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Restricted HIV-1 Env glycan engagement by lectin-reengineered DAVEI protein chimera is sufficient for lytic inactivation of the virus

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Abstract

We previously reported a first-generation recombinant DAVEI construct, a dual action virus entry inhibitor composed of cyanovirin-N (CVN) fused to a membrane proximal external region or its derivative peptide Trp3. DAVEI exhibits potent and irreversible inactivation of HIV-1 (human immunodeficiency virus) viruses by dual engagement of gp120 and gp41. However, the promiscuity of CVN to associate with multiple glycosylation sites in gp120 and its multivalency limit current understanding of the molecular arrangement of the DAVEI molecules on trimeric spike. Here, we constructed and investigated the virolytic function of second-generation DAVEI molecules using a simpler lectin, microvirin (MVN). MVN is a monovalent lectin with a single glycan-binding site in gp120, is structurally similar to CVN and exhibits no toxicity or mitogenicity, both of which are liabilities with CVN. We found that, like CVN-DAVEI-L2-3Trp (peptide sequence DKWASLWNW), MVN-DAVEI2-3Trp exploits a similar mechanism of action for inducing HIV-1 lytic inactivation, but by more selective gp120 glycan engagement. By sequence redesign, we significantly increased the potency of MVN-DAVEI2-3Trp protein. Unlike CVN-DAVEI2-3Trp, re-engineered MVN-DAVEI2-3Trp(Q81K/M83R) virolytic activity and its interaction with gp120 were both competed by 2G12 antibody. That the lectin domain in DAVEIs can utilize MVN without loss of virolytic function argues that restricted HIV-1 Env (envelope glycoprotein) glycan engagement is sufficient for virolysis. It also shows that DAVEI lectin multivalent binding with gp120 is not required for virolysis. MVN-DAVEI2-3Trp(Q81K/M83R)

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Author Contribution

B.P. conceived the idea, designed clones, designed ELISA and SPR-binding studies, performed these experiments and wrote the manuscript. B.P. and K.A. worked together on ITC experiments and data analysis. K.A. assisted with SPR optimization experiments on Biacore3000 and helped in manuscript preparation. H.C.B. and S.Z. assisted with virus characterization work and B*.P. assisted with protein expression and purification. C.F.A., A.B.S. and I.C. provided overall guidance for the project.

Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

provides an improved tool to elucidate productive molecular arrangements of Env-DAVEI enabling virolysis and also opens the way to form DAVEI fusions made up of gp120-binding small molecules linked to Trp3 peptide.

Introduction

The Env (envelope glycoprotein) of HIV-1 (human immunodeficiency virus) responsible for host cell receptor engagement and entry is a highly glycosylated trimeric protein complex composed of non-covalently associated outer gp120 and transmembrane gp41 subunits [1,2]. There are over 20 N-linked and O-linked glycosylation sites in gp120, which account for ~50% of its molecular mass [1–3]. These glycans act as a barrier that protects virus from recognition by the human immune system [4]. The conserved high mannose-binding epitopes of Env, especially those in gp120, have themselves been used as target sites for infection inhibition. Glycan-binding lectin proteins, such as the cyanovirin-N (CVN) family [5–7] and antibodies such as 2G12 [8] and PGTs [9–11], have been found to be potent entry blockers.

We previously found that the high mannose-binding sites of Env can be used by bifunctional chimeras denoted DAVEIs (dual action virus entry inhibitors) to lytically inactivate HIV-1 virions independent of host cell encounter [12]. The initial DAVEIs were composed of CVN for gp120 binding linked covalently to a HIV-1 membrane proximal external region (MPER) now known to bind gp41 [13]. The appealing virolytic activity of the DAVEIs has been proposed to be due to the ability of this class of molecules, upon dual site Env contact, to hijack the metastability intrinsic in the Env trimer and to disrupt the trimer-containing virus envelope membrane [13].

While the results with CVN-DAVEIs have been encouraging for defining new classes of HIV-1 antagonists, the complexity of the CVN component itself has limited our understanding of the mechanism of Env engagement and consequent inactivation [14]. CVN has a large glycan-binding footprint on Env (Figure 1A), with multiple possible binding modes [14]. CVN also contains both high- and low-affinity glycan-binding sites (Supplementary Figure S1B) [15–17] that allow it to associate multivalently with gp120 protein [18,19]. A multivalent interaction with gp120 has been shown to be important for anti-HIV-1 activity of CVN [20]. Additionally, CVN is known to aggregate at modest concentration [21,22] and to form a domain-swapped dimer [23]. These properties of CVN limit efforts to localize DAVEI interaction with the Env trimer.

In contrast with CVN, the related lectin microvirin (MVN) has only a single glycan-binding site and a simpler footprint of gp120 glycan binding (Figure 1B) [24,25]. Additionally, MVN has a similar structure as CVN despite having only 30% sequence identity [24,25] (Supplementary Figure S1A,B). In this study, we asked if the CVN component of CVN-DAVEI could be substituted with MVN as an alternative gp120-binding domain to steer the MPER component to interact with gp41 and enable virolysis. By NMR structure analysis, MVN has been found to contain residues similar to those in the glycan-binding site of CVN, with minor variations (Supplementary Figure S1A) [24,25]. Other properties, including MVN activity against all HIV-1 group M clades with nanomolar potency (2–187 nM) [24],

lower mitogenicity than CVN and no cell toxicity in contrast with the toxicity of CVN [24,26], also make MVN an attractive candidate for construction of DAVEI fusions. In addition, as a monovalent glycan binder, MVN provides a simplified molecular tool for mechanistic investigations.

In this study, we designed and constructed second-generation DAVEI molecule MVN(Q81K/M83R)-DAVEI2-3Trp (peptide sequence DKWASLWNW), and found that it acts via a similar HIV-1 lysis mechanism as CVN-DAVEI. The lytic functionality of MVN-DAVEIs ruled out the need for multivalent lectin association with gp120 as a requirement for virolysis and demonstrated that restricted glycan engagement is sufficient for lytic inactivation. We envision that MVN fusions can be used as a tool for deconvolution of glycan engagement of DAVEI with the envelope spike protein and therein to better define binding stoichiometry and molecular arrangements that these classes of compounds employ to inactivate HIV-1. Moreover, this work also opens up possibilities for the development of small molecule fusions containing gp120-binding small molecules linked to MPER-like sequences to irreversibly destroy HIV-1 viruses.

Materials and methods

Reagents

HEK293F (HEK, human embryonic kidney cells) cells were purchased from ATCC. HIV-1 YU2 gp120 and HIV-1 YU2 gp160 plasmids were gifts from Joseph Sodroski. Modified human osteosarcoma cells (HOS.T4.R5) were a gift from Nathaniel Landau. HEK293T cells were purchased from ATCC. The DNA purification was conducted using the Promega Miniprep Kit (Cat. No. A1222). BamHI (Cat. No. R0136S) and NdeI (R0111S) restriction enzymes and DNA ligase (Cat. No. M0202S) were purchased from New England Biolabs for plasmid digestion ligation. Mutagenesis was conducted using the Quik Change II XL Site-Directed Mutagenesis Kit (Cat. No. 200522), and reaction was conducted using the PFU Ultra Polymerase (Cat. No. 600380-51) provided by Agilent Technologies. All other reagents for protein purification were purchased from Sigma-Aldrich unless otherwise specified. Biotinylated-Trp3 peptide was purchased from Scilight-Peptide, Inc. The integrity of the purified peptide was confirmed by mass spectrometry, observed mass of 1561.11 Da versus expected mass of 1559.82 Da (Supplementary Figure S9).

Plasmid construction

Sub-cloning of MVN-DAVEI2-3Trp and MVN-DAVEI4-3Trp fusion constructs—

The MVN sequence construct was obtained from Carole Bewley's lab. The MVN-coding gene sequence is present in the pET15b plasmid. NdeI and BamHI restriction sites were introduced on pET15b plasmid at the N-terminus and C-terminus of MVN plasmid. Forward primers 5'-GAAGGAGATATACCATATGCATGGGCCATCATC-3', 5'-CCACTGGAAATTGGATCCGATCCGGCTGCTAAC-3' and their reverse complement primers were used to insert NdeI and BamHI sites, respectively, on N- and C-termini of the MVN-coding sequence. Corresponding sites were also introduced in the CVN-DAVEI-L4 plasmid before and after the CVN-coding sequence. Primer 5'-GCTACCCAGCCGGCGATGCATATGGGTAAATTCTCCCAGACC-3' and its reverse

complement were used to introduce NdeI site before the CVN sequence in CVN-DAVEI-L4. Primer 5'-CTGAAATACGAAGGGGGATCCGGCGGAGGGTCGGGCGGAGG-3' and its reverse complement were used to introduce the BamHI site after the CVN sequence in CVN-DAVEI-L4. Plasmid CVN-DAVEI-L4 and MVN were digested with BamHI and NdeI. The CVN-coding region was removed from DAVEI-L4 constructs and MVN insert was inserted in the corresponding region. A follow-up PCR was performed post ligation to remove serine from the first linker using forward primer: 5'-CCACTGGAAATTGGAGGGGGCGGAGGGTCGGGCGGAGG-3' and its reverse complement. MVN DAVEI fusions with shorter MPER sequences post linker were constructed using primers 5'-GGTGGAGGCGGGTCCGACAAATGGGCAAG-3' and 5'-GGGCAAGTTTGTGGAATTGGTAGGAAATAACAGAATGGC-3' and their reverse complements. Corresponding constructs were designated as MVN-DAVEI2-3Trp and MVN-DAVEI4-3Trp. All plasmids were validated by sequencing using GeneWiz sequencing services using T7 forward and reverse primers.

Introduction of G43E, Q54S, Q81K/M83R and G85Q mutations on MVN fusions

—Five mutations were introduced onto the glycan-binding site of MVN (PDB ID: 2YHH) and MVN-DAVEI2-3Trp based on its structural alignment with the high-affinity binding site of CVN (PDB ID: 1IIY). G43E, Q54S, Q81K/M83R and G85Q mutations were introduced using forward primers 5'-GAGGCTTAGTGACCATATCGAAAATATAGATGGGGAATTGC-3', 5'-GCAGTTCGGGGATTCAAACCTCCAAGAAACC-3', 5'-GGTGTGTACTTGTAACAAGGGATGGGGAATGG-3' and 5'-GGTGTGTACTTGTAACAAGGGATCAGGAATGGAAATCTACC-3' and their reverse complements, respectively. Q81K and M83R mutations were separately introduced using forward primers 5'-GGTGTGTACTTGTAACAATGGATGGGGAATGG-3' and 5'-GGTGTGTACTTGTAACAAGGGATGGGGAATGG-3' and their reverse complements, respectively. Plasmids were validated by sequencing using T7 forward and reverse primers post mutagenesis.

Expression, purification and validation of MVN, MVN mutants and MVN fusion proteins

MVN protein was purified based on a protocol reported earlier [25]. Purified MVN-DAVEI2-3Trp and MVN-DAVEI4-3Trp plasmids were transformed on Rosetta (DE3) competent cells (Novagen) and plated on LB-ampicillin agar plates for 16 h at 37°C. Positive colonies were isolated and inoculated on 1 ml of LB ampicillin for 16 h at 37°C. One milliliter culture was subcultured to 4 l culture and grown for 4 h at 37°C, followed by induction with IPTG when the optical density was in the range of 0.6–0.8. Cells were induced for 16 h at 16°C at 225 rpm shaking speed. Grown cells were pelleted by centrifugation and lysed by sonication in the presence of Buffer A (50 mM Na₂HPO₄, 300 mM NaCl and 10 mM imidazole, pH 7.5). Samples were centrifuged and filtered supernatant was loaded onto a Ni-NTA column, followed by a 26/60 Superdex 200 prep-grade column (GE Healthcare) using AKTA FPLC. Homogeneity was assessed with protein eluates ran on 18% SDS-PAGE (Supplementary Figure S2), followed by MALDI analysis (Wistar Institute, Supplementary Figure S3).

Binding characterization of MVN fusion constructs

The presence of functional MVN and fully exposed 3Trp moieties on the fusion protein were validated by ELISA-binding experiments. In brief, varying concentrations (1000–0.001 ng) of MVN or MVN-DAVEI-3Trp fusion proteins were immobilized on high-binding polystyrene ELISA plates overnight at 4°C followed by blocking with 3% BSA for 2 h at room temperature on a rocker. The blocked plates were rinsed three times with PBS-T (PBS and 0.05% Tween 20). All subsequent incubation steps were done in 0.5% BSA in 1× PBS.

Validation of functional MVN component of fusion proteins—For investigating the functionality of MVN moiety on MVN-DAVEI-3Trp fusions, recombinantly purified gp120 protein (50 ng) was loaded onto the plate and incubated for an hour followed by three PBS-T washes. About 50 µl of sheep anti-gp120 (dilution factor 1: 3000; Aalto Bioreagents, D7324) was added to the plates for an hour at room temperature. The plates were washed with PBS-T followed by the addition of rabbit anti-sheep secondary antibody (dilution factor 1: 3000; Life Technologies PJ209733). The plates were incubated for an additional 1 h at 25°C and washed three times with PBS-T and one more time with PBS before the addition of OPD (ortho-phenylenediamine dihydrochloride). Plates were developed for 30 min in the dark and the final absorbance measured at 450 nm using an Infinite m50 (Tecan) plate reader. Immobilized biotinylated-Trp3 peptide was taken as a negative control and MVN was taken as a positive control for this experiment.

Validation of functional Trp3 component of fusion proteins—For investigating the functionality and full exposure of the 3Trp moiety in MVN fusions, 50 ng of 2F5 (AIDS Repository: Cat. No.1475), 4E10 (AIDS Repository: Cat. No. 10091) and 10E8 (AIDS Repository: Cat. No. 12294) antibodies were added onto the plate and incubated for an hour, followed by three PBS-T washes. Anti-human HRP (horseradish peroxidase; dilution factor 1: 3000; Millipore — AP101P) was added onto the plate and incubated for an hour. The plate was washed three times with PBS-T and one more time with PBS before the addition of OPD. Plates were developed for 30 min in the dark and the final absorbance measured at 450 nm using an Infinite m50 (Tecan) plate reader. Immobilized MVN protein was taken as a negative control, and full-length CVN-DAVEI-L4 protein containing a full-length MPER sequence was taken as a positive control for this experiment.

Expression and purification of YU2 gp120 protein

A pcDNA3.1 vector containing YU2 gp120-coding gene was prepared using a Qiagen MaxiPrep Kit (Qiagen). Plasmids were transiently transfected into HEK 293F cells according to the manufacturer's protocol (Invitrogen). Five days post transfection, cells were harvested and spun down, and the supernatant was filtered through 0.2 µ filters. Purification was performed over a 17b antibody column prepared using an NHS-activated Sepharose, HiTrap HP column (GE Healthcare). Gp120 was eluted from the column using 0.1 M glycine buffer (pH 2.4). The pH of the eluted protein was rapidly neutralized by the addition of 1 M Tris (pH 9.0). Purified proteins were run on 10% SDS gel for detecting the expression of protein. Eluted protein was immediately dialyzed onto PBS. All the proteins were fractionated by size exclusion on a HiLoad 26/60 Superdex 200 HR prepacked gel filtration column (GE Healthcare). Eluted fractions of protein were run on 10% SDS-PAGE

gel. The purified, monomeric fractions of gp120 were concentrated and flash-frozen at -80°C .

Surface plasmon resonance analysis

CM5 chip preparation—Surface plasmon resonance (SPR) experiments were performed on a Biacore 3000 Optical Biosensor (GE Healthcare). Experiments were carried out at 25°C using standard PBS buffer (pH 7.4) with 0.005% surfactant Tween. Initially, CM5 sensor chips were activated using a standard 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide (EDC)/N-hydroxysuccinimide (NHS) cocktail, and coupling was performed through ligand amine groups. Gp120 protein was covalently captured on a CM5 chip using standard EDC/NHS chemistry. Post gp120 capture, the chip was deactivated using 1 M ethanolamine (pH 8.5). Antibody Vectibix (Amgen; NDC 55513-954-01) was used as a negative control surface since it showed no detectable binding to MVN at its highest concentration (50 μM) (data not shown).

Control experiments for SPR optimization

1. *Control experiment to identify adequate flow rate for avoiding mass transport effect:* The effect of the flow rate on the apparent reaction rate constants was investigated. The amount of gp120 immobilized onto the CM5 chip was fixed at 700 RU (response unit), and 400 nM MVN solutions were passed over the resulting gp120 chip. Binding data were collected at flow rates of 10, 25, 50 and 100 $\mu\text{l}/\text{min}$. The association curves were overlaid to identify any mass transport effect.
2. *Control experiment to rule out the mass transport effect due to immobilized ligand density:* To assess the effect of the level of immobilization on the apparent reaction rate constants, different amounts of gp120 (350, 540 and 680 RU) were immobilized onto the CM5 chip. MVN solutions (400 nM) were flowed (50 $\mu\text{l}/\text{min}$) over each chip surface. Binding sensorgrams generated from various amounts of immobilized gp120 were superimposed by normalizing the R_{max} values to detect and avoid any change in association or dissociation constants caused by higher ligand density.

Direct binding SPR of MVN and MVN fusions with gp120 proteins—Post optimization, SPR experiments were performed to measure binding properties of MVN, MVN fusions and their mutants. Recombinantly purified gp120 protein was immobilized on the CM5 surface and the amount of gp120 immobilized was strictly limited to ~ 500 RU to avoid mass transport effect. Varying concentrations of MVN, Cyanovirin, MVN mutants, MVN fusions and cyanovirin fusions were passed over the surface as an analyte at a flow rate of 50 $\mu\text{l}/\text{min}$ to avoid mass transport effect. Surface regeneration was achieved by two 10 μl injections of 15 mM HCl solution at a flow rate of 50 $\mu\text{l}/\text{min}$. All analyses were performed in triplicates.

SPR direct binding data analysis—The sensorgrams for direct binding were analyzed using BIAEvaluation v.4.0 software provided by Biacore (GE Healthcare). Prior to

calculation, the binding data were corrected for nonspecific interaction by subtracting the reference surface data from the reaction surface data and were further corrected for buffer effects by subtracting the signal due to buffer injections from those due to protein sample injections [27]. The sensorgrams for CVN and gp120 interaction or CVN-DAVEI-L2-3Trp and gp120 interaction were globally fitted using a multivalent binding model with the following equation:

In this model, multiple CVN or CVN-DAVEI-L2-3Trp associates with gp120 to form a final complex, $\text{gp120} \cdot (\text{CVN})_n$ or $\text{gp120} \cdot (\text{CVN-DAVEI-L2-3Trp})_m$, where $n \geq 2$. The dissociation constants were derived by fitting data according to a multivalent binding model. For simple Langmuir binding model, dissociation constant was calculated as:

$$K_d = \frac{k_{\text{off}}}{k_{\text{on}}} \quad (2)$$

The sensorgrams for MVN and gp120 interaction were analyzed using a 1: 1 binding model with a conformational change of gp120: MVN complex as shown below:

In this model, gp120 binds to MVN to form an intermediate complex gp120: MVN with a simple 1: 1 binding. The complex subsequently undergoes conformational rearrangement to form a final complex gp120·MVN. We used symbols k_1 and k_{-1} to denote association and dissociation rates for gp120: MVN complex from free proteins MVN and gp120, and k_2 and k_{-2} to denote the forward and reverse rate constants for the formation of structurally altered conformation gp120·MVN from gp120: MVN. The equilibrium constant using the two-step model was determined as described elsewhere [28]. The equation for the two-step model is shown below:

$$K_d = \frac{k_{-1}k_{-2}}{k_1(k_2 + k_{-2})} \quad (4)$$

For analysis of MVN mutants using two-step model, k_{on} (association rate constant) and k_{off} (dissociation rate constant) were calculated as follows:

$$K_d = \frac{k_{-1}k_{-2}}{k_1(k_2 + k_{-2})} = \frac{k_{\text{off}}}{k_{\text{on}}} \text{ (from eqns 2 and 4)} \quad (5)$$

$$k_{\text{off}} = k_{-1}k_{-2} \quad \text{and} \quad k_{\text{on}} = k_1(k_2 + k_{-2})$$

Individual kinetic parameters were obtained from three independent experiments.

Isothermal titration calorimetry-binding analysis

To determine the binding stoichiometry and equilibrium dissociation constant, we performed isothermal titration calorimetry (ITC)-binding analyses for the following — MVN: YU2-gp120; MVN-DAVEI2-3Trp: YU2-gp120; CVN-DAVEI2-3Trp: YU2-gp120 and a series of MVN mutants with YU2 gp120 protein. The analyses were carried out at 25°C on a VP-Isothermal Titration Calorimeter (VP-ITC) system (MicroCal™, GE Healthcare, Freiburg). In brief, 30 μM of MVN, 30 μM of MVN-DAVEI2-3Trp, 25 μM of MVN(Q81K/M83R), 40 μM of MVN-DAVEI2-3Trp(Q81K/M83R), 300 μM of MVN(G43E/Q54S), 300 μM of MVN_PENTA(G43E/Q54S/Q81K/M83R/G85Q) and 20 μM of CVN-DAVEI2-3Trp, dialyzed into 1× PBS, were added into 2 μM of WT YU2 gp120, all dialyzed into 1× PBS. A total of 40 injections were performed, each containing 8 μl of solution. All experiments were performed at 25°C using 1× PBS buffer at pH 7.4. The resulting heat change upon injection was integrated over a time range of 300 s. For all the MVN, MVN fusions or MVN mutants, the values obtained were fit to a standard single site-binding model. CVN-DAVEI2-3Trp thermograms were fit to a multisite-binding model.

Protein ELISA with CVN-DAVEI-L2-3Trp

gp120 (50 ng) was immobilized on high-binding polystyrene ELISA plates overnight at 4°C followed by blocking with 3% BSA for 2 h at room temperature on a rocker. The blocked plates were rinsed three times with PBS-T and the following experiments were performed.

Direct binding of CVN and CVN-DAVEI-L2-3Trp to gp120 in the presence of 2G12

Plates were preincubated with 150 ng of 2G12 (1: 1000 fold dilution; AIDS repositary — Cat. No. 1476) for 2 h followed by three PBS-T washes. Varying concentrations of CVN and CVN-DAVEI-L2-3Trp protein (1000–0.001 nM) were added to the plate and incubated for an additional hour followed by three PBS-T washes. Rabbit anti-CVN (Biosyn, Inc.; dilution 1: 3000) was added as a primary antibody followed by two washes with 1× PBS-T. The donkey anti-rabbit HRP conjugate (GE Biosciences, GE NA934V; dilution factor 1: 3000) was used as the secondary antibody, which was detected using an OPD solution. Plates were developed for 30 min in the dark and the final absorbance was measured at 450 nm using an Infinite m50 (Tecan) plate reader. 2G12 binding was also detected using anti-human HRP (dilution factor 1: 3000; Millipore — AP101P) to ensure it is not lost during the wash process (data not shown).

Competition of 2G12 binding by CVN and CVN-DAVEI-L2-3Trp—2G12 (50 ng; dilution factor: 1: 3000 dilution) was simultaneously loaded onto the plate in the presence of

increasing concentrations of CVN and CVN-DAVEI-L2-3Trp (50–0.03 nM) and incubated for 2 h followed by three PBS-T washes. Anti-human HRP (dilution factor 1: 3000; Millipore — AP101P) was added onto the plate and incubated for 1 h. The plate was washed three times with PBS-T and one more time with PBS before the addition of OPD. 2G12 loaded alone (without CVN-DAVEI-L2-3Trp) was used as a positive control.

Competition of CVN-DAVEI-L2-3Trp by 2G12—Recombinantly purified CVN and CVN-DAVEI-L2-3Trp proteins (50 ng) were loaded onto the plate simultaneously in the presence of increasing concentrations of 2G12 (10 000–0.03 nM) and incubated for 2 h followed by three PBS-T washes. Rabbit anti-CVN (Biosyn, Inc.; dilution 1: 3000) was added as a primary antibody followed by two washes with 1× PBS-T. The donkey anti-rabbit HRP conjugate (GE Biosciences, GE NA934V; dilution factor 1: 3000) was used as the secondary antibody, which was detected using an OPD solution. CVN and CVN-DAVEI-L2-3Trp proteins loaded alone (without 2G12 added) were used as positive controls.

Protein ELISA for 2G12 competition by MVN-DAVEI2-3Trp and MVN-DAVEI2-3Trp (Q81K/M83R) proteins

Protein ELISA was performed by two different methods to confirm binding site specificity of MVN-DAVEIs and their competition by 2G12.

Competition ELISA with gp120 immobilization—Initially, 50 ng of gp120 was immobilized on high-binding polystyrene ELISA plates overnight at 4°C followed by blocking with 3% BSA for 2 h at room temperature on a rocker. The blocked plates were rinsed three times with PBS-T. 2G12 (50 ng; dilution factor 1: 3000) was added onto the plate with increasing concentrations of MVN-DAVEI2-3Trp and MVN-DAVEI2-3Trp (Q81K/M83R) (400–0.4 nM) and incubated for 2 h followed by two PBS-T washes. Anti-human HRP was added onto the plate and incubated for another hour. The plate was washed three times with PBS-T and one more time with PBS before the addition of OPD. 2G12 loaded alone (without MVN-DAVEI2-3Trp) was used as a positive control.

Competition ELISA by MVN-DAVEI immobilization—Owing to the unavailability of MVN-specific mAb and the potential for cross-reactivity of secondary antibodies, a second set of ELISA assays was optimized to confirm the overlapping binding site of 2G12 and MVN-DAVEI fusions in gp120. In this method, 100 ng of MVN-DAVEI2-3Trp and MVN-DAVEI2-3Trp(Q81K/M83R) were immobilized on ELISA plates overnight at 4°C followed by blocking with 3% BSA for 2 h at room temperature on a rocker. The blocked plates were rinsed three times with PBS-T. Recombinantly purified YU2 gp120 protein (50 ng) was added onto the plate and incubated for 2 h followed by two PBS-T washes. Increasing concentrations of 2G12 were added onto the plate and allowed to incubate for an hour followed by two PBS-T washes. Anti-human HRP (dilution factor 1: 3000; Millipore — AP101P) was added onto the plate and incubated for an hour. The plate was washed three times with PBS-T and one more time with PBS before the addition of OPD. CVN-DAVEI-L2-3Trp (50 ng) immobilized on the ELISA plate was used as a positive control.

Production and characterization of YU2 gp160 pseudotype viruses

Pseudoviruses were produced by co-transfection of two plasmids: (1) an envelope plasmid that codes for YU2 gp160 region and (2) a backbone sequence corresponding to envelope deficient pNL4-3 Luc⁺ Env⁻ [29]. Three million HEK293T cells were plated on T75 flask (Corning, Inc.). Cells were co-transfected 24 h after plating with 4 µg of envelope DNA and 8 µg of backbone DNA, using Fugene as the transfection reagent. Medium was changed 24 h post transfection, and the cells were allowed to grow for 48 more hours. After 48 h, cell supernatant containing virus was collected and filtered using a 0.45 µm filter (Corning, Inc.). The filtered supernatant was loaded onto an Iodixanol gradient (gradient range: 6–20%; Optiprep, Sigma–Aldrich) and centrifuged in a Sw41 Ti rotor (Beckman Coulter) at 110 000×g for 2 h at 4°C. The lower three fractions were pooled together [30] and 400 µl of aliquots were frozen at –80°C. Purified pseudoviruses were validated for infectivity and p24 content post production.

Viral infection inhibition assay

Infection inhibition assays were conducted for all YU2 viruses produced, as described earlier [31,32]. Approximately 7000–8000 HOS-T4-R5 cells were seeded in 96-well plates on day 1. Twenty-four hours later, YU2 viruses were added onto the plated cells in the presence or absence of MVN and MVN fusion compounds at various dilutions. The amount of viruses to be added to each well was determined based on a titer experiment. For optimal signal, virus stocks were diluted in growth media such that the final dilution of virus showed signal of 10⁵ luminescence counts. Post virus and compound addition, the plates were incubated for 24 h at 37°C before the medium was changed. Cells were allowed to grow for 24 more hours after which the medium was removed and the cells were lysed using Passive Lysis Buffer (Promega). Cell lysates were transferred to a white well plate (Greiner) and mixed with 1 mM Luciferin salt (Anaspec) diluted in 0.1 M potassium phosphate buffer containing 0.1 M magnesium sulfate. The luminescence was measured using a Wallac 1450 Microbeta Luminescence reader at 490 nm wavelength.

Sandwich ELISA for p24 release detection

p24 release from viruses was measured using the protocol described before [30]. In brief, a p24 titer experiment was conducted to estimate the dilution factor of virus required for observing the optimal p24 signal, equivalent to signal shown by 50 ng of recombinantly purified p24. The final dilution factor for the viral stock used was 1/40. Post titer experiment, high-binding polystyrene ELISA plates were coated overnight at 4°C with 50 ng of mouse anti-p24 and blocked with 3% BSA for 2 h at room temperature on a rocker. The blocked plate was rinsed three times with PBS-T. Viral stocks were diluted 10-fold using PBS (7.2) and 100 µl of diluted stocks were treated with 100 µl of serially diluted MVN or MVN-DAVEI2-3Trp fusions. Recombinant p24 (50 ng) was used as a positive control. The virus/compound stocks were incubated at 37°C for half an hour and were spun down in 1.5 ml tubes for 2 h at 4°C at 21 130 g. The top 100 µl of supernatant was removed carefully without disturbing the pelleted virus fractions. This supernatant was then diluted (1: 1, v/v) in PBS containing 1% BSA and 1% Triton X-100. A sample (50 µl/well) was then loaded onto the prepared ELISA plates. The plate was incubated at 4°C overnight (16 h) on a rocker

before being washed three times with PBS-T. Primary rabbit anti-p24 primary antibody (dilution factor 1: 3000; Abcam ab63913) was added to the plate, followed by secondary antibody, donkey anti-rabbit HRP conjugate (dilution factor 1: 3000; GE Biosciences, GE NA934V). Both antibodies were incubated for 1 h at room temperature. The plate was washed three times with PBS-T and one more time with PBS before the addition of OPD. Plate was developed for 30 min in the dark and the final absorbance was measured at 450 nm using an Infinite m50 (Tecan) plate reader.

Competition cell-based ELISA

Our previous study showed direct binding of Bt-Trp3 (biotinylated-Trp3 peptide) peptide to the HIV-1 envelope glycoprotein trimers expressed on HEK293T cell surfaces [13]. The protocol was based on earlier experiments published by Haim et al. [33] and was optimized for our assay with minor modification. Briefly, HEK293T cells (30 000) were seeded on 24-well plates. Cells were transfected the next day with 0.4 µg of a plasmid expressing the 'uncleaved' envelope glycoprotein trimers [Env(–) CT] using the Fugene transfection reagent (Promega, Cat. No. E2311). Medium was changed the following day. Two days later, cells were used for the cell-based ELISA assay. Initially, cells were blocked once with blocking buffer (0.5 mM BSA, 140 mM NaCl, 1.8 mM CaCl₂, 1 mM MgCl₂ and 25 mM Tris, pH 7.5) for 30 min. Post blocking, cells were buffer-washed (140 mM NaCl, 1.8 mM CaCl₂, 1 mM MgCl₂ and 25 mM Tris, pH 7.5). Cells were pretreated with 2500 nM, 500 nM and 100 and 20 nM of F105, 2G12, MVN(Q81K/M83R) and MVN-DAVEI2-3Trp(Q81K/M83R) (purified in laboratory) proteins for 45 min at 37°C followed by two buffer washes. Biotinylated-Trp3 (200 nM) was added to the cells. The concentration of biotinylated-Trp3 was chosen based on prior analysis to achieve ~80% binding to the transfected cells [13]. Plates were buffer-washed twice, 15 min each. Streptavidin HRP (dilution factor 1: 3000; Anaspec, 0668) was added onto the cells and incubated for 45 min. Plates were buffer-washed twice, OPD solution was added and end point absorbance measured at 450 nm.

Competition of MVN-DAVEI2-3Trp(Q81K/M83R) induced virolysis by 2G12

The p24 lysis titer experiment was first performed with MVN-DAVEI2-3Trp(Q81K/M83R) protein to determine the minimal concentration of protein required for observing maximal (~80%) lysis. This concentration was determined to be 150 nM. Purified YU2 virus was incubated with 150 nM of MVN-DAVEI2-3Trp(Q81K/M83R) protein in the presence of 0, 100 and 1000 nM concentrations of F105 and 2G12 for 30 min at 37°C. The incubated mixture was centrifuged for 2 h at 4°C and 21 130g. The top 100 µl of supernatant was carefully removed without disturbing the virus pellet and then further diluted [1: 1 (v/v)] in PBS containing 1% BSA and 1% Triton X-100. This sample was used to conduct the sandwich p24 ELISA as described above. Viruses treated with PBS were used as a negative control. A p24 standard (50 ng) was also used as a positive control in each experiment. CVN-DAVEI-L2-3Trp (180 nM) was also competed against various concentrations of 2G12 as a control experiment for MVN-DAVEI2-3Trp(Q81K/M83R) protein. Experiments were performed in triplicate, and the percentage of p24 released when compared with the positive control was plotted against the antibody concentration used. Data were plotted using Origin version 8.1 for the determination of IC₅₀.

Results

Purification and functional analysis of MVN–DAVEI fusion protein

Expression and purification of MVN-DAVEI2-3Trp and MVN-DAVEI4-3Trp proteins

—Since MVN was expected to monovalently interact with gp120 [24], we designed and produced two fusion proteins that consist of hexa-histidine, MVN, glycine₄serine linker and 3Trp sequence from N- to C-terminus (Supplementary Figure S2A). The two MVN-DAVEI-3Trp fusions, namely MVN-DAVEI2-3Trp and MVN-DAVEI4-3Trp, containing two and four glycine₄serine linker repeats, respectively, were purified in moderate yield (0.4 mg/l culture) when compared with MVN (4–6 mg/l). The purity of all recombinant proteins was assessed by SDS–PAGE (18% gel) post Ni-NTA and Superdex 200 separations, revealing a single band for all proteins between 11 and 17 kDa (Supplementary Figure S2B). Chemical compositions of the purified proteins were validated by MALDI analysis (Table 1 and Supplementary Figure S3). The masses of 14 056.7 Da (MVN), 15 690.7 Da (MVN-DAVEI2-3Trp) and 16 391.5 Da (MVN-DAVEI4-3Trp) were acceptably close (<0.025% SD) to the predicted molecular mass of 14 059.21, 15 693.95 and 16 395.5 Da, respectively. These results confirmed expression of the desired recombinant protein fusions.

Functional validation of purified proteins—A protein ELISA was performed to validate the binding functionality of purified fusion proteins. ELISA result showed that MVN-DAVEI2-3Trp [effective concentration for half maximal response (EC₅₀): 86 nM] and MVN-DAVEI4-3Trp (EC₅₀: 79 nM) bound to gp120 with potency similar to wild-type MVN (EC₅₀: 48 nM) (Figure 2A). Since the 3Trp moiety of MVN-DAVEI2-3Trp and MVN-DAVEI4-3Trp has an expected epitope sequence for 2F5 antibody (Figure 2E), we also performed protein-binding ELISA with 2F5, using 4E10 and 10E8 antibodies as negative controls. The ELISA results showed binding of 2F5 antibody to immobilized fusion proteins MVN-DAVEI2-3Trp (EC₅₀: 35 nM) and MVN-DAVEI4-3Trp (EC₅₀: 28 nM) protein but not 4E10 and 10E8 (Figure 2B–D). Immobilized recombinant MVN, an additional negative control, did not bind to any of these antibodies. CVN-DAVEI-L4, containing full-length MPER and used as a positive control, bound to all the antibodies (2F5, 4E10 and 10E8) as expected [13]. The ELISA results confirmed the presence and binding-competent exposure of both the 3Trp and MVN moieties on MVN-DAVEI2-3Trp and MVN-DAVEI4-3Trp fusions.

SPR kinetic binding analyses of fusion proteins—To compare the binding affinity of MVN against MVN fusions (MVN-DAVEI2-3Trp and MVN-DAVEI4-3Trp) and to understand the binding kinetics, we performed SPR kinetic binding analyses with these proteins.

Optimization of SPR-binding experiment

Before determining the interaction kinetics for MVN and MVN-containing fusions, we screened for regeneration conditions to ensure complete removal of MVN bound to gp120 on chip. MVN was injected as an analyte at 50 µl/min flow rate onto gp120 immobilized on the CM5 chip. We observed a complete regeneration of CM5 with two 10 µl injections of 15

mM HCl (Supplementary Figure S4A) at a flow rate of 50 $\mu\text{l}/\text{min}$. We optimized experimental conditions to eliminate mass transport artifacts, which can occur in SPR analyses involving fast association rates or a too-large amount of immobilization. To assess the effect of the immobilization level on the kinetics, 400 nM of MVN solutions were injected onto various amounts of immobilized gp120. R_{max} values were normalized and sensorgrams were superimposed. No significant differences in the binding kinetics were observed at the gp120 immobilization levels tested (Supplementary Figure S4B). We further examined the presence of the mass transport effect by measuring the association rates at varying flow rates (10, 25, 50 and 100 $\mu\text{l}/\text{min}$). The data obtained, Supplementary Figure S4C, showed a minor decrease in association rate at a decreasing flow rate, indicative of the mass transport effect at the slower flow rates. Since the flow rates of 50 and 100 $\mu\text{l}/\text{min}$ resulted in the same measured association rates, further kinetic experiments were performed at a flow rate of 50 $\mu\text{l}/\text{min}$. The aforementioned experiments provided us with optimal conditions for SPR-binding analysis.

SPR kinetic interaction analysis of MVN, MVN-DAVEI2-3Trp, CVN and CVN-DAVEI2-3Trp proteins

The interaction kinetics were measured for CVN, MVN and their respective fusions against gp120. The biosensor surface was prepared by immobilization of gp120 (~500 RU), and binding of MVN or MVN-DAVEI2-3Trp was initially measured when flowed over the chip surface as the analyte. MVN and MVN-DAVEI2-3Trp bound to immobilized gp120 with a K_D (equilibrium constant) value of 120 and 160 nM, respectively (Tables 2 and 3 and Figure 3A,B). The rate constants were calculated by globally fitting data sets using a 1: 1 binding model with a conformational change (Supplementary Figure S5 and Figure 7B). Fit for the two-step binding model with a conformational change was chosen based on the goodness of fit, which is reflected by the lowest χ^2 values (Supplementary Figure S5).

Experiments were performed with CVN or CVN-DAVEI-L2-3Trp identically with the same amount of gp120 immobilization and similar flow rates. CVN and CVN-DAVEI-L2-3Trp bound to gp120 with a K_D value of 8.65 and 6.9 nM, respectively (Figure 3C,D). The rate constants were calculated by globally fitting the association and dissociation phases to a multivalent binding model, where at least two CVN proteins can bind to one gp120 [34]. No significant difference was seen for the goodness of fit for the binding of CVN or its fusion, regardless of what binding model was used for fitting. Since a ~3.4: 1 stoichiometry was determined by ITC analysis (Figure 4), the corresponding multivalent binding model was used to report the binding kinetics. Association rates of $1.71 \pm 0.36 \times 10^3$ and $5.45 \pm 0.11 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ and dissociation rates of $1.48 \pm 0.59 \times 10^{-5}$ and $3.77 \pm 0.9 \times 10^{-5} \text{ s}^{-1}$ were measured for CVN and CVN-DAVEI-L2-3Trp proteins, respectively (Table 2). Binding kinetics have not been reported for MVN against HIV-1 gp120 previously. However, interestingly, there is precedent for a protein interaction system in which 1: 1 stoichiometry and conformational change SPR data fits have been observed for the same protein system depending on extent of mutation [35].

ITC analysis of MVN, MVN-DAVEI2-3Trp and CVN-DAVEI2-3Trp with gp120—

Although results from escape analysis [24] suggested that MVN has a localized and

monovalent interaction with gp120 (Figure 1B), we performed ITC analysis to confirm its binding stoichiometry with gp120. WT MVN (30 μ M) was titrated with 2 μ M WT YU2 gp120 in the VP-ITC system (MicroCal™, GE Healthcare, Freiburg). An exothermic binding profile was obtained with close to 1: 1 stoichiometry (Figure 4, center). A similar binding interaction analysis was performed for CVN-DAVEI2-3Trp with YU2 gp120 (Figure 4, left). Again, an exothermic profile was observed, with a stoichiometry of ~3.4: 1 for CVN-DAVEI2-3Trp:gp120, which confirms the multivalent association of CVN [17,36]. The binding affinity for CVN-DAVEI2-3Trp was much greater (K_d = 18 nM) than that of MVN (K_d = 225 nM). Similarly, purified MVN-DAVEI2-3Trp fusion was titrated with YU2 gp120 protein to ensure that its monovalent 1: 1 binding functionality was retained. The data obtained showed a 1: 1 binding stoichiometry with gp120, and a K_d value of 305 nM (Figure 4, right).

Comparison of infection inhibition and virolytic potencies of MVN fusion proteins with CVN fusion proteins

—We compared the infection inhibition and virolytic activities of MVN- and CVN-based constructs. Both MVN and MVN-DAVEI2-3Trp inhibited viral infection at sub-micromolar concentrations [MVN IC_{50} : 180 $\pm$ 5 nM; MVN-DAVEI2-3Trp IC_{50} : 149 $\pm$ 3 nM] (Figure 5A), which were significantly larger than that of CVN-DAVEI2-3Trp [IC_{50} : 2.1 $\pm$ 0.3 nM] [13]. Nonetheless, and most importantly, MVN-DAVEI2-3Trp, but not MVN, exhibited virolytic activity [MVN-DAVEI2-3Trp EC_{50} : 510 $\pm$ 55 nM] (Figure 5B). This result confirmed that MVN-DAVEI fusions can mimic CVN-DAVEI fusions lytic function when fused to Trp3 sequence, though with a lower potency.

Mutations in MVN glycan-binding domain to increase binding affinity and potency

Assessment of relative binding affinities—We assessed the extent to which the large decrease in potency of antiviral and virolytic potencies of MVN-DAVEI2-3Trp versus CVN-DAVEI2-3Trp was due to the lower gp120-binding affinity of the MVN domain. To compare their binding affinities, binding sensorgrams for relative responses were overlaid for MVN, MVN-DAVEI2-3Trp, CVN and CVN-DAVEI2-3Trp proteins by normalizing their R_{max} values. Both MVN and MVN-DAVEI2-3Trp were found to associate at similar rates (Figure 6A and Table 2). On the other hand, sensorgram overlays showed faster dissociation of MVN-DAVEI2-3Trp than MVN alone (Figure 6A). Interestingly, MVN and MVN-DAVEI2-3Trp showed faster association rates than CVN or CVN-DAVEI2-3Trp. However, CVN and CVN-DAVEI2-3Trp dissociated more slowly than MVN and MVN-DAVEI2-3Trp (Figure 6A), probably accounting for the higher observed affinities and hence greater antiviral potencies [CVN K_D = 8.65 nM; CVN-DAVEI2-3Trp K_D = 6.9 nM].

Structure and sequence comparison of amino acids at the mannobiose-binding site of CVN and MVN—To improve upon the lower potency of MVN, we implemented a sequence-guided protein engineering approach. Structure and sequence comparison of the high-affinity glycan-binding sites of CVN [15] and MVN [25] showed similarity in a small subset of amino acids directly involved in glycan interaction, though many variations were seen between the overall sequences of these two proteins (Figure 6B). CVN is able to form four strong hydrogen-bonding interactions, directly with Man-Alpha1

(Asn42, Ser52, Thr57 and Gln78), that provide for a tight binding affinity [15] (Figure 6C). Despite similarity in some of these residues (Asn44, Thr59), MVN does not form direct hydrogen-bonding interactions between mannobiose and these residues [25] (Figure 6D). Additionally, the positioning of these amino acids and their distances from mannobiose are also different for MVN *versus* CVN. For example, Man-Alpha1 is stabilized by a hydrogen bond with Ser52 in CVN–ligand structure [15] (Figure 6C). Gln54, which is present in the corresponding position in MVN structure, does not contribute to a direct hydrogen bond, but instead stabilizes the neighboring Asn55 [25] (Figure 6D) and acts as its chaperone to form the cavity for mannobiose binding.

SPR kinetic interaction analysis for MVN variants—To increase the affinity for glycans, five mutations were introduced at various positions (G43E, Q54S, Q81K, M83R and G85Q) (Figure 7A) of MVN based on its structure and sequence alignment with CVN. SPR analysis was performed for all mutants, and sensorgrams were fitted using a conformational change model. Results showed that the G43E mutation led to increased K_D by almost three-fold [Table 3, Supplementary Figure S6 and eqn (3)]. No detectable binding signal was observed for single mutant Q54S, double mutants (G85Q/Q54S), (M83R/Q54S), (Q81K/Q54S), (G43E/Q54S), quadruple (Q54S/Q81K/M83R/G85Q) or penta (G43E/Q54S/Q81K/M83R/G85Q) mutants (Table 3 and Supplementary Figure S6). In contrast, Q81K and M83R mutation lowered the K_D by three- and two-fold, respectively. G85Q mutation lowered the K_D by nearly 1.5-fold. Double mutation (G43E/Q81K; no change), (G43E/M83R; no change) and (G43E/G85Q; 1.5-fold higher K_D) did not show a significant change in binding affinity (Table 3 and Figure 7C). Pleasingly, the double-mutation Q81K/M83R lowered K_D by almost six-fold (Table 3). In this case, we observed a two-fold decrease of k_{-2} (rate of MVN·gp120 to MVN:gp120 transition) and three-fold decrease of k_{-1} (rate of MVN: gp120 dissociation into free MVN and gp120). Overall, a two-fold decrease in k_{on} and ~13-fold decrease in k_{off} were observed when compared with MVN(WT) (Table 3). Thus, the double mutation (Q81K/M83R) significantly increased the stability of the complex, with a slower dissociation rate (Table 3 and Figures 7C and 8A). Since lysine and arginine are both positively charged amino acids that can readily form stabilizing hydrogen bonds, we surmise that the significant gain in affinity is possibly due to the introduction of new hydrogen bonds to stabilize the mannobiose–MVN complex. This is consistent with a recent computational study that showed Q81 in MVN contributing strongly to the binding free energy with di-mannose [37]. The triple mutant (Q81K/M83R/G85Q) had 1.5-fold lower K_D when compared with wild-type protein (Table 3). The loss of binding potency from double (Q81K/M83R) to triple (Q81K/M83R/G85Q) mutant may be due to the larger glutamine side chain than glycine at position 85, which we speculate could have a destabilizing steric effect on mannobiose binding [25]. The quadruple mutant, G43E/Q81K/M83R/G85Q, did not show a significant change in K_D value (Table 3).

Since the double mutation (Q81K/M83R) significantly increased the binding affinity of MVN with gp120 (Figure 8A), we introduced the corresponding double mutation into MVN-DAVEI2-3Trp and assessed binding of this fusion protein to immobilized gp120 (500 RU). The globally fit data set showed a confirmed 1: 1 binding stoichiometry of MVN-DAVEI2-3Trp(Q81K/M83R) and gp120 (Table 5 and Figure 8B). A nearly eight-fold lower

K_D (20 nM) when compared with wild-type MVN-DAVEI2-3Trp (160 nM) was also observed for the MVN-DAVEI2-3Trp (Q81K/M83R) mutant (Table 5 and Figure 8B).

ITC binding analyses were performed for some of the representative mutants, namely G43E/Q54S, PENTA mutant (G43E/Q54S/Q81K/M83R/G85Q) and Q81K/M83R (Figure 9 and Supplementary Figure S10) to confirm the SPR-binding results. Consistent with SPR studies, G43E/Q54S and PENTA mutant showed at most only weak binding to gp120 (Figure 9). MVN and MVN-DAVEI mutants with mutation Q81K/M83R showed lower K_d values of 75 and 77 nM, respectively, which was consistent with the SPR-binding results (Figure 9, Table 4 and Supplementary Figure S10). In summary, binding analysis helped identify a double mutant that could significantly enhance MVN-gp120 interaction.

Antiviral, virolytic and Env engagement properties of re-engineered MVN-DAVEI

Antiviral and virolytic potency of MVN(Q81K/M83R) and MVN-DAVEI2-3Trp(Q81K/M83R) post mutational optimization—

We assessed the antiviral and virolytic activities of the double-mutant Q81K/M83R in both MVN and MVN-DAVEI2-3Trp backgrounds. Infection inhibition assays showed that both the MVN(Q81K/M83R) [IC_{50} : 29 ± 3 nM] and MVN-DAVEI2-3Trp(Q81K/M83R) [IC_{50} : 34 ± 2 nM] inhibited pseudoviral infection with a higher potency when compared with wild-type MVN [IC_{50} : 180 ± 5 nM] and MVN-DAVEI2-3Trp [IC_{50} : 149 ± 3 nM], respectively (Figure 10A). In addition, viral stocks treated with MVN-DAVEI2-3Trp(Q81K/M83R) exhibited increased virolytic potency [EC_{50} : 70 ± 18 nM] than MVN-DAVEI2-3Trp [EC_{50} : 510 ± 55 nM] (Figure 10B). MVN(Q81K/M83R) did not show any virolytic activity (Figure 10B). The overall results demonstrate that MVN-DAVEI2-3Trp(Q81K/M83R) is potently active as an HIV-1 virolytic inhibitor.

Competition of biotinylated-Trp3 peptide binding to Env trimer by optimized MVN-DAVEI fusion—

In our previous study, we showed by cell and virus ELISA that binding of biotinylated-Trp3 peptide to Env-expressing cells and HIV-1 viruses could be inhibited by the 3Trp moiety of CVN-DAVEI-L4-3Trp, gp41-specific antibodies such as 10E8 and 4E10, and gp120/gp41 interface specific antibody 35O22, but not by 2G12 and F105 antibodies [13]. We also did not observe direct binding of biotinylated peptide to these antibodies (2G12, F105, 4E10 and 10E8) in that study [13]. Since CVN was substituted with MVN for generating MVN-DAVEI2-3Trp, we sought to confirm that the fundamental binding mechanism of the lectin-simplified DAVEI was retained. A cell-based competition ELISA was performed, where Bt-Trp3 peptide binding to the Env trimer was competed with 2G12, MVN(Q81K/M83R), MVN-DAVEI2-3Trp(Q81K/M83R) and F105 antibody. We found that F105 and 2G12 did not compete with binding of Bt-Trp3 (Supplementary Figure S7), consistent with earlier observations [13]. On the other hand, binding of Bt-Trp3 peptide was competed by MVN-DAVEI2-3Trp(Q81K/M83R), but not by MVN(Q81K/M83R) protein (Supplementary Figure S7). The observation that Bt-Trp3 binding is competed by MVN-DAVEI2-3Trp(Q81K/M83R) [$>60\%$ inhibition at 500 nM concentration] protein, but not by MVN(Q81K/M83R), confirms that the 3Trp moiety of MVN-DAVEI2-3Trp(Q81K/M83R) protein is exposed and, as Bt-Trp3 peptide alone, can interact with Env gp41 (Supplementary Figure S7).

2G12 competition analysis against MVN–DAVEI fusion and CVN–DAVEI fusion

The binding epitope for the mAb 2G12 has been identified previously [8] and it overlaps the glycan sites expected for MVN, also validated in competition ELISA studies [24]. To localize the binding footprint of re-engineered MVN–DAVEI fusions to Env protein, we tested if 2G12 could compete with MVN-DAVEI2-3Trp (Q81K/M83R) after prebinding to gp120. These experiments were essential because mutations in MVN were dictated based on its sequence alignment with CVN, which has a larger glycan footprint.

Competition ELISA of CVN–DAVEI fusion binding to Env against 2G12—We first evaluated 2G12 competition of CVN–DAVEI interaction, the latter of which has a more extensive glycan footprint as mentioned earlier. We found that, despite 2G12 prebinding, gp120 could still bind to CVN-DAVEI-L2-3Trp and CVN (Figure 12A) in a dose-dependent manner, with EC_{50} values of 1.0 ± 0.12 and 0.47 ± 0.08 nM, respectively. These results argued that CVN and CVN-DAVEI-L2-3Trp could engage other glycosylation sites of gp120 apart from 2G12-specific glycans.

We also carried out analysis in which 2G12 and CVN/CVN-DAVEI-L2-3Trp were added at the same time to enable simultaneous competition of gp120 binding (Figure 12B). When 2G12 was competed against an increasing concentration of CVN and CVN-DAVEI-3Trp, we observed dose-dependent loss of 2G12 binding, with IC_{50} values of 0.90 ± 0.17 and 1.91 ± 0.31 nM, respectively (Figure 12B). These results suggested that CVN and CVN-DAVEI-3Trp could also interact with 2G12-specific glycosylation sites. Furthermore, we performed a competition ELISA experiment in which we competed CVN and CVN-DAVEI-L2-3Trp with increasing concentrations of 2G12 (Figure 12C). CVN/CVN-DAVEI-L2-3Trp protein and 2G12 were simultaneously allowed to interact with gp120 immobilized on ELISA plates. This experiment showed that 2G12 even at its highest concentration (10 000 nM) was not able to compete out CVN (<15%) and CVN-DAVEI-L2-3Trp (<5%) binding to gp120 (Figure 12C), suggesting a much stronger binding affinity of CVN and CVN-DAVEI-L2-3Trp with gp120 when compared with 2G12.

Competition ELISA of MVN–DAVEI(Q81K/M83R) re-engineered fusion binding to Env against 2G12—Competition experiments analogous to those described above were performed with engineered MVN-DAVEI2-3Trp(Q81K/M83R) protein to evaluate if 2G12 binding was altered as a result of mutational changes in MVN component. 2G12 was simultaneously added to immobilized gp120 in the presence of increasing concentration of MVN, MVN-DAVEI2-3Trp and MVN-DAVEI2-3Trp(Q81K/M83R) (Figure 12D). We observed a dose-dependent loss of 2G12 binding with an increasing concentration of MVN (IC_{50} : 68 ± 8 nM), MVN-DAVEI2-3Trp (IC_{50} : 71 ± 13 nM) and MVN-DAVEI2-3Trp(Q81K/M83R) (IC_{50} : 8 ± 1 nM) (Figure 12D). This result argues that the MVN, MVN-DAVEI2-3Trp and MVN-DAVEI2-3Trp (Q81K/M83R) binding epitopes still overlap with the glycan epitope of 2G12.

We then carried out a competition experiment in which MVN, MVN-DAVEI2-3Trp and MVN-DAVEI2-3Trp(Q81K/M83R) and CVN-DAVEI-L2-3Trp were immobilized on an ELISA plate and gp120 was captured (Figure 12E). Increasing concentrations of 2G12 were

then loaded onto the plate and its binding was detected using anti-human HRP. We found that 2G12 did not bind gp120 when MVN, MVN-DAVEI2-3Trp and MVN-DAVEI2-3Trp(Q81K/M83R) were used for gp120 capture (Figure 12E). In contrast, dose-dependent 2G12 binding was observed when CVN-DAVEI-L2-3Trp was used for gp120 capture (Figure 12E). These two experiments (Figure 12D,E) argue that, despite inclusion of the double mutation, MVN-DAVEI2-3Trp(Q81K/M83R) still binds in the vicinity of the 2G12-binding site and competes with 2G12 binding, similarly to MVN and MVN-DAVEI2-3Trp (Figure 11A,B). Because CVN-DAVEI-L2-3Trp could bind to sites other than 2G12-binding region, dose-dependent binding of 2G12 persisted with gp120 captured by CVN-DAVEI-L2-3Trp (EC_{50} : 12 ± 2 nM) (Figure 12E).

Inhibition of MVN DAVEI-induced virolysis by 2G12 and other mAbs—A 2G12 competition virolysis experiment was performed for MVN-DAVEI2-3Trp (Supplementary Figure S8 and Figure 11A,B). The results obtained showed that 2G12 competes with MVN-DAVEI2-3Trp(Q81K/M83R)-induced virolysis in a dose-dependent fashion (Figure 13) [$>75\%$ inhibition by 2G12 (red bar)]. F105, used as a negative control [13], did not compete with MVN-DAVEI2-3Trp(Q81K/M83R)-induced virolysis (blue bar). Alternately, when 2G12 was competed against CVN-DAVEI-L2-3Trp-induced virolysis, 2G12 did not compete with CVN-DAVEI2-3Trp-induced virolysis (pink bar) (Figure 13). These results argue that CVN-DAVEI-L2-3Trp can employ glycosylation sites other than 2G12-binding site to induce virolysis, whereas MVN-DAVEI2-3Trp engages a specific site that overlaps the 2G12-binding site to induce virolysis.

Discussion

We previously reported [12,13] that simultaneous chimera protein engagement of the gp120 and gp41 subunits of the HIV-1 Env spike complex causes lytic inactivation of the metastable virus. The prototype chimera identified, denoted CVN-DAVEI, contained a fusion of the gp120-binding lectin CVN covalently connected through $[Gly_4Ser]_n$ linkers to a gp41-binding polypeptide sequence derived from the MPER domain of HIV-1 Env. The current work is part of an effort to better define the spatial arrangement by which the DAVEI molecules encounter Env to exert the irreversible HIV-1 inactivating function. Until now, a major limitation in understanding the encounter complex has been the multiple glycan-binding sites in CVN. In addition, CVN can promiscuously bind to a relatively large set of glycans in gp120 (Figure 1A), and this limits the ability to define spatially the location of its binding to the Env spike complex. Additionally, it has been unresolved whether a multivalent engagement is a necessity for virolytic function. We answered some of these questions in this work. Here, we replaced CVN with a lectin, MVN, that uses a simpler gp120-binding footprint. MVN has a single glycan-binding site (compared with two in the CVN protein) that interacts with a restricted subset of gp120 glycans (Figure 1B). Using MVN allowed us to determine if monovalent 1:1 engagement of gp120 can still exhibit a DAVEI-like virolytic function. Since MVN is structurally similar to CVN, it was also a good starting point to test this idea.

MVN is almost two orders of magnitude weaker than the CVN (Figure 3 and Table 2) [24]. Therefore, a sequence-guided approach was used to re-engineer the MVN construct. By

sequence re-engineering, we showed a significant improvement in its gp120-binding affinity and antiviral potency. The engineered, enhanced-affinity MVN [Q81K/M83R] was obtained by comparing mannobiose–lectin structures of lower affinity MVN and higher affinity CVN, and then grafting sequence elements from the high-affinity binding site of CVN into the single lower affinity site of MVN. In CVN, the high-affinity glycan-binding site (Figure 6C) contains two positively charged residues, R76 and K74. While the MVN glycan-binding site does not contain positively charged residues, two residues are positioned similarly to R76 and K74, namely M83 and Q81, respectively. Mutating certain non-charged to positively charged residues led to an MVN[Q81K/M83R] mutant with higher affinity *versus* wild-type MVN (Figure 7C and Table 3) and ultimately to the potent MVN-DAVEI2-3Trp(Q81K/M83R) (Figure 8B and Table 5). The resulting DAVEI construct pleasingly had desired enhanced antiviral and virolytic potencies (Figure 10) closer to those observed before with the CVN-DAVEI2-3Trp construct (Table 6) [13]. Of note, the importance of the single MVN glycan-binding site also was highlighted in the current study by the finding that mutation G54S alone led to complete loss of Env binding (Figure 7C and Table 3).

We further demonstrated by antibody competition measurements that the MVN[Q81K/M83R]-binding epitope in gp120 is restricted and overlaps the known site of mAb 2G12 binding (Figure 11 and Supplementary Figure S8). This restricted glycan footprint was expected from prior escape analysis (Figure 1 and Supplementary Figure S8), our ITC analysis (Figures 4 and 9) and also validated in the current study by binding cross-competition of MVN-DAVEI2-3Trp(Q81K/M83R) with the monoclonal antibody 2G12 (Figure 12). The overlap of MVN- and 2G12-binding sites in gp120, initially predicted by glycan footprint comparison (Figure 11), in turn has enabled a simplified spatial model for productive MVN-DAVEI2-3Trp (Q81K/M83R) engagement with HIV-1 Env protein, as shown in Figure 14. In this model, we envision that the lectin domain of the MVN-DAVEI construct interacts with a restricted glycan patch encompassing components that are common to both 2G12 and MVN, namely N386 and N392. This interaction enables the linker-connected Trp3 domain to interact with gp41, and it is the latter interaction that we know to be the driving force for the virus lysis function of DAVEI. Overall, the results lead to a potent antiviral composition, and an improved hypothesis for how this composition can bind to the Env complex to transform the Env. The engagement of a restricted gp120-binding site by the MVN domain in DAVEI points to the possibility of further simplification of DAVEI inactivators of HIV-1 by, among other strategies, using smaller surrogates of 2G12 itself, for example a stabilized construct of a 2G12 CDR region obtained by approaches that have been used previously [38] to form small antibody-mimicking surrogates.

An unexpected observation in this study was that, in SPR interaction analysis of lectin–gp120 interactions, sensorgram data for all MVN derivatives were best fit to a conformational change model. It must be borne in mind that such SPR data cannot distinguish explicitly between MVN alone, gp120 alone and MVN–gp120 together as the source of the conformational change. Nonetheless, the fact that the conformational change fit preference was not observed with CVN–gp120 interaction suggests that the conformational effect is in some way derived from intrinsic differences in binding properties of the two lectins. The most obvious difference for the latter is that MVN has a single glycan-binding site [24,25] (also shown in this study), while CVN has two [20,39]. One may

speculate that two-site binding of CVN to multiple glycans in gp120 [20,39,40] could impose greater conformational constraint in the CVN component. In contrast, single site binding of MVN that in the current work appears to enable faster on and off rates than observed for CVN, at the same time, allows for greater conformational adaptation in MVN upon gp120 binding. Importantly, more direct biophysical/structural analysis will be required to improve our understanding of the role of conformational changes in MVN and MVN–DAVEI interactions with Env gp120 [35].

Simplified DAVEI structures, such as MVN-DAVEI2-3Trp(Q81K/M83R) and smaller DAVEI constructs developed in the future, will help address several remaining mechanistic questions of DAVEI function and lead to a better understanding of how this class of molecules is able to irreversibly inactivate HIV-1. Mechanistically, it remains unclear how simultaneous engagement of gp120 and gp41 by DAVEI constructs causes viral membrane transformation and lysis. We have previously established [12,13] that lysis is not exhibited by the lectin alone and that this action requires the co-presence of the covalently linked MPER/Trp3 domain and its binding with gp41 [13]. Thus, it is the dual engagement that is necessary and sufficient to hijack the intrinsic metastability of the Env spike to transform the Env protein spike, disrupt the membrane in which the spike is embedded and results in virus lysis. Looking ahead, the derivation and synthesis of more simplified and smaller DAVEIs will reduce the sets of both DAVEI and Env structural elements that are participating in DAVEI–Env lytic encounter, making it more tractable to use mutational variation as an approach to identify the structural elements driving inactivation. In turn, smaller DAVEIs will help define the spatial requirements for dual domain binding and consequent lysis. The size limits of DAVEIs, and the extents to which small molecule domains can be employed that bind to specific epitopes in both the gp120 and gp41 domains of Env, remain open questions for further investigations into development of improved rational approaches to the design of specific HIV-1 inactivators.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

3Trp	peptide sequence DKWASLWNW
Bt-Trp3	biotinylated-Trp3 peptide

CVN	cyanovirin-N
DAVEI	dual action virus entry inhibitor
EC₅₀	effective concentration for half maximal response
EDC	1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide
Env	envelope glycoprotein
HEK	human embryonic kidney cells
HIV-1	human immunodeficiency virus
HRP	horseradish peroxidase
ITC	isothermal titration calorimetry
K_D	equilibrium constant
k_{off}	dissociation rate constant
k_{on}	association rate constant
MPER	membrane proximal external region
MVN	microvirin
NHS	<i>N</i> -hydroxysuccinimide
OPD	ortho-phenylenediamine dihydrochloride
PBS-T	PBS and 0.05% Tween 20
RU	response unit
SPR	surface plasmon resonance
VP-ITC	VP-Isothermal Titration Calorimeter

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Figure 1. Literature-derived maps [24] of glycosylation sites that developed mutations in NL4.3 virus escape studies as viruses were treated with increasing concentrations of CVN and MVN (A) Structural locations of five of the eight N-linked glycosylation sites (N160, N339, N386, N392 and N448) in gp120 protein that were deleted in CVN-based passaging studies (represented as blue spheres) are shown in one monomeric subunit of BG505.SOSIP.664 [14]. Three N-linked glycosylation sites associated with asparagine (N136, N230 and N332) are not seen in the solved crystal structure (5CJX); therefore, closest residues (N133, V333 and N234) are highlighted as blue spheres. Glycans are arranged in four different clusters shown by dotted circles. (B) Structural locations of four N-linked glycosylation sites (N295, N339, N386 and N392) in gp120 protein [24] that were deleted in MVN passaging studies (represented as blue spheres). All four residues lie in one cluster shown by dotted circle, consistent with the monovalent interaction of MVN to gp120.

Figure 2. Validation of the presence of gp120-binding component (MVN) and Trp component in MVN fusions by protein ELISA

(A) Protein ELISA showing direct binding of MVN, MVN-DAVEI2-3Trp and MVN-DAVEI4-3Trp with YU2 gp120. MVN fusions were immobilized onto ELISA plates followed by the addition of gp120. D7324 and rabbit anti-sheep were used as primary and secondary antibodies. Biotinylated-Trp3 peptide was taken as a negative control, and MVN was used as a positive control. Experiments were done in triplicate, and graph was plotted using Origin v.8.1. (B–D) Protein ELISA showing a direct binding interaction of MVN-DAVEI2-3Trp and MVN-DAVEI4-3Trp with MPER-specific antibodies (2F5, 4E10 and 10E8). MVN fusions were immobilized on the plates, blocked with BSA followed by the addition of 50 ng of antibodies (2F5, 4E10 and 10E8). Antibodies were detected with anti-human HRP. Recombinant full-length CVN-DAVEI-L4 containing full-length MPER was used as a positive control for the experiment, and MVN was used as a negative control ($n = 3$). (E) Comparison of MVN fusion sequence with MPER, highlighting the mAb (2F5, 4E10 and 10E8) epitopes. MVN fusion contains epitopes only for 2F5 binding but not for 4E10 and 10E8 binding.

Figure 3. SPR kinetic analysis of MVN-DAVEI2-3Trp, MVN, CVN-DAVEI-L2-3Trp and CVN proteins

Various concentrations of (A) MVN-DAVEI2-3Trp (50, 100, 150 and 200 nM) and (B) MVN (50, 100 and 150 nM) were passed over gp120 immobilized on CM5 surface. Vectibix was taken as a negative control surface. Protein was allowed to associate for 300 s followed by injection of running buffer after 300 s. Binding analysis was performed by globally fitting sensorgrams using a 1: 1 binding, two-step model with a conformational change of gp120: MVN-DAVEI2-3Trp complex. Overlay of real-time sensorgram shows sequential injections of 2, 4, 8 and 12 nM of (C) CVN-DAVEI-L2-3Trp and (D) CVN proteins to immobilized gp120 proteins (500 RU) on a CM5 chip, followed by the injection of running buffer after 300 s. The rate constants were calculated by globally fitting the association and dissociation phases to a model for multivalent binding for CVN and CVN-DAVEI-L2-3Trp. Black and colored lines show calculated and experimental sensorgrams, respectively.

Figure 4. ITC analysis of MVN, MVN-DAVEI2-3Trp and gp120 and its comparison with CVN-DAVEI: gp120

Exothermic thermograms show the binding of MVN, MVN-DAVEI2-3Trp and CVN-DAVEI2-3Trp to gp120. YU2 Gp120 protein (2 μ M) (1.5 ml) in a sample cell was titrated with WT MVN (30 μ M), WT MVN-DAVEI2-3Trp (30 μ M) and CVN-DAVEI2-3Trp (20 μ M) at 25°C. Experiments were performed on the VP-ITC system (MicroCal™, GE Healthcare, Freiburg) and data were integrated using a one site model for MVN and multisite model for CVN-DAVEI2-3Trp.

Figure 5. Antiviral and virolytic properties of MVN and MVN-DAVEI2-3Trp fusions before mutation

(A) HOS-T4-R5 cells were exposed to HIV-1 YU2 pseudovirus with serial dilutions of the MVN and MVN-DAVEI2-3Trp protein. The inhibitory potency of MVN and MVN-DAVEI2-3Trp were 180 ± 5 and 149 ± 3 nM, respectively. IC₅₀ values were calculated using Origin v.8.1. (B) Release of p24 from HIV-1 YU2 pseudovirus upon incubation with MVN-DAVEI2-3Trp derivatives. A sandwich ELISA was carried out in which experimental p24 release was background-subtracted using PBS-treated virus and then compared with virus lysed with 1% Triton X-100 (means $\pm$ SD; $n = 3$). The EC₅₀ value for MVN-DAVEI2-3Trp was 510 ± 55 nM. MVN did not show p24 release. EC₅₀ values of virolysis of HIV-1 YU2 pseudovirus were determined with Origin v.8.1.

Figure 6. Structure and sequence comparison between CVN and MVN and their interactions with mannose

(A) Binding kinetics of CVN, MVN, CVN-DAVEI-L2-3Trp and MVN-DAVEI2-3Trp proteins, when injected as an analyte onto the YU2 gp120 protein (500 RU) immobilized on a CM5 chip. The association (0–300 s) and dissociation (300–600 s) were measured and compared. Each sensorgram was normalized. (B) Sequence alignment comparing the amino acids in mannose-binding site of CVN (highlighted in pink color) and MVN (highlighted in orange color). (C) Close-up view of interacting residues in the high-affinity binding site of CVN with Man-Alpha1 (PDB ID: 1IIY). Four important hydrogen-bonding interactions were seen with residues Asn42, Ser52, Thr57 and Glu78 as shown by red dotted lines. Additional bonds are formed with water molecules (not shown in figure). (D) Close-up view of interacting residues in the binding site of MVN with mannobiose (PDB ID: 2YHH). Hydrogen bonds were not observed but several polar bonds were formed with water molecules (not shown in the figure).

Figure 7. Domain constructs of MVN and its derivatives used and their biosensor interaction analyses

(A) Sequence of MVN domain involved in gp120 interaction (shown in blue). The sequence is homologous to the high-affinity binding site of CVN. Amino acids involved in glycan interaction are highlighted (shown in orange). Mutations introduced based on sequence comparison with CVN are highlighted in red. Constructs derived are named based on the corresponding mutations inserted. (B) Layout of SPR experimental design. Gp120 was immobilized on a CM5 chip, and MVN and its derivatives were injected as analytes. MVN–gp120 interaction curves were fit to a 1: 1 binding model with a conformational change. (C) The gp120-coupled surface challenged with 100 nM of each mutational variant of MVN. In this assay, a rapid increase in the association phase (0–300 s) indicated fast association in the interaction of MVN and gp120, while a rapid decrease in the dissociation phase (300–700 s) indicated fast dissociation of the complex. Sensorgrams of MVN-WT, Q81K, G43E, G85Q, M83R, Q54S, Q81K/M83R, G43E/Q54S and G43E/Q54S/Q81K/M83R/G85Q (MVN_penta) are shown in pink, gray, blue, green, red, cyan, purple, brown and maroon lines, respectively.

Figure 8. SPR sensorgrams of MVN (Q81K/M83R) and MVN-DAVEI2-3Trp (Q81K/M83R) interactions with gp120

Various concentrations of MVN(Q81K/M83R) (50, 100, 150 and 200 nM) and MVN-DAVEI2-3Trp (Q81K/M83R) were passed over gp120 immobilized on a CM5 surface. Vectibix was taken as a negative control surface. Protein was allowed to associate for 300 s followed by the injection of running buffer after 300 s. Binding analysis was performed by globally fitting sensorgrams using a 1: 1 binding, two-step model with a conformational change of gp120: MVN-DAVEI2-3Trp complex.

Figure 9. ITC binding analysis of MVN variants with gp120

YU2 gp120 protein (2 μ M) (~1.5 ml) in sample cell was titrated with MVN-DAVEI2-3Trp(Q81K/M83R) (40 μ M) and MVN mutants G43E/Q54S (300 μ M) and PENTA (G43E/Q54S/Q81K/M83R/G85Q) (300 μ M) at 25°C. Experiments were performed on the VP-ITC system (MicroCal™, GE Healthcare, Freiburg) and data were integrated using a one site-binding model.

Figure 10. Concentration dependence of antiviral and virolytic activities of MVN and MVN-DAVEI2 fusion protein constructs prior and post mutations

(A) HOS-T4-R5 cells were exposed to HIV-1 YU2 pseudovirus with serial dilutions of the MVN, MVN(Q81K/M83R), MVN-DAVEI2-3Trp and MVN-DAVEI2-3Trp(Q81K/M83R) proteins. The inhibitory potency of MVN, MVN(Q81K/M83R), MVN-DAVEI2-3Trp and MVN-DAVEI2-3Trp(Q81K/M83R) proteins were 180 ± 5 , 29 ± 3 , 149 ± 3 and 34 ± 2 nM, respectively. IC_{50} values were calculated using Origin v.8.1. (B) Release of p24 from HIV-1 YU2 pseudovirus upon incubation with MVN and MVN-DAVEI2-3Trp derivatives. A sandwich ELISA was carried out in which experimental p24 release was background-subtracted using PBS-treated virus and then compared with virus lysed with 1% Triton X-100 (means $\pm$ SD; $n = 3$). The EC_{50} values for MVN-DAVEI2-3Trp and MVN-DAVEI2-3Trp(Q81K/M83R) were 510 ± 55 and 70 ± 18 nM, respectively. MVN and MVN(Q81K/M83R) did not show p24 release. EC_{50} values for virolysis of HIV-1 YU2 pseudovirus were determined with Origin v.8.1.

Figure 11. Comparison of 2G12-specific [41] and putative MVN-specific glycan sites [24] (A) Gp120 subunit is shown as blue surface color, and gp41 subunit is shown as pink surface. Figure on the left shows five 2G12-specific glycosylation sites (N295, N332, N386, N392 and N448), highlighted in red color (PDB ID: 5FUU). (B) Figure on the right shows glycan patches that developed mutations in gp120 treated with an escalating concentration of MVN, highlighted in green.

Figure 12. Competition ELISA analysis of interactions of lectin and lectin-DAVEI proteins
(A) Direct binding of CVN and CVN-DAVEI-L2-3Trp to gp120 immobilized on ELISA plate in the presence of 2G12 prebound to gp120. 2G12 bound to gp120 was detected using anti-human HRP (data not shown). **(B)** Loss of binding of 2G12 to gp120 immobilized in the presence of increasing concentrations of CVN and CVN-DAVEI-L2-3Trp. **(C)** Binding of CVN and CVN-DAVEI-L2-3Trp to immobilized gp120 in the presence of increasing concentrations of 2G12. No loss of CVN and CVN-DAVEI-L2-3Trp was seen. **(D)** Loss of binding of 2G12 to gp120 immobilized in the presence of increasing concentrations of MVN, MVN-DAVEI2-3Trp and MVN-DAVEI2-3Trp(Q81K/M83R). **(E)** Detection of binding of 2G12 (with increasing concentrations) to gp120 captured with immobilized CVN-DAVEI-L2-3Trp (positive control), MVN, MVN-DAVEI2-3Trp and MVN-DAVEI2-3Trp(Q81K/M83R) on ELISA plate ($n = 3$).

Figure 13. Inhibition of DAVEI-induced virolysis by 2G12 and F105

MVN-DAVEI2-3Trp(Q81K/M83R) protein (150 nM) was incubated with YU2 virus in the presence and absence of F105 (blue color), 2G12 (red color), antibodies at various concentrations (0, 100 and 1000 nM). 2G12 inhibited virolysis (~50% 2G12) at 100 nM concentrations. CVN-DAVEI-L2-3Trp protein was used as a control (pink color). 2G12 showed no inhibition of CVN-DAVEI-L2-3Trp-induced virolysis up to 1000 nM concentrations ($n = 3$).

Figure 14. Possible glycan engagement by lectins in MVN- versus CVN-DAVEI

Cartoon representation on the left shows possible gp120 glycan association by first-generation CVN-DAVEI molecule. CVN can engage multiple glycosylation sites of gp120 [14]. CVN can also engage multiple glycan sites at the same time by showing a multivalent association. Figure on the right shows restricted glycan site engagement of second-generation MVN-DAVEI molecule that utilizes MVN as a substitute for CVN. MVN associates with the outer domain residues and binds in the vicinity of 2G12-binding site and also competes with 2G12 binding. MVN also shows monovalent association with gp120. Closed black circles show the glycan sites of gp120 that are engaged by DAVEIs and open black circles on the right show glycan sites in gp120 that are not engaged by MVN-DAVEI.

Table 1
Validation of purified proteins by MALDI analysis

The expected mass (based on sequence information) and observed molecular mass (based on MALDI analysis) of purified MVN and MVN fusions used in this study.

Proteins	Mass spectrometry results	
	Predicted mass (Da)	Observed mass (Da)
Microvirin (MVN)	14059.21	14056.7
MVN-DAVEI2-3Trp	15693.95	15690.7
MVN-DAVEI4-3Trp	16395.50	16391.50

Table 2
Interaction kinetics of CVN, MVN and their respective fusions with gp120

Global fitting analysis of the interaction kinetics of CVN, MVN, CVN-DAVEI-L2-3Trp and MVN-DAVEI2-3Trp with gp120. CVN and CVN-DAVEI-L2-3Trp were analyzed using a global fit to a multivalent binding model, whereas that of MVN and MVN-DAVEI2-3Trp were fit to a 1: 1 binding/conformational change model for gp120: MVN complex.

Protein	$k_1 \times 10^5$ (M ⁻¹ s ⁻¹)	$k_{-1} \times 10^{-2}$ (s ⁻¹)	$k_2 \times 10^{-3}$ (M ⁻¹ s ⁻¹)	$k_{-2} \times 10^{-3}$ (s ⁻¹)	$k_{on} \times 10^3$ (M ⁻¹ s ⁻¹)	$k_{off} \times 10^{-5}$ (s ⁻¹)	$K_D \times 10^{-7}$ (M)	R_{max} (RU)	χ^2
MVN_WT	2.4 ± 0.6	9 ± 3.2	7.8 ± 2.0	2.4 ± 0.5	—	—	1.2 ± 0.9	65.4	0.24 ± 0.21
MVN_DAVEI2-3Trp	2.37 ± 0.3	12.5 ± 2.2	6.62 ± 1.15	1.9 ± 0.4	—	—	1.6 ± 0.45	68.8	0.38 ± 0.13
CVN-DAVEI2-3Trp	—	—	—	—	5.45 ± 0.11	3.77 ± 0.9	0.0697	66.4	0.21 ± 0.4
CVN	—	—	—	—	1.71 ± 0.36	1.48 ± 0.59	0.0865	55.8	0.23 ± 0.28

Table 3

Kinetic parameters for the interaction of MVN and its mutational variants to gp120, as determined by global fit of SPR-binding data

Protein	$k_1 \times 10^5$ (M ⁻¹ s ⁻¹)	$k_{-1} \times 10^{-2}$ (s ⁻¹)	$k_2 \times 10^{-3}$ (M ⁻¹ s ⁻¹)	$k_{-2} \times 10^{-3}$ (s ⁻¹)	$k_{on} \times 10^2$ (M ⁻¹ s ⁻¹)	$k_{off} \times 10^{-5}$ (s ⁻¹)	$K_D \times 10^{-7}$ (M)	R_{max} (RU)	χ^2
MVN_WT	2.4 ± 0.6	9 ± 3.2	7.8 ± 2.0	2.4 ± 0.5	41.6 ± 5.4	44.5 ± 2.8	1.2 ± 0.9	65	0.24 ± 0.21
MVN_G43E	3.4 ± 1.2	24.4 ± 5.1	4.9 ± 1.3	2.5 ± 0.3	20.16 ± 1.9	61 ± 1.5	3.6 ± 1.4	56	0.433 ± 0.04
MVN_Q54S	—	—	—	—	—	—	ND	—	—
MVN_Q81K	17.3 ± 4.3	16 ± 4.9	4.8 ± 0.8	2.3 ± 0.35	91.8 ± 4.7	36.8 ± 1.7	0.45 ± 0.06	82	0.499 ± 0.35
MVN_M83R	2.9 ± 1.25	5 ± 1.4	7.6 ± 1.1	2.9 ± 0.45	29.9 ± 1.9	14.5 ± 0.6	0.52 ± 0.2	69	0.454 ± 0.11
MVN_G85Q	2.9 ± 0.5	7.1 ± 0.5	8.9 ± 2.2	2.7 ± 0.65	29.6 ± 1.4	19.1 ± 0.3	0.72 ± 0.4	60	0.728 ± 0.30
MVN_G43E/Q54S	—	—	—	—	—	—	ND	—	—
MVN_G43E/Q81K	1.8 ± 0.35	6.4 ± 2.6	8.2 ± 2.0	2.7 ± 0.5	16.6 ± 0.9	17.3 ± 1.3	1.2 ± 0.7	79	1.21 ± 0.56
MVN_G43E/M83R	1.1 ± 0.25	4.5 ± 1.3	7.5 ± 1.6	2.3 ± 0.4	9.8 ± 0.5	10.3 ± 0.5	1.3 ± 0.85	88	1.07 ± 0.27
MVN_G43E/G85Q	0.9 ± 0.16	5.1 ± 0.7	7.7 ± 3.3	2.4 ± 0.9	7.1 ± 0.2	12.2 ± 0.6	1.8 ± 0.6	74	1.18 ± 0.49
MVN_Q54S/Q81K	—	—	—	—	—	—	ND	—	—
MVN_Q54S/M83R	—	—	—	—	—	—	ND	—	—
MVN_Q54S/G85Q	—	—	—	—	—	—	ND	—	—
MVN_Q81K/M83R	3.1 ± 1.8	3 ± 0.55	5.1 ± 1.9	1.1 ± 0.1	18.8 ± 3.6	3.3 ± 0.05	0.21 ± 0.01	58	0.401 ± 0.20
MVN_Q81K/G85Q	1.6 ± 0.45	5.3 ± 0.43	4.1 ± 0.75	1.78 ± 0.3	8.8 ± 0.5	9.4 ± 0.1	1.01 ± 0.27	54	0.552 ± 0.26
MVN_Q81K/M83R/G85Q	3.4 ± 0.90	5.8 ± 1.7	5.5 ± 0.9	4.1 ± 0.2	32.4 ± 4.6	23.2 ± 0.3	0.77 ± 0.12	81	0.895 ± 0.54
MVN_G43E/Q81K/M83R/G85Q	2.4 ± 0.75	7.8 ± 1.8	6.3 ± 1.3	2 ± 0.4	14.9 ± 1.3	15.6 ± 0.7	1.0 ± 0.25	70	0.645 ± 0.18
MVN_Q54S/Q81K/M83R/G85Q	—	—	—	—	—	—	ND	—	—
MVN_G43E/Q54S/Q81K/M83R/G85Q	—	—	—	—	—	—	ND	—	—

Abbreviations: ND: not detected.

Table 4

Summary of K_d values obtained via SPR and ITC experiments and binding stoichiometries of corresponding proteins determined by ITC

Proteins	SPR K_d (μ M)	ITC K_d (μ M)	ITC analysis N (protein:gp120)
CVN-DAVEI2-3Trp	0.006 ± 0.0005	0.018 ± 0.012	3.384 ± 0.028
MVN(WT)	0.120 ± 0.055	0.225 ± 0.010	1.29 ± 0.040
MVN (Q81K/M83R)	0.021 ± 0.005	0.075 ± 0.011	1.31 ± 0.132
MVN-DAVEI2-3Trp	0.160 ± 0.03	0.305 ± 0.050	1.11 ± 0.060
MVN-DAVEI2-3Trp (Q81K/M83R)	0.020 ± 0.006	0.077 ± 0.011	1.345 ± 0.112

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Kinetic parameters, obtained via global fitting, for MVN-DAVEI2-3Trp and MVN-DAVEI2-3Trp(Q81K/M83R) protein binding to gp120

Table 5

Protein	$k_1 \times 10^5 \text{ (M}^{-1} \text{ s}^{-1})$	$k_{-1} \times 10^{-2} \text{ (s}^{-1})$	$k_2 \times 10^{-3} \text{ (M}^{-1} \text{ s}^{-1})$	$k_{-2} \times 10^{-3} \text{ (s}^{-1})$	$K_D \times 10^{-7} \text{ (M)}$	$R_{\text{max}} \text{ (RU)}$	χ^2
MVN-DAVEI2-3Trp	2.37 ± 0.3	12.5 ± 2.2	6.62 ± 1.15	1.9 ± 0.4	1.6 ± 0.45	68.8	0.38 ± 0.13
MVN-DAVEI2-3Trp (Q81K/M83R)	3.47 ± 0.7	3.49 ± 0.88	6.11 ± 1.59	0.64 ± 0.25	0.2 ± 0.065	74	0.46 ± 0.28

Table 6

Antiviral and virolytic potency of first-generation recombinant CVN–DAVEI and its comparison with second-generation MVN–DAVEI

Generation	Proteins	K_d (nM)	Antiviral activity (nM)	Virolytic (nM)
I	Cyanovirin (CVN)	1.2 ± 0.2	0.9 ± 0.2	No effect
I	CVN-DAVEI4-3Trp	2.6 ± 0.8	2.2 ± 0.4	36.1 ± 5.1
II	Microvirin (MVN)	120 ± 90	180 ± 5	No effect
II	MVN-DAVEI2-3Trp	160 ± 45	149 ± 3	510 ± 55
II [*]	MVN (Q81K/M83R)	21 ± 1	29 ± 3	No effect
II [*]	MVN-DAVEI2-3Trp (Q81K/M83R)	20 ± 12	34 ± 2	70 ± 18

* Denotes re-engineered constructs.