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Opening the HIV Envelope: Potential of CD4 mimics as multifunctional HIV entry inhibitors

Annemarie Laumaea[1,2], **Amos B Smith III**[3], **Joseph Sodroski**[4,5], **Andrés Finzi**[1,2,6]

[1.]Centre de Recherche du CHUM, Montreal, Quebec, Canada

[2.]Département de Microbiologie, Infectiologie et Immunologie, Université de Montréal, Montreal, Quebec, Canada

[3.]Department of Chemistry, School of Arts and Sciences, University of Pennsylvania, Philadelphia, PA 19104-6323, USA

[4.]Department of Cancer Immunology and Virology, Dana-Farber Cancer Institute, Boston, Massachusetts, USA

[5.]Department of Microbiology, Harvard Medical School, Boston, Massachusetts, USA

[6.]Department of Microbiology and Immunology, McGill University, Montreal, Quebec, Canada

Abstract

Purpose of Review—Close to two million individuals globally become infected with human immunodeficiency virus (HIV-1) each year and just over two thirds will have access to life-prolonging antivirals. However, the rapid development of drug resistance creates challenges, such that generation of more effective therapies is not only warranted but a necessary endeavour. This review discusses a group of HIV-1 entry inhibitors known as CD4 mimics which exploit the highly conserved relationship between the HIV-1 envelope glycoprotein (Env) and the receptor, CD4.

Recent Findings—We review the structure/function guided evolution of these inhibitors, vital mechanistic insights that underpin broad and potent functional antagonism, recent evidence of utility demonstrated in animal and physiologically relevant *in vitro* models, and current progress towards effective new-generation inhibitors.

Summary—This review highlights the promising potential of CD4 mimetics as multifunctional therapeutics.

Keywords

HIV entry inhibitors; CD4 mimics; CD4 mimetics; HIV Env; ADCC; Phe43 cavity; gp120

Corresponding Author: Andrés Finzi, Centre de Recherche du CHUM, Montreal, Quebec, Canada, Ph: 514-890-8000 ext: 35264, andres.finzi@umontreal.ca.

Competing interests

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INTRODUCTION

Developing a therapeutic effective against human immunodeficiency virus (HIV-1) remains the goal for successfully halting an epidemic that has spanned multiple decades, killing more than 770 000 individuals in 2018 (1). Vaccines represent the central strategy for combating a wide range of pathogens; however despite ongoing efforts, the recent premature termination of the HIV vaccine clinical trial, HVTN 702 in South Africa due to futility (2, 3) highlights the ongoing challenges faced by the field. Though Highly Active Antiretroviral Therapy (HAART) targeting viral replication has helped prolong the life of HIV-1-infected individuals, emergence of resistance and the inability of HAART to purge the latent viral reservoir is an unresolved problem, calling for approaches that are aimed at eradicating or functionally curing HIV infection. Entry inhibitors, in particular CD4 mimics, have gained significant traction due to their potential utility as multifunctional therapeutics that can be employed for both prevention and treatment.

HIV-1 entry is mediated by the interaction of gp120, the surface subunit of its Envelope glycoprotein complex (Env), with the primary receptor CD4 and one of two chemokine coreceptors (CXCR4 or CCR5) (Reviewed in (4)). In its unliganded form, the Env trimer exists in a structurally constrained ‘closed’ conformation. Receptor engagement initiates a series of structural rearrangements within gp120 that result in the sequential opening of the trimer into an asymmetric intermediate and finally into an “open” conformation (5–7). CD4 engagement is a major driving force for Env structural rearrangements. CD4 is comprised of four extracellular immunoglobulin-like domains (D1-D4) linked to a short cytoplasmic tail through its transmembrane domain. Early work identified D1 and D2 as the active regions of CD4 that interacted with gp120 (8), with key structural analysis clearly demonstrating the critical interaction between gp120 and D1 (9). Importantly, this showed that the CD4 binding site (CD4bs) formed by the inner domain, outer domain and bridging sheet of gp120 accommodated a large hydrophobic cavity known as the Phe43 cavity. The side chain of Phe43 of D1 of CD4 is inserted into the opening of this cavity, allowing for a critical electrostatic interaction between the cavity lining residue Asp368 of gp120 and Arg59 of CD4 (9). This and other interactions within and surrounding the cavity modulate infection (10, 11) and as discussed by a detailed study conducted by Prevost *et al,.*(12), influence recognition by CD4bs antibodies (12, 13). Importantly in the context of this review, changes in the Phe43 cavity affect the binding and potency of small-molecule CD4 mimetic compounds (CD4mc)(12). Furthermore, Env-CD4 interaction at the surface of HIV-producing cells is critical for exposure of epitopes for CD4-induced (CD4i) antibodies that mediate Antibody-dependent cellular cytotoxicity (ADCC) (14, 15). Incidentally, these epitopes are recognized by non-neutralizing antibodies (nnAbs) present in natural infection (7, 16). However since the accessory proteins Nef and Vpu play active roles in downregulating membrane-bound CD4 as part of HIV’s evasion mechanism (Reviewed in (17–19), CD4 mimics can act as a surrogate to ‘open’ cell-surface Env (Figure 1 and Table 1). All these reasons provide the impetus for targeting the Phe43 cavity with rationally designed CD4 mimics.

Brief history of CD4 mimics

The earliest evidence of CD4 mimics as potential HIV therapies was the discovery that soluble forms of CD4 (sCD4) were able to neutralize HIV-1 (20–24). Unfortunately, these early promising *in vitro* studies yielded disappointing results in clinical studies. Despite showing anti-viral activity, administration of sCD4 resulted in rapid viral rebound; alarmingly, suboptimal concentrations were observed to enhance infection (25, 26). Although widespread clinical application of sCD4 was promptly abandoned, it was evident from these early studies that sCD4 preserved high affinity binding and conformational and functional characteristics similar to that of native membrane-bound CD4 (reviewed in (27)), encouraging the development of CD4 mimics with more favourable qualities. Currently CD4 mimics fall into the broad categories of; (i) CD4 immunoadhesins, (ii) miniproteins, and (iii) small-molecule CD4 mimetics (CD4mc) (Fig 1; Table 1). This review discusses the functional evolution of these inhibitors and current progress.

CD4 IMMUNOADHESINS

CD4 immunoadhesins (CD4-Ig), are antibody-like chimeric proteins typically comprising the immunoglobulin (Ig) constant domain fused with the D1 and D2 domains of CD4. A first-generation tetrameric CD4 immunoadhesin, CD4-IgG_2, demonstrated that replacement of the variable fragment (Fv) portions of IgG_2 with D1 and D2 afforded a longer half-life than sCD4, exhibited cross-clade neutralization (28), and blocked HIV-1 Env-mediated syncytium formation (29). Importantly both tetrameric (28, 29) and dodecameric CD4-Ig (D1D2-Igαtp) (30) lacked the unfavourable feature of viral enhancement inherent to sCD4 derivatives. The tetrameric immunoadhesin PRO542, the first of this class to be approved for clinical trials, was shown to be safe and effectively reduced plasma viremia (31), with especially pronounced effects in patients with advanced disease (32). Additionally, the unique ability to crosslink multiple gp120s afforded immunoadhesins potent avidity, which translated to heightened viral clearance *in vitro* (30). Fusion of sulfated peptide sequences corresponding to the amino terminus of the CCR5 coreceptor to the carboxy termini of tetrameric CD4-Ig (33), generated the bi-specific immunoadhesin, eCD4-Ig (34). This molecule demonstrated unmatched breadth and potency, neutralizing all HIV-1, HIV-2 and SHIV strains tested with IC_{50} lower than some broadly neutralizing CD4bs antibodies (34, 35). While CD4-Ig proved inefficient at eliciting antibody-mediated antiviral effector functions, the inclusion of the sulfated peptide afforded eCD4-Ig the capability to promote ADCC (34–36), in particular by enhancing recognition by otherwise occluded CD4i V3 antibodies; eCD4Ig decreased the binding of CD4bs, V2-apex and interface antibodies (35). Davis-Gardner *et al.* (35) showed that eCD4-Ig synergised with patient sera to kill reactivated latently infected primary cells (35). Interestingly, eCD4-Ig has minimal propensity to allow the generation of escape mutants (37), highlighting that in addition to its potential to purge the latent reservoir it may also be potentially utilised as a long-acting antiviral.

Further variations of eCD4-Ig, excluding D2, were developed to improve specificity for the Phe43 cavity (36); however, were shown to have reduced stability and often enhanced CD4-independent infection (36). Others have also explored conjugation of D1D2 with single-

chain Fv domains of CD4i antibodies such as the co-receptor binding site (CoRBs) antibody 17b. These proteins showed potent neutralization; however, data on their ADCC potential is currently lacking (38), though the absence of the Fc portion of the antibody likely precludes efficient effector cell engagement.

Finally, current eCD4-Ig approaches include passive immunization through gene therapy utilising Adeno-associated viral (AAV) vectors following proof of concept studies highlighted by Gardner *et al.,*(39) in a macaque model. These studies showed durable antibody production, and unlike AAV-delivered broadly neutralizing antibodies (bNAbs) (40), did not induce anti-drug antibodies. Immunoadhesins such as eCD4-Ig hold promise as multifunctional antivirals.

MINIPROTEINS

In the early 1990s, toxicology research highlighted the intriguing therapeutic potential of the cysteine stabilized alpha-beta motif (CSα/β) of scorpion toxins (41). Work showed that these highly stable biologically active structures could accommodate large sequence substitutions from unrelated proteins, with little to no impact on overall structure while adopting the functionality of the transplanted protein (41, 42). The scorpion toxins could therefore act as appropriate molecular scaffolds onto which the binding surfaces of proteins could be transplanted. Interestingly, the complementarity determining region (CDR)-2 loop of CD4 D1 bore a striking structural similarity to the scorpion toxin's β-hairpin protrusion, consequently inspiring the generation of CD4-miniproteins.

CD4 miniproteins are typically ~27-31 amino acids in length comprising the critical gp120-interacting residues of CD4 D1 grafted onto the β-hairpin region of a short scorpion toxin, scyllatoxin (43). Through a series of rational engineering approaches, Vita and colleagues generated the M-series of miniproteins, of which CD4M9 (M9) was the most potent; CD4M9 retained a native-like conformation, bound gp120 as well as CD4, and exhibited antagonism of HIV-1 infection at μM concentrations. The generation of bivalent (44) and trivalent (45) reconstructions of M9 designed to target multiple CD4 binding sites effectively enhanced inhibitor potency. Further critical improvements of M9 designed for deeper Phe43 cavity penetration, through the replacement of residue 23 (analogous to CD4 Phe43) with a non-natural amino acid, biphenylalanine (Bip) (M33) (46), and later with more flexible groups (47), generated the M48 series of peptides. Amongst these peptides, M48U1 showed the most potent antiviral activity, with neutralization of almost all HIV-1 strains tested; only those HIV-1 strains with atypical Phe43 cavities occluded by larger residues, such as the CRF_01 A/E strains, were resistant to M48U1 (48). Nonetheless, M48U1 has been shown to retain its potent antiviral activity in hydrogels in macaque models of transmission expanding its utility as a topical microbicide (49).

More recent approaches include vaccination with chimeric immunogens of CD4 miniproteins crosslinked to gp120 or gp140. However, despite eliciting high titres of CD4i Abs in rabbits (50, 51) and strong ADCC responses in macaques (52, 53), the high avidity due to multivalent antibody binding was short-lived (51, 53). Additional structure-guided engineering could one day see the promises of this approach achieved.

SMALL MOLECULES (CD4mc)

A screen of chemical libraries looking for inhibitors of the gp120-CD4 interaction by Debnath and colleagues led to the discovery of NBD-556 and NBD-557; these two lead *N*-phenyl-*N*′-(2,2,6,6-tetramethyl-piperidin-4-yl)-oxalamide analogues bound within the gp120 Phe43 cavity with specific, μM activity against the gp120-CD4 interaction, for both lab-adapted and primary isolates (54). The NBD series of compounds were subsequently characterized as comprising 3 distinct regions: a para-halide substituted aromatic ring (Region I) connected to a tetramethyl piperidine heterocyclic ring moiety (Region III) by an oxalamide linker (Region II) (Fig 1). Subsequent structure-guided re-engineering by the Sodroski/Smith and Matsushita/Tamamura groups (55, 56) aimed at deeper penetration of the compound into the Phe43 cavity showed that region I modifications incorporating additional halide groups on the phenyl ring improved gp120 binding compared to the miniprotein M33 and native CD4 (55), while methyl additions demonstrated favourable toxicity profiles in rodents and non-human primates (57).

Further structural analysis by Debnath's group (58, 59) suggested that the lack of region III insertion into the Phe43 cavity by NBD analogues likely reduced binding efficiency. Consequent replacement of the piperidine moiety in Region III showed improved inhibitory activity and potent neutralization against diverse strains, although still lacking efficient cavity penetration and surface complementarity. Importantly, these Region III modifications provided an alternate scaffold to improve on, yielding a new generation of small molecules. In addition to replacing the piperidine group with an indane, inclusion of a guanidinium moiety to the five-membered ring (60–62) altered positioning within the cavity allowing for deeper penetration that facilitated unique binding characterised by additional H-bonding networks spanning residues Met426 and Asp368 (61, 63). This translated to highly potent functional antagonism and diminished CD4-independent infection for compounds such as AWS-I-169, DMJ-I-228 and DMJ-II-121; subsequent studies by Madani and colleagues showed that the activated Env intermediate induced by these compounds is very short lived (64). Further reconstructions by Matsushita (65) and Sodroski/Smith (64) groups took advantage of region III as a scaffold (YIR-821; BNM-III-147, BNM-III-170 and JP-III-048 respectively). Despite failing to replicate the crucial Arg59 (CD4)-Asp368 (gp120) interaction, these compounds exhibited greater potency against primary strains of HIV-1. The Sodroski/Smith groups reported a pivotal discovery that CD4mc strongly induced conformational changes in gp120 that expose otherwise-hidden highly conserved epitopes (62). This translated to sensitization of viral particles to neutralization by otherwise non-neutralizing CD4i antibodies; such antibodies are easily generated through vaccination with CD4-bound stabilised gp120s (62, 66, 67), leading to the concept that CD4mc could be used to improve the vaccine efficacy of weak immunogens.

Further, work by our group (15, 68) showed that, independent of their region III modifications (JP-III-048 and DMJ-I-228 and BNM-III-170) or class (M48U1 or sCD4), CD4 mimics "opened" Env on cells infected with primary isolates and sensitized them to ADCC mediated by antibodies present in biologically relevant body fluids (ie. sera, cervicovaginal lavage and breast milk) from HIV-1-infected individuals. Structural differences in the CD4mc influenced the magnitude of Env opening and thereby potency at

activating ADCC, with more superior responses observed for the indane derivative, JP-III-048 against transmitted/founder HIV-1 in primary cells; both JP-III-048 and DMJ-I-228 exhibited better responses compared to the miniprotein M48U1 and sCD4, suggesting that small CD4 mimetics could avoid conformational constraints imposed by primary Envs on larger protein ligands (15, 68).

Importantly, Richard *et al,.*(15), presented evidence that CD4mc were able to sensitize *ex vivo*-amplified primary $CD4^+$ T cells to ADCC mediated by autologous sera and effector cells. This sensitization required synergy between CD4mc and CoRBS Abs, which "open" the Env trimer and facilitate recognition by anti-cluster A antibodies; this results in the stabilization of the asymmetric State 2A Env conformation, which is vulnerable to ADCC (7). Subsequent work by Richard *et al.,*(68) and Anand *et al.,*(69) confirmed sequential and synergistic action of CoRBS antibodies and anti-cluster A antibodies to promote efficient FcγRIII engagement, enhanced by CD4mc. These studies provided an otherwise unrecognised biological and mechanistic explanation for the potency of CD4mc in influencing ADCC responses. These results suggested that CD4mc might be useful in the "kill" part of the "shock and kill" strategy being pursued to eliminate the HIV-1 reservoir (70). In these approaches, latently infected cells are activated and subsequently killed by host immune responses. Preclinical studies are ongoing to establish the value of the CD4mc BNM-III-170 to decrease the size of the viral reservoir in humanized mice and non-human primates.

By allowing 'easy to elicit' nnAbs to target both viral particles and infected cells, CD4mc represent an alternative approach to prevention of HIV-1 transmission. Recently the small CD4mc JP-III-048 was shown to protect BLT-humanized mice from HIV-1 challenge (71). BNM-III-170 was demonstrated to augment the protective efficacy of an otherwise weak gp120 immunogen in non-human primates stringently challenged with a heterologous Tier 2 SHIV (66, 72). On the backdrop of this functional attribute of CD4mc, the Finzi and Smith Groups (73) using a carefully designed high-throughput cell-based ELISA (CBE) aimed at detecting small molecules that exposed vulnerable HIV-1 Env epitopes, identified and developed a novel family of small molecules from a chemical library of over a hundred thousand compounds (73, 74). This family of compounds, though resembling a binding structure similar to that of BNM-III-170, differ in two respects: (i) they exhibit a smaller, more compact structure, comprising an amide bond linking a halogenated aromatic ring to a piperidine core; (ii) they can easily be synthesized. Structurally, these differences translate to deeper cavity filling anchored by the aromatic halogen ring; this in turn allows for a closer distance between the piperidine core and Asp368, particularly members with sulfonamide extensions. Furthermore, positioning of the halogenated aromatic ring also facilitates direct interactions with another important Phe43 cavity residue, Glu370, as well as close packing against residue 375.

The lead compound, (*S*)MCG-IV-210, from this series was shown to have the most contacts with gp120, is able to expose Env to nnAbs, and can sensitize infected cells to ADCC (73). In addition, it efficiently sensitized viral particles to neutralization by otherwise non-neutralizing antibodies, consistent with previous studies with NBD analogues (15, 68). Furthermore, as reported previously for other CD4mc (75), this new family was able to

protect uninfected cells from bystander killing by preventing shed gp120 from interacting with CD4 on uninfected cells. This mechanism potentially reduces the detrimental depletion of uninfected immune cells, particularly $CD4^+$ T cells during natural course of infection.

Interestingly, Prevost *et al*,.(12) recently showed that natural polymorphisms of residue 375 and a series of six co-evolving gp120 inner domain residues (12, 76) can structurally remodel the Phe43 cavity and directly impact CD4mc affinity. For instance, the presence of Thr375 (in ~17% of clade B HIV-1 strains) in place of the Ser375 found in the majority of M group HIV-1 isolates, increased affinity for all small CD4mc tested. Furthermore, changes in residue 375 and its coevolving gp120 inner domain residues restructure the CD4bs and thus influence the binding mode, occupancy and potency of the different CD4mc classes, which modulates virus neutralization and ADCC. These findings are especially pivotal for the design and synthesis of next-generation compounds with broader activity. As suggested, shaping of the Phe43 cavity by these residues has major implications for efficient targeting of CRF_01.A/E as well as strains with alternate Phe43 cavity residues.

A mechanistic understanding of how CD4mc engage the functional HIV-1 Env trimer has begun to emerge. Indeed, computational prediction suggests that potent functional antagonism mediated by CD4mc can be achieved by i) engagement of two or three protomers; or ii) high-affinity interactions demonstrated by the different conformational states induced by the different CD4 mimics (64). Overall, these studies show that the ability of CD4mc to allosterically transform Env structure to conformations susceptible to the vast majority of antibodies present in sera from HIV-1-infected individuals promises novel prevention, treatment and cure strategies.

CONCLUSION

Major strides have been made in the development of CD4 mimetics, which has been aided immensely by structure-guided bioengineering. Importantly, each category has provided promising candidates, currently undergoing evaluation, or as scaffolds on which rationally designed enhancements are being considered and pursued.

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KEY POINTS

- The need for more effective HIV therapies is driven largely by the emergence of resistance and the inability of HAART to target the latent viral reservoir
- Among current therapies, CD4 mimetics, which target the highly conserved CD4-gp120 interaction have demonstrated broad functional potency
- Several potent CD4 mimetics have been identified that have shown promise as multifunctional therapeutics that could also be utilized to purge the latent reservoir

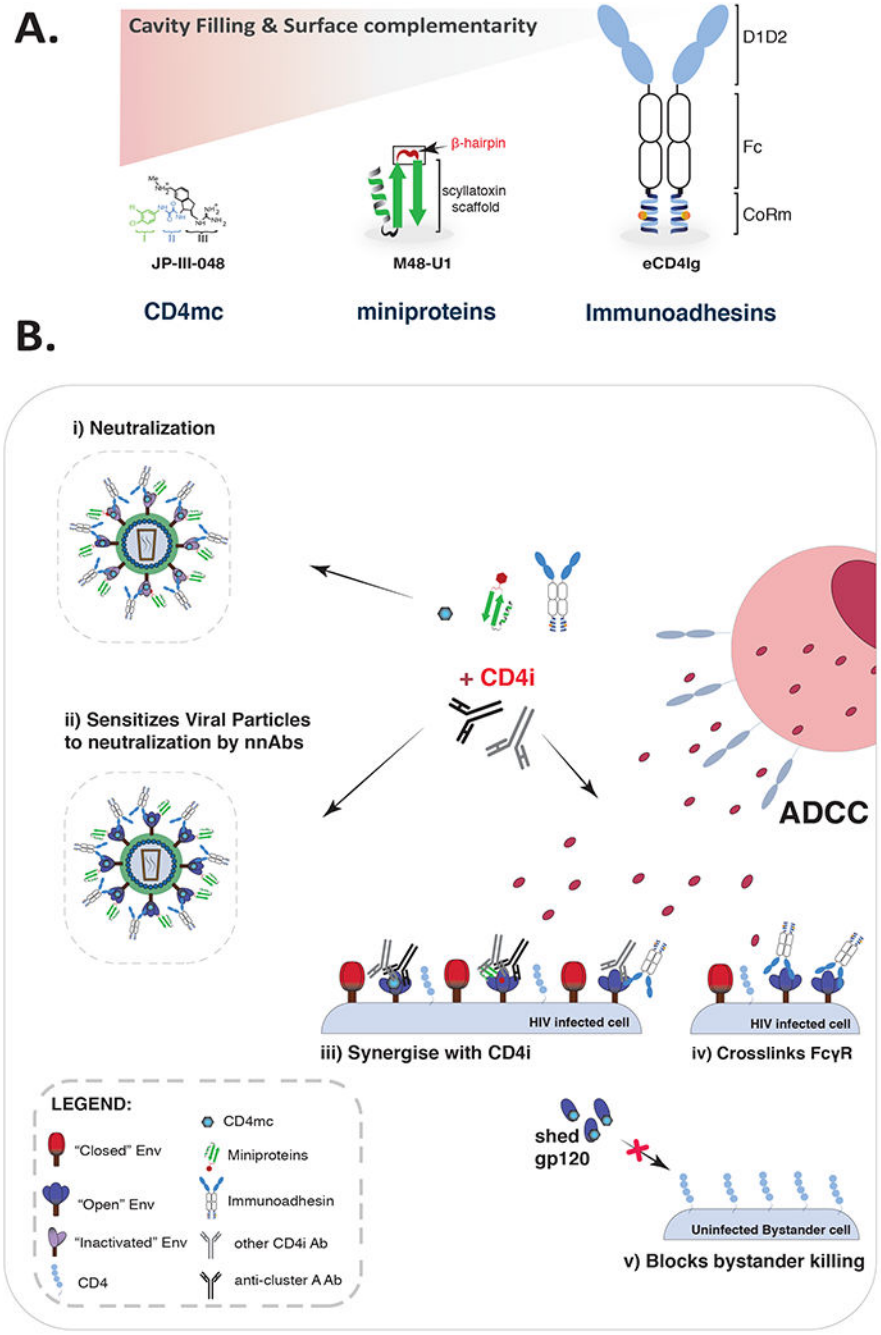

Figure 1: Functional attributes of CD4 mimetics.

(A) Comparison of cavity filling and surface complementarity relative to CD4 size. (B) Env-CD4mimetic ligation elicits distinct responses. With differing potency all CD4 mimetics are able to (*i*) inactive viral particles, (ii) facilitate neutralization of viral particles by non-neutralizing antibodies (nnAbs) and (iii) synergize with CD4i antibodies to mediate ADCC. However, only eCD4Ig (immunoadhesin)(iv) is able to independently cross-link FcγR. Only small molecule CD4mc (vi) are able to prevent bystander killing.

Table 1:

Functional characteristics of lead/potent CD4 mimics

Class	Compound	Description	Functional properties	Therapeutic potential	References
Immunoadhesins					
	CD4Ig (PRO542)	D1D2 of CD4 fused to Fc portion of Ig	*i, ii*	***Prevention*** Passive immunization	Jacobsen JM et al, 2000 Jacobsen JM et al, 2004
	eCD4Ig	CD4Ig fused with CoR mimic	*i, ii, iii, iv, v*	***Treatment*** Antiviral ***Cure*** Kill part of 'Shock and kill'	Davis-Gardner ME et al, 2017 Fellinger CH et al, 2019 Gardner MR et al, 2019
Miniproteins					
	M48U1	Select CD4 β-hairpin residues transplanted on scyllatoxin scaffold	*ii, iv, v*	***Prevention*** Topical microbicide	Acharya P et al, 2015
	gp120-S-S-M46U1	M46U1 fused with gp120	*i, ii, iii, iv, vi*	***Treatment*** Antiviral ***Cure*** Kill part of 'Shock and kill'	Martin G et al, 2011 Dey AK et al, 2012
	gp140-S-S-M46U1	M46U1 fused with gp140	*i, ii, iii, iv, vi*		
CD4mc					
	NBD Series *NBD14110*	Analog of NBD556 modified to contain bulky benzodioxole moiety in region I	*ii, iv, v*		Curelli F et al, 2018
	DMJ-II-121 Analogs *BNM-III-170*	Analog of NBD556 modified in region III to an indane core with guanidinium moiety	*ii, iv, v, vi*	***Prevention*** As part of vaccination ***Treatment*** Antiviral	Melillo B et al, 2016; Madani N et al, 2018; Prevost J et al, 2020
	YYA-021 Analogs *YIR-821*	Analog of NBD556 structurally modified in region III to consist of a mono-cyclohexy with long guanidino extension attached to piperidine core	*ii*	***Cure*** Kill part of 'Shock and kill'	Ohashi N et al, 2016
	MCG Series *MCG-IV-210*	Novel molecule containing a short amide linker between piperidine core and halogenated aromatic ring	*ii, iv, v, vi*		Ding S et al, 2019; Grenier MC et al, 2018 Prevost J et al, 2020

i) High avidity binding to gp120

ii) Inactivates viral particle

iii) Mediates ADCC of infected cells

iv) Sensitizes infected cells to ADCC

v) Sensitizes viral particle to neutralisation by nnAbs

vi) Protects against bystander killing