Ig was 20-fold more potent at inhibiting HIV entry than sCD4-IgG1. Furthermore, eCD4-Ig with an optimized CCR5-mimetic peptide (alanine at position 40 instead of glutamine) was highly effective against isolates that were resistant to the CD4-binding site mAbs 3BNC117 and VRC01. Introducing three mutations present in D1.22 into eCD4-Ig further increased its potency 9-fold.[159]

Overall, fusing sCD4 to coreceptor binding-site-targeting moieties seems to be a highly promising approach. Especially eCD4-Ig exhibits highly potent antiviral activity against a broad range of HIV isolates. The high potency of eCD4-Ig in comparison to the other bAVPs based on the same concept is probably related to use of the small coreceptor-mimicking peptide as opposed to antibody fragments, which may have problems reaching the coreceptor-binding site due to steric hindrance or improper folding. However, one disadvantage of eCD4-Ig is the requirement for the co-expression of a tyrosylprotein sulfotransferase, which increases the sulfation of the CCR5 mimetic peptide. Furthermore, it could be possible that the CCR5 mimetic peptide binds to natural CCR5 ligands. Nevertheless, eCD4-Ig has a high potential for HIV prophylaxis and therapy and can be expected to advance to testing in clinical trials.

In a different approach, scFvs of the glycan-targeting bnAbs PG9 or PG16 were fused to the N terminus of mAb ibalizumab, which binds to CD4 but interferes with coreceptor binding.[160] The bispecific antibodies PG9-iMab and PG16-iMab inhibited 118 different HIV isolates at picomolar concentrations. The remarkable antiviral effect was likely due to bringing the scFv moiety close to its site of action, the CD4 receptor on the surface of HIV target cells. Antiviral activity was optimal when a 15-residue flexible linker was used as shorter and longer linkers decreased the potency. In a follow-up study, bispecific antibodies were designed in which one arm was derived from ibalizumab or PRO140, and the other arm was derived from bnAbs targeting HIV Env. The best inhibitors combined one arm of the MPER-targeting bnAb 10E8 with one arm of either ibalizumab or PRO140 (10E8/iMab or 10E8/P140, respectively) and showed similar antiviral effects as PG9-iMab and PG16-iMab.[161]

Bifunctional Antiviral Proteins Targeting Receptor Binding and Membrane Fusion

The HR1 is inaccessible in native HIV Env trimers, but it has been shown that fusion inhibitors can bind to HIV Env in the presence of sCD4 in the absence of HIV target cells.[162,163] We have covalently linked sCD4 to the third-generation fusion inhibitor FI$_{T45}$ by using a seven-repeat GGGGS linker (Figure 3C). The resulting fusion protein sCD4-FI$_{T45}$ was a potent inhibitor of HIV entry and neutralized isolates that were resistant to sCD4, FI$_{T20}$, or VRC01.[164,165] Lu et al.[166] have also fused sCD4 to the second-generation FI$_{T1144}$ by using a seven-repeat GGGGS linker (2DLT). The sCD4 moiety in 2DLT was shown to expose the HR1 of HIV Env. 2DLT could inactivate cell-free HIV isolates, whereas the fusion inhibitors FI$_{T20}$ or FI$_{T1144}$ did not. Therefore, combining FIs with sCD4 addresses one of the major drawbacks of FIs, namely their inability to bind to virus particles in the absence of target cell binding. Both sCD4 and FIs have been tested in clinical trials and were found to be safe. The bAVPs could be used to augment the effect of existing therapies in individuals on ART who fail to suppress viremia. For example, the 2DLT worked synergistically with antiretroviral drugs, including nucleoside and non-nucleoside reverse-transcriptase inhibitors as well as protease inhibitors.[167] 2DLT was also effective against single or multiple reverse transcriptase inhibitor-resistant HIV strains.[167] However, one drawback of the fusion proteins is their potentially low *in vivo* half-life (less than 24 hr for sCD4 and FIs). Therefore, it might be necessary to adapt an antibody-based design, e.g., combining sCD4-IgG1 with fusion inhibitors, for further development as alternative therapeutics.

The lectin GRFT binds to high mannose glycans on gp120 and interferes with binding to the CD4 receptor. To inhibit both HIV gp120 and gp41, GRFT was fused to FI$_{C37}$ with a linker sequence consisting of 16 G and S residues in random order (Griff37; Figure 3C).[168] Griff37 was 9-fold more potent than GRFT in cell fusion assays with HIV target cells and R5 HIV-Env-expressing cells. However, Griff37 was only two- to four-fold more effective than GRFT in single-round infection assays and with R5 HIV$_{ADA}$ and R5HIV$_{JRFL}$, likely because of the strong inhibition mediated by GRFT alone against these strains and because griffithsin does not work synergistically with FIs, e.g., GRFT does not allow binding of FIs to virions in the absence of CD4 engagement. Additionally, GRFT originates from

algae and may therefore induce immune responses. Further research into this area will be necessary to evaluate the usefulness of either GRFT or Griff37 for HIV therapy or prophylaxis.

Bifunctional Antiviral Proteins Targeting Coreceptor Binding and Membrane Fusion

In order to increase the potency of 5P12-RANTES and 5P14-RANTES, the chemokine analogs were fused with FI_{C37} using a 2-repeat GGGGS linker (5P12-linker-C37 and 5P14-linker-C37, respectively; Figure 3D).[169] 5P12-linker-C37 showed exceptional antiviral activity against six R5 HIV isolates and was over ten-fold more potent than 5P12-RANTES. Interestingly, 5P12-linker-C37 was also more active than FI_{C37} when $CD4^+$ $CCR5^+/CXCR4^+$ cells were infected with X4 HIV, suggesting that CCR5-bound 5P12-linker-C37 affects entry of X4 HIV. As expected in the absence of CCR5 interaction, the activity of 5P12-linker-C37 was similar to FI_{C37} when $CD4^+$ $CCR5^-CXCR4^+$ cells were infected with X4 HIV. The antiviral activity of 5P12-linker-C37 was similar to that observed for 5P14-linker-C37.

Based on a similar principal, a mAb targeting CCR5 was fused to two molecules of FI_{T2635} with a 3-repeat linker comprised of one glycine residue and four glutamine residues (CCR5mAb-FI; Figure 3D).[170] The CCR5 mAb was comprised of the variable regions of the mouse anti-human CCR5 mAb ROAb14 inserted into a human IgG1 scaffold.[171] CCR5mAb-FI mediated 30-fold better inhibition of HIV_{BaL} in comparison to the CCR5 mAb and 100-fold better inhibition than the fusion inhibitor T_{2635}. Similar to what was observed for 5P12-linker-C37, CCR5mAb-FI also inhibited X4 HIV_{NL4-3} with 400-fold increased potency in comparison to FI_{T2635} when $CD4^+$ $CCR5^+CXCR4^+$ cells were infected. However, CCR5mAb-FI was only 2-fold more potent than the CCR5 mAb or FI_{T2635} when peripheral blood lymphocytes were infected with five R5 HIV strains.

CD4-BFFI is comprised of the anti-CD4 mAb 6314 covalently linked to FI_{T651} (Figure 3D).[172–174] The mAb 6314 is derived from ibalizumab, binds to the D2 of CD4, and interferes with coreceptor binding without causing the depletion of $CD4^+$ T cells by ADCC. When tested against a panel of 20 HIV isolates, CD4-BFFI inhibited HIV entry with 3-fold higher potency than the mAb 6314.

5P12-linker-C37, CCR5mAb-FI, and CD4-BFFI increase the activity of FIs by bringing them to the surface of target cells, their site of action. All three bAVPs have the disadvantage that they cannot engage virions in the absence of target cell binding. Furthermore, 5P12-linker-C37 and CCR5mAb-FI offer no advantage over FIs when CCR5-negative cells are infected with CXCR4-tropic HIV. Here, CD4-BFFI has an advantage as it binds to CD4, resulting in similar antiviral effects irrespective of coreceptor expression or coreceptor tropism. Development of CD4-BFFI continues, and it was shown that intravenous administration of CD4-BFFI was safe in monkeys and can result in therapeutically relevant serum concentrations.[175] Nevertheless, 5P12-linker-C37 is exceptionally potent at inhibiting CCR5-tropic HIV isolates and is currently tested as a topical microbicide.

Trifunctional Antiviral Proteins

In order to increase the potency of sCD4-$scFv_{17b}$ and sCD4-FI_{T45}, we designed a trifunctional entry inhibitor by linking sCD4-$scFv_{17b}$ to FI_{T45}.[164] However, the resulting fusion protein, sCD4-$scFv_{17b}$-FI_{T45}, did not show increased antiviral potency in comparison to either sCD4-$scFv_{17b}$ or sCD4-FI_{T45}. This was likely due to the lack of synergy between the $scFv_{17b}$ moiety and the FI_{T45} moiety as linking FI_{T45} to $scFv_{17b}$ alone also did not increase the potency of $scFv_{17b}$. Furthermore, secretion of the trifunctional inhibitor was markedly reduced in comparison to sCD4-$scFv_{17b}$ and sCD4-FI_{T45} when produced in human cells.

In another study, trispecific antibodies were designed that target the CD4-binding site, the MPER, and the V1V2 glycan site.[176] One of these antibodies, VRC01/PGDM1400-10E8v4, was tested in macaques and provided complete protection to infection, and VRC01 or PGDM1400 alone only provided partial protection. However, the trifunctional antibody was not compared to mixtures of the single moieties. One arm of VRC01/PGDM1400-10E8v4 consisted of the heavy and light chains of VRC01. The other arm was generated by linking the V_L domain of PGDM1400 to the V_L and C_L of 10E8v4 and by linking the V_H of 10E8 to the V_H of PGDM1400 and the C_H1-3 of 10E8. Despite this unnatural design, the trispecific antibody had a similar plasma half-life to VRC01 in macaques. The trifunctional antibody could be a good candidate for HIV prevention, as it was effective against HIV isolates that were resistant to the individual antibodies. Further studies are necessary to evaluate the capability of the trispecific antibody to bind to HIV Env on the surface of infected cells and induce ADCC.

Advantages and Limitations of Bifunctional Antiviral Proteins

The *in vitro* data show that bAVPs are potent inhibitors of HIV entry and have a remarkable neutralization breadth (Table 1). Among bnAbs targeting HIV Env, CD4-binding site bnAbs mediate the broadest inhibition across different strains. For example, the recently identified CD4-binding site bnAb N6 neutralizes 97% of circulating HIV isolates at a concentration of 50 μg/mL.[77,177] However, several HIV strains that require high concentrations of N6 for inhibition have been described, e.g., the N6 IC_{50} for the clade C isolate CAP210.2.00.E8 is 12.2 μg/mL.[177] In contrast, the bAVPs eCD4-Ig and sCD4-FI_{T45} are significantly more effective against HIV strains that are relatively resistant to CD4-binding site bnAbs; the eCD4-Ig and sCD4-FI_{T45} IC_{50}s for the same clade C isolate are below 0.1 μg/mL.[158,165]

The broad neutralization activity of bAVPs is likely due to their unique mechanism of action. Because HIV needs to maintain its ability to interact with surface CD4 on target cells, mutations that affect binding to sCD4 inevitably reduce viral fitness.[178] However, some HIV isolates require relatively high concentrations of sCD4 for inhibition without an evident loss of fitness.[63] Further analyses revealed no consistent correlation between resistance to sCD4-mediated inhibition and the affinity of sCD4 to HIV Env.[179,180] sCD4 interaction with gp120 prematurely triggers the entry process in the absence of

target cells and induces extensive conformational changes in HIV Env. The sCD4-induced conformational state of HIV Env is transient and is followed by the permanent loss of infectivity of the virus.[163] Interestingly, the sCD4-induced conformational state of resistant isolates was shown to be more stable than that of sensitive isolates.[163] Therefore, HIV Env trimers of resistant isolates likely undergo conformational changes upon binding of one sCD4 molecule but remain infectious despite binding of sCD4. Combining sCD4 with inhibitors that target conserved sCD4-induced epitopes, such as CCR5-mim1/2 or FIs, may therefore exploit the sCD4 resistance path.

Entry inhibitors that target CCR5 work by reducing the density of available CCR5 on the cell surface. Low levels of surface CCR5 lead to slower kinetics of fusion, which in turn has been shown to increase susceptibility to FIs.[181,182] Combining CCR5-targeting entry inhibitors with FIs not only reduces the number of available CCR5 molecules but also brings the FIs directly to the surface of HIV target cells. Resistance to small-molecule CCR5 inhibitors is typically conferred by mutations that allow interaction of HIV Env with CCR5 despite binding of the inhibitors.[183] In contrast, resistance to protein- and peptide-based CCR5 inhibitors seems to require a more difficult switch to utilize CXCR4 as a coreceptor, which requires more mutations than the resistance path to small-molecule inhibitors, such as maraviroc.[184] Third-generation FIs also have the advantage that, for resistance to develop, multiple mutations within gp41 are required, which is accompanied with a dramatic loss of viral infectivity.[185] Although pre-existing resistance to bAVPs has extensively been investigated, emergence of resistance to bAVPs has not been examined. As resistance to bAVPs would require multiple mutations in different regions of HIV Env, resistance to bAVPs would likely cost a severe loss of viral fitness.

Despite advantages, bAVPs have limitations in comparison to mAbs. For example, bnAbs have an exceptional serum half-life of 2–4 weeks.[1] Although the *in vivo* stability of bAVPs has not been extensively tested, data from clinical trials with sCD4 and FIs suggest that the half-life is less than 24 hr.[64,65,114] Furthermore, bnAbs have the potential to mediate the destruction of infected cells via ADCC.[99] Both limitations can at least partially be overcome by designing bAVPs that contain the Fc region of antibodies. For example, sCD4-IgG1 has an increased serum half-life in comparison to sCD4 and can mediate ADCC.[66] Interestingly, fusing a linker sequence and CCR5mim1/2 to the Fc region of sCD4-IgG1 reduced neither the *in vivo* stability nor the ability to mediate ADCC.[158]

Delivery of Bifunctional Antiviral Proteins for HIV Prevention and Treatment

Due to their broad neutralization breadth, bAVPs could be used for HIV prevention and treatment. An overview of different routes of delivery is given in Figure 4. Like bnAbs, the simplest method of delivering bAVPs for either prevention or treatment would be via injection. However, bnAbs or bAVPs would require frequent injections to maintain inhibitory serum concentrations. There are multiple possibilities to increase their serum half-life. Generally, serum proteins are continuously endocytosed and degraded in lysosomes. IgG and serum albumin have a prolonged half-life in comparison to the majority of serum proteins and are abundantly present in serum. Upon endosome acidification, they can bind to the neonatal Fc receptor (FcRn), which mediates their transcytosis and recycling back to the membrane surface.[186] Fusing sCD4 and human serum albumin increased the serum half-life of the fusion protein by over 100-fold in a rabbit model system in comparison to sCD4 alone.[187] The bnAb VRC01 was modified by site-directed mutagenesis to increase its binding affinity for FcRn (VRC01-LS).[188] The modified bnAb retained the functions of the parental bnAb, including ADCC activity, but had a three-fold longer serum half-life and mediated protection superior to VRC01 against intrarectal infection with simian-HIV (SHIV) in non-human primates.[188] But even if half-life is prolonged, production of protein-based therapeutics is costly, which limits their use in resource-limited settings. In comparison, *in vitro* production of mRNA is simple and cost effective. Furthermore, mRNA is more stable at higher temperatures and does not require constant cooling. Administration of nucleoside-modified mRNAs encoding the light and heavy chains of VRC01 resulted in the translation of functional VRC01 in humanized mice and protected them from infection.[189] Therefore, mRNA delivery may represent an alternative delivery method for proteins with antiviral activity. However, further research in that area is needed to prove the feasibility of this method. Long-acting injectable antiretroviral small-molecule inhibitors that require an intramuscular injection only every 4–8 weeks have shown promising results in clinical trials.[190,191] The safety, benefit, and cost of injecting bnAbs or bAVPs will therefore have to be carefully compared to long-acting injectable small-molecule drugs. At least for therapeutic applications, bnAbs and bAVPs that mediate ADCC have an advantage over small-molecule inhibitors, which are unable to remove infected cells.

bAVPs that do not require extensive post-translational modifications can be produced in bacteria. For prevention, a cost-effective alternative would be to utilize probiotic bacteria of the gastrointestinal and genital tract, such as lactobacilli, to produce bAVPs. Lactobacilli are naturally present in the vagina and rectum of healthy individuals. Certain strains have been associated with health benefits, and their presence can promote a healthy microbiota.[192] Oral administration of probiotic lactobacilli results in the vaginal and rectal colonization.[193] Several lactobacilli strains have been engineered to secrete single-entry inhibitors, such as sCD4, CV-N, or FIs.[121,194,195] *Lactobacillus jensenii* secreting CV-N colonized the vagina of macaques after oral administration and 63% of all animals were protected from infection after repeated virus challenges.[196] Further research will be necessary to determine the level of colonization and inhibitor concentration that is necessary for increased protection.

Another method to deliver protein-based therapeutics is via gene therapy. Basically any cell type can be modified to produce and secrete antiviral proteins. For prevention, it is possible to use gene modification of cells of the genital tract to locally produce the bAVPs at the site of virus entry. In one study, human vaginal epithelial cells were

modified using adeno-associated viral (AAV) vectors to secrete a bnAb b12 derivative that blocked virus transfer *in vitro*.[197] Furthermore, systemic delivery of proteins via AAV vector-mediated muscle gene therapy is currently under extensive investigation. Multiple studies have shown that antibodies and antibody-based antiviral proteins are secreted in significant quantities from gene-modified muscle cells in macaques and can result in partial protection of animals from infection.[198–200] In another pre-clinical macaque model of HIV prevention, AAV-mediated genetic modification of muscle cells was used to deliver the bAVP eCD4-Ig, which protected all tested macaques from infection.[158] However, these studies have also shown that significant immune responses can be mounted to the secreted inhibitors, which interfered with the effectiveness of the approach. Clinical trials will show what role immune responses in humans will play.

A genetic approach could also be used to deliver bAVPs for treatment. Conventional HIV gene therapy strategies are based on rendering HIV target cells non-permissive for viral replication. The gene products can generally be classified into RNA-based and protein-based therapeutics that need to be expressed in HIV target cells in order to be effective.[4,8,201–214] Because basically any cell type can function as producer cells, gene-modified muscle cells, $CD34^+$ hematopoietic stem and progenitor cells, $CD4^+$ and $CD8^+$ T cells, or B cells could be used to secrete bAVPs for HIV treatment.[215–217] Hematopoietic cells have already been engineered to produce single-entry inhibitors. For example, FI_{C46} was fused to a signal peptide and a transmembrane domain (maC46). Expression in T cells and hematopoietic stem cells resulted in high concentrations of maC46 on the surface of gene-modified cells and protected them from infection.[218] Based on the lack of a therapeutic effect of gene-modified cells expressing maC46 in a clinical trial,[8] secreted FI_{C46} was designed.[215] Although FI_{C46} was efficiently secreted from gene-modified T cell lines and prevented HIV infection of unmodified cells, secretion of FI_{C46} by gene-modified primary $CD4^+$ T cells was low (below the limit of detection) and only partial inhibition of HIV replication was observed.[215] We have shown that human hematopoietic stem cells and their progeny can be modified to secrete sCD4. sCD4 was chosen because recombinant sCD4 was safe in clinical trials and repeated administration of high doses of sCD4 resulted in complete suppression of viremia in patients.[65] Although some clinical isolates require relatively high concentrations of sCD4 for effective inhibition, it is not possible for HIV to escape binding to sCD4 without reducing binding to CD4 on the surface of HIV target cells. Secretion of sCD4 from gene-modified hematopoietic stem cells and their progeny inhibited HIV replication and protected $CD4^+$ T cells from HIV-mediated destruction in a humanized mouse model over time.[181] In an individual on ART with suppressed viremia, continuous production of bAVPs from gene-modified cells would ideally prevent the emergence of virus from latently infected cells upon cessation of ART and lead to a functional cure.

Conclusions

bAVPs exhibit exceptional antiviral potency, which surpasses even the latest generation of bnAbs. Due to their unique mechanism of action, there is an extremely low level of pre-existing resistance and likely also a high barrier to developing resistance. However, their use as injectable drugs remains to be determined, as repeated injections would be required to maintain bAVP concentrations for prevention and treatment. A one-time gene delivery procedure may offer a viable alternative to injecting bAVPs and could be cost saving in comparison to lifelong ART or PrEP.[219] Considerable challenges exist before genetic approaches could be translated into the clinic. Based on the recent successes of genetic therapies,[220–226] it is foreseeable that technical and financial barriers will be further reduced and genetic approaches will become a treatment and prevention option for a larger population.

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