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Abstract: Here, we applied the designed ankyrin repeat protein (DARPin) technology to develop novel gp120-directed binding molecules with HIV entry-inhibiting capacity. DARPins are interesting molecules for HIV envelope inhibitor design, as their high-affinity binding differs from that of antibodies. DARPins in general prefer epitopes with a defined folded structure. We probed whether this capacity favors the selection of novel gp120-reactive molecules with specificities in epitope recognition and inhibitory activity that differ from those found among neutralizing antibodies. The preference of DARPins for defined structures was notable in our selections, since of the four gp120 modifications probed as selection targets, gp120 arrested by CD4 ligation proved the most successful. Of note, all the gp120-specific DARPin clones with HIV-neutralizing activity isolated recognized their target domains in a conformation-dependent manner. This was particularly pronounced for the V3 loop-specific DARPin 5m3$_D$12.$InstarkcontrasttoV3-specificantibodies, 5m3_D12preferentiallyrecognizedtheV3loopinaspecificconformation, asprobedbystructurallyarrestdependentV3recognitionallowed5m3_D12tobypasstheV1V2shieldingofseveraltier2HIVisolatesandtoneutralizethesevi$

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Conformation-dependent recognition of HIV gp120 by Designed Ankyrin Repeat Proteins provides access to novel HIV entry inhibitors

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Abstract

Here we applied the *D*esigned *A*nkyrin *R*epeat *P*rotein (DARPin) technology to develop novel gp120-directed binding molecules with HIV entry-inhibiting capacity. DARPins are interesting molecules for HIV envelope inhibitor design, as their high affinity binding differs from that of antibodies. DARPins in general prefer epitopes with a defined folded structure. Here we probed whether this capacity favors the selection of novel gp120-reactive molecules with specificities in epitope recognition and inhibitory activity that differ from those found amongst neutralizing antibodies. The preference of DARPins for defined structures was notable in our selections as of the four gp120 modifications probed, gp120 arrested by CD4 ligation proved the most successful panning target. Of note, all gp120 specific DARPin clones with HIV neutralizing activity we isolated, recognized their target domains in a conformation-dependent manner. This was particularly pronounced for the V3-loop-specific DARPin **5m3_D12.** In stark contrast to V3-specific antibodies, 5m3_D12 preferentially recognized the V3 loop in a specific conformation, as probed by structurally arrested V3 mimetic peptides, but bound linear V3 peptides only very weakly. Most notably, this conformation-dependent V3 recognition allowed **5m3_D12** to bypass V1V2 shielding of several tier 2 HIV isolates and to neutralize these viruses. These data provide a proof of concept that the DARPin technology holds promise for the development HIV entry inhibitors with unique mechanism of action.

Introduction

The highly specific interaction of the HIV envelope spike, a trimer composed of gp120 and gp41 heterodimers, with the cellular receptor CD4 and a co-receptor (typically CCR5 or CXCR4) initiates HIV entry and is indispensable for infection (reviewed in (1)). Although, the potential of HIV entry blocking agents for intervention strategies has been acknowledged for long and a number of agents targeting the viral envelope proteins and the cellular receptors CD4, CCR5 or CXCR4 have been developed (2, 3), thus far only two entry inhibitors, the peptidic fusion inhibitor T-20 targeting gp41 (4) and the small molecule CCR5 inhibitor Maraviroc (5), have been used clinically.

Design of appropriate inhibitors, in particular those which target the viral envelope proteins, faces challenges which are in many aspects similar to those encountered by natural and vaccine-induced antibody responses. Both neutralizing antibodies and inhibitors need to access vulnerable sites on the HIV envelope trimer. The complex quaternary structure of the trimeric envelope spike, however, efficiently shields functionally important domains. Conserved domains are positioned facing inwards in the trimeric complex, only to be exposed transiently upon receptor engagement (6-8), while the outer trimer surface is protected by flexible, variable loops and extensive glycosylation (7, 9-11). These strategies act in concert to shield the envelope complex from immune recognition and inhibitor attack (8, 12). Most of the gp120 outer domain is functionally not important (7, 13) and allows the virus to rapidly mutate and evade any envelope targeting agent (12). Antibody-based vaccines and entry inhibitors thus need to act against a wide spectrum of genetically divergent HIV strains to be effective, ideally by targeting conserved, yet easily accessed sites on the viral envelope, which are important for the function of the envelope trimer and cannot be altered without significant fitness costs. Rare, broadly active and potent neutralizing antibodies can emerge in natural infection (14). Vaccine immunogens, however, capable of eliciting such responses, still need to be developed and, likewise, means to design and select inhibitors, which target conserved and accessible domains, still need to be created.

Here we employed the *D*esigned *A*nkyrin *R*epeat *P*rotein (DARPin) technology (15-19) to derive HIV envelope-specific entry inhibitors. We have previously utilized the technology successfully

to develop DARPins specific for CD4, which proved to be highly potent in inhibiting entry of divergent HIV strains (18). DARPins, like the natural ankyrin repeat proteins they are derived from (20), have exceptional binding properties and recognize particularly well targets with a defined rigid surface, such as folded proteins. Although they share many properties with antibodies, most noteworthy their high target specificity and affinity, DARPins differ from antibodies in size, structure and preference for folded proteins as targets (19). In the current study we sought to explore whether these distinct properties of DARPins allow selection of binding molecules which recognize epitopes on gp120 differing from those targeted by antibodies and, which upon binding to virions, interfere with HIV entry.

To this end, we performed four independent series of ribosome display selections of gp120-specific DARPins, utilizing different gp120 molecules and epitope display approaches (Figure 1A, B). Our selections yielded a variety of gp120-directed DARPin molecules which all proved to depend to a higher degree on a structural conservation of the envelope protein than gp120-specific antibodies recognizing overlapping domains. Most noteworthy, DARPin **5m3_D12** proved to recognize the V3 loop in a highly conformation dependent manner. This clone recognized the V3 loop on wild-type gp120 protein as well as in the context of the trimeric spike on virus particles and bound to structurally arrested V3 loop mimetic peptides, but unlike V3 loop antibodies, showed dramatically reduced capacity to bind to linear V3 loop peptides. Importantly, V3 loop antibodies largely fail to interact with the V3 loop on envelope trimers in the presence of active V1V2 shielding on tier 2 viruses and thus have in most cases only marginal or no neutralization activity (21). On the contrary, DARPin **5m3_D12** was in part capable of bypassing V1V2 shielding of tier 2 HIV isolates, albeit in a strain-dependent manner, enabling it to block entry of these virus isolates.

Materials and Methods

Reagents

Reagents were kindly provided by following groups: soluble CD4 (sCD4) by W. Olson (Progenics Pharmaceuticals Inc., Tarrytown, New York, USA); mAbs IgG1b12 (22), b6 (23), PGT128 (24) by D. Burton (The Scripps Research Institute, La Jolla, USA); mAb 1-79 (25) by M. Nussenzweig (Rockefeller University, New Yor, NY); mAb 2G12 (26) by H. Katinger (Polymun, Vienna, Austria); mAbs 17b, 19b, 48D, A32 (27) by J. Robinson (Tulane University, USA); 447-52D (28) by S. Zolla Pazner and F425 B4e8 (29) by Dr. Marshall Posner and Dr. Lisa Cavacini via the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. CD4M47 was synthesized as described (30). Linear and cyclic V3 mimetic peptides from strains JR-FL and MN were synthesized as described (31). The following V3 loop peptides were used (sequences are indicated in brackets).

- Linear peptides: linear (JR-FL) (NNTRKSIHIGPGRAFYTTGEIIG); linear (MN) (GGGGYNKRKRIHIGPGRAFYTTKNIIG).
- Structural V3 loop mimetics which were cyclized by a D-Pro-L-Pro (^{D}PP) dipeptide which stabilizes the hairpin conformation: cyclic IY (MN) (KRIHIGPGRAFYTT^{D}PP); cyclic IY (MN^{mut}) (KRIHIGAGRAFYTT^{D}PP); cyclic IY (JR-FL) (KSIHIGPGRAFYTT^{D}PP); cyclic IF (MN) (KRIHIGPGRAFYT^{D}PP); cyclic IF (JR-FL) (TCKSIHIGPGRAFYTCG^{D}PP) with the Cys residues disulfide bonded; cyclic HY (JR-FL) (SIHIGPGRAFYTT^{D}PP); cyclic HF (MN) (RIHIGPGRAFYT ^{D}PP); cyclic HF1 (JR-FL) (SIHIGPGRAFYT^{D}PP); cyclic HF2 (JR-FL) (RCSIHIGPGRAFYTCG^{D}PP) with the Cys residues disulfide bonded; cyclic HF3 (JR-FL) (RKSIHIGPGRAFYTTG^{D}PP).
- A cyclic peptide cyclized by a disulfide bond between the two cysteines: cyclic SS (JR-FL) (GNCRKSIHIGPGRAFYTTGCG).

For the peptide ELISA, biotinylated peptides were used. The linear (MN) peptide was biotinylated directly and the cyclic IY (MN) had a PEG08 linker between the mimetic and biotin (see (31)). All synthetic peptides were ≥95% pure by analytical HPLC and gave electrospray MS spectra consistent with the expected masses.

Gp120 proteins

Codon-optimized sequences of strain JR-FL gp120 wild type, $gp120^{D368R}$, $gp120^{I420R}$ and the loop deletion mutants (32, 33) were custom synthesized (GeneArt, Germany), fused to a C-terminal AviTag and cloned into the expression vector CMV/R (34). Recombinant gp120 was produced by transient transfection of HEK 293T Freestyle suspension cells maintained in serum-free medium as described by the manufacturer (Invitrogen, USA). Gp120 was purified from culture supernatants using *Galantus nivalis* lectin resin (Vector Laboratories) as described (35). Retrieved gp120 was mono-biotinylated using BirA as described by the manufacturer (Roche) followed by Superdex200 size exclusion chromatography (GE Healthcare, USA) to derive monomeric gp120. To obtain deglycosylated gp120, 500 µg of gp120 were subjected to 0.15 U α(1-2,3,6)-mannosidase (from jack bean; Europa Bioproducts) for 30 h at 37°C in the presence of protease inhibitors (Complete Mini EDTA-free Protease Inhibitor Cocktail Tablets, Roche Applied Science, Switzerland). The $gp120^{\Delta V1V2}$ construct was created as previously described (21). The ΔV1 and ΔV3 gp120 loop deletions were created with the following linker sequences for deleted loops (HXB2 numbering) ΔV1 linker: C_{131}KDVNAGEIKNC_{157}; ΔV3 linker: C_{296}TGAGHC_{331} .

DARPin selection by ribosome display

Selection with the N2C and N3C DARPin libraries was performed essentially as described (36-38). During ribosome display, a library of DARPins is translated *in vitro* without stop codons, such that a ternary complex of ribosome, mRNA and the still attached, newly translated DARPin is formed. Thereby, the genotype is coupled to the phenotype, allowing the enrichment of DARPins which specifically bind to the respective target (Figure 1A). After each selection round RNA of bound DARPin species is amplified by RT-PCR. The obtained cDNA constitutes a sub-library of the starting library. Selection in absence of target protein is performed in parallel to monitor the extent of background (off-target) cDNA amplification. Comparisons of the quantity of amplified RT-PCR products from target and background selection indicate when enrichment of target-specific DARPins occurs (38). Commonly, several consecutive rounds (>3) of selection

are necessary to derive sub-libraries which are highly enriched for target-specific DARPins. During this process, a balance between maintaining diversity in the library (in order not to lose specific binders with low affinity too early), and stringency of panning (to deselect nonspecific binders) has to be achieved (37). Stringency can be modulated by the intensity of the washing steps, tuning of RT-PCR cycles or depletion of low-affinity binders by addition of excess, non-immobilized target (off-rate selection, see below). Prior to the experiments described here we performed a series of analyses to probe which selection conditions prove best for our different targets (data not shown).

In summary, we found that in order to achieve selection of gp120-specific binders, adding the target in solution rather than immobilized on a surface was key, as previously described for other targets (38). Biotinylated gp120 was bound to streptavidin-coupled magnetic microbeads (MyOne-T1 Dynabeads, Invitrogen, USA) and panning was performed in microtubes. Early rounds of selection (1-3) were kept at low stringency with typically 3-5 short washing steps (5 min), while intensity of washing was increased during subsequent rounds of selection (typically 8-10 wash steps of 10 min each). The most important adaptation to the standard ribosome display protocol previously described (18, 36, 37) was the omission of heparin in the panning buffer. Heparin is usually added during panning to prevent non-specific RNA binding to protein or surfaces. Since gp120 has been described to have multiple heparin binding sites (39) and *in vitro* efficiently interacts with heparin (40), addition of heparin during the selection process could block otherwise accessible sites on gp120. Indeed, we found that addition of heparin completely obliterated gp120-specific DARPin selections (data not shown). Thus, while omission of heparin can lead, to some extent, to false positive binder enrichment as judged by RT-PCR, selections of DARPins in the absence of heparin proved the only possible approach to select gp120-specific DARPins (data not shown).

The number of selection rounds performed in each respective selection is stated in Figure 1B. Sub-libraries for which a target-specific enrichment by RT-PCR was evident were screened for DARPin molecules that specifically bind to gp120 by ELISA (see below). In an alternate screening approach N3C sub-libraries from *Selection II,* which yielded 1st and 2nd generation binders based on ELISA gp120, were re-probed and tested directly for inhibition of HIV by pseudovirus

inhibition screening against strains JR-FL, JR-FLΔV1V2, NL4-3, SHIV SF162 P3 and MuLV on TZM-bl cells (see below).

Detection of DARPin and mAb binding to target protein and peptides by ELISA

20 nM biotinylated gp120 or peptide was immobilized to white high binding microplates (Costar) that had been coated with 60 nM Neutravidin (Pierce) either over-night at 4°C or for 1 h at 37°C, followed by three wash steps with TBST (Tris buffered saline containing 0.1% Tween 20), pH 7.5.

Gp120 proteins were either probed unliganded or triggered with 50 nM sCD4 or CD4M47. Serial dilutions of either purified DARPins or crude extract from DARPin-producing *E. coli* were added in TBSTB (TBST with 0.5% bovine serum albumin), pH 7.5. Unbound material was washed off in TBST and bound DARPins were detected via their His-tag using a mouse anti-polyHistidine–alkaline phosphatase antibody (clone HIS-1; Sigma-Aldrich) and Tropix CDP-star chemiluminescent substrate (Applied Biosystems). MAbs were detected with polyclonal Anti-Human IgG (Fc specific)–Alkaline Phosphatase antibody produced in goat (Sigma-Aldrich). Emission of relative light units was detected on a Dynex Technologies Luminometer. For competition ELISA, gp120 was pretreated with 33 nM of the respective mAb or sCD4 and the subsequent ELISA steps were performed as described above. The percentages of binding in the presence of competitor were determined at saturating DARPin concentrations. For peptide competition ELISA, the DARPin **5m3_D12** or mAbs 447-52D and F425-4e8 were pretreated with indicated concentrations of peptide and the subsequent ELISA steps were performed as described above.

Affinity maturation of DARPin binders by diversification by error-prone PCR and off-rate selection

Sub-libraries of interest were randomized by amplification via error-prone PCR (41), using a dNTP-Mutagenesis Kit (Jena Biosciences) including 50 µM of each of the nucleotide analogs 8-

oxo-dGTP and dPTP in the RT-PCR reaction of ribosome display. For the following panning round a one-to-one mix of error-prone and original PCR product was used as a template for RNA transcription.

To select clones with improved affinity, DARPin-ribosome complexes were first allowed to bind to bead-immobilized target before an 100 to 300-fold molar excess of non-biotinylated gp120 was added as competitor for 1-2 h, followed by 10-15 times 10 min washing steps. DARPin binders with a fast off-rate are captured by the non-biotinylated competitor target in solution and will be lost during subsequent washing steps. In contrast, high-affinity binders with a slow off-rate remain bound to bead-coupled target protein and their mRNA will eventually be preferentially eluted and amplified. This off-rate selection was then followed by a ribosome display round in the absence of competitor and subsequent washing for 10 times 5 min, to re-enrich for specific binders, since the proportion of specific binders is lowered in resulting sub-libraries by the off-rate selection step (42). Afterwards, individual clones from the sub-libraries were screened by binding ELISA as described above.

Molecular phylogenetic analysis by the Maximum Likelihood method

Phylogenetic analyses were conducted in MEGA5 (43) using the Maximum Likelihood method based on the JTT matrix-based model (44). The trees with the highest log likelihood are shown. Initial tree(s) for the heuristic search were obtained automatically employing the BIONJ method with MCL distance matrix.

DARPin purification and analysis

DARPins were produced in *E. coli* XL10 Gold strain via the qQE30 system with Isopropyl β-D-1-thiogalactopyranoside (IPTG) induction and purified using Ni-NTA affinity chromatography as described (45). Obtained DARPin proteins were checked by SEC-MALS (Size Exclusion Chromatography coupled to Multi Angle Light Scattering) for oligomerization state. DARPins that exhibited a tendency to form dimers or higher order aggregates were excluded from

further analysis. For direct neutralization screening, small scale DARPin preparations were purified from 400 µl bacterial lysate in 96-well plates using Ni-NTA-coated magnetic beads (His Mag Sepharose Ni; GE Healthcare, USA).

Virus preparation

Env-pseudotyped viruses were prepared by cotransfection of HEK 293-T cells with plasmids encoding the respective Env genes and the luciferase reporter HIV vector pNLluc-AM as described previously (46). The following envelope genes were used: NL4-3 (47), JR-FL (48), SF162-LS (49), NAB1pre-cl39x, NAB2pre-cl_3, NAB3pre-cl_43, NAB4pre-cl_1, NAB05.1, NAB10pre-cl_2 and NAB12pre-cl_7 (50), ZA110.C.10.14 and V1V2-deleted envs (21). ZA110.C.10.14 (21) was isolated in the frame of the Zurich Primary HIV Infection Study (ZPHI), Division of Infectious Diseases and Hospital Epidemiology supported by the University of Zurich's Clinical research Priority Program (CRPP).

The panel of reference subtype B env clones comprising the following envelopes, 6535.3, AC10.0.29, CAAN5342.A2, PVO.4, QH0692.42, REJO4541.67, RHPA4259.7, SC422661.8, THRO4156.18, TRO.11 and WITO4160.33 (51) was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH.

Neutralization assay using Env-pseudotyped virus

The neutralization activity of DARPins and mAbs was evaluated on TZM-bl cells as described previously (46). Virus input was chosen to yield virus infectivity corresponding to 5,000–20,000 RLU (relative light units) in the absence of inhibitors. The antibody concentrations or reciprocal plasma titers causing 50% reduction in viral infectivity (inhibitory concentration IC_{50} or neutralization titer NT_{50}) were calculated by fitting pooled data from two to three independent experiments to sigmoid dose–response curves (variable slope) using Prism software (GraphPad Software). If 50% inhibition was not achieved at the highest or lowest drug or plasma concentration, a greater than or less than value was recorded.

For competition inhibition assays with V3-petides, mAb and DARPin inhibitor concentrations were chosen that inhibit the env-pseudotyped viruses to 95%. The respective inhibitor at the fixed dose and increasing serial dilutions of the different V3-petides were preincubated for 1 h, then virus was added and the entire mixture incubated for an additional hour before TZM-bl cells were added. Infection was measured after 48 h as described (46). Percent inhibition of DARPin and mAb in presence of competing peptide was calculated in relation to control wells containing the corresponding concentration of the peptide in the absence of inhibitor.

Results

Selection of gp120 specific DARPin molecules

The high structural flexibility of the surface unit gp120 within the viral envelope trimer and its extensive glycosylation are considered major barriers for the selection of neutralizing antibodies, both in natural infection and upon vaccination (8, 10, 52). In *Selection I* (Figure 1B) we sought to define whether DARPins can overcome these barriers and efficiently bind to this flexible, highly glycosylated target. DARPin DNA libraries encoding either two (N2C) or three (N3C) internal randomized ankyrin repeats between an N- and C-terminal capping repeat (15) were subjected to ribosome display selections and panned against recombinant gp120 from strain JR-FL (Figure 1B). Enrichment of specific binders in both the N2C and the N3C libraries was observed based on RT-PCR products after four rounds of ribosome display (data not shown). 285 individual clones of each library were expressed in *Escherichia coli,* crude extracts screened for gp120 binding by ELISA and reactive clones sequenced. None of the N2C clones and only one single clone from the N3C library proved to encode a DARPin which specifically bound to JR-FL gp120 (Figure 1B), indicating that DARPins have a limited capacity in recognizing the wild-type, conformationally flexible gp120 envelope glycoprotein.

DARPin panning on gp120 target proteins with decreased flexibility and degree of glycosylation

This low frequency in selecting gp120-specific DARPin binders prompted us to probe three alternative selection strategies to determine whether modified gp120 proteins with reduced structural flexibility or decreased camouflage by glycosylation prove better selection targets. In a first approach we limited the structural flexibility of gp120 by ligation with the CD4 mimetic mini-protein CD4M47, which arrests gp120 in the CD4-bound conformation (30) (Figure 1B, *Selection II*). In a further strategy, we probed whether removal of the V1V2 domain, the largest and most flexible of the gp120 variable loop domains (53, 54), improves the efficiency of gp120-specific DARPin selection (gp120ΔV1V2, *Selection III*). Additionally, we explored whether de-glycosylated gp120 allows selection of gp120-specific DARPins at higher frequency (Figure 1B, *Selection IV*) as sites vulnerable to neutralizing antibody attack are known to be efficiently shielded by the Env protein's heavy glycosylation (9-11).

After three to five rounds of ribosome display, 95 clones from each sub-library were screened by ELISA for reactivity with the respective target protein used during panning. Gp120 conformationally arrested by ligation with the CD4 mimetic proved the most effective target as already after three rounds of selection, we derived two specific binders from the N2C library and nine specific binders from the N3C library of *Selection II*. In *Selection III* (target: gp120ΔV1V2) and *Selection IV* (target: deglycosylated gp120) the derived N2C and N3C sub-libraries yielded no gp120-specific clones. Although a few N2C clones (3 clones in *Selection III* and 1 clone in *Selection IV*) with low reactivity with gp120 were detected, these had, however, too low affinity ($\geq$10µM) for gp120 to allow further follow-up analysis (data not shown).

Affinity maturation of gp120-specific DARPins

Our primary screen of sub-libraries from the four independent selection strategies yielded in total 12 gp120-specific clones (referred to as 1st generation binders, Figure 1B and Figure 2), which all originated from *Selections I* and *II*. Of note, specific binding of these 1st generation binders in ELISA to the respective gp120 target protein used in the selection was only detected

at high nanomolar to micromolar concentrations, indicative of relatively low affinities of these DARPins for gp120 (Figure 3 and data not shown). In order to select for gp120-specific DARPins with improved affinities, the sub-libraries of all four selection strategies were subjected to additional selection rounds with the aim to diversify specific binders and to specifically select for high-affinity binders using off-rate selection. Clones obtained after this affinity maturation step are referred to as "2nd generation binders" (Figure 1B and Figure 2). Overall the affinity maturation and off-rate selection step proved successful. *Selection I* yielded four improved derivatives of the N3C clone **4a3_H3,** selected in the primary screen, and four additional N2C binders whereas *Selection II* yielded 5 further N3C clones upon affinity maturation.

For *Selections III* and *IV,* which only yielded particularly weak binders in the 1st generation, we retrieved several 2nd generation binders with improved reactivity for the respective target (13 N2C from *Selection III* and 10 N2C binders from *Selection IV*; Figure 1B).

Characterization of gp120-specific 1st and 2nd generation DARPins

Sequence analysis of gp120-specific DARPins obtained in the 1st and 2nd generation of selections revealed that individual clones derived from the same N2C or N3C sub-library were in most cases highly related (Figure 2 and Supplement Figure S1). Based on the sequence analysis and binding efficacy in the initial screen, distinct clones from all selections were chosen for detailed binding and inhibitory activity analysis. To this end, DARPin preparations were purified and checked by SEC-MALS for oligomeric state. DARPins which had a tendency to form dimers or higher order aggregates were excluded from further analysis (data not shown). The 10 N2C DARPin clones from *Selection IV* only differed in a few amino acids (Figure 2). Unfortunately, all clones needed to be excluded from follow up as they formed higher order oligomers. For all other clones from *Selection I*, *II* and *III* selected for follow-up, binding properties of purified DARPins to (i) full length, wild-type (wt) gp120 of strain JR-FL, (ii) JR-FL gp120 liganded to CD4M47, (iii) JR-FL gp120 liganded to soluble CD4, and (iv) V1V2-loop-deleted JR-FL gp120 were determined. Figure 3 depicts gp120 binding data for representative clones of each selection

series. In general, the derived DARPin clones portrayed gp120-binding patterns that were in line with their respective selection strategy.

Selection I

Clones derived from the N2C and N3C sub-libraries of *Selection I* were in both cases closely related (Figure 2). While both N2C and N3C DARPins bound wt gp120 efficiently, this binding was abolished in the presence of sCD4, but remained comparable to wt gp120 when gp120 was complexed with CD4M47 (Figure 3). Of note, the N2C clone **6a2_A1** bound V1V2-deleted gp120 efficiently, while the N3C clone **H3_1B3** failed to do so, indicating that these clones recognize distinct epitopes.

Selection II

S*election II* (CD4M47-liganded gp120) yielded clones with substantially divergent sequences and binding reactivities within the N3C sub-library (Figure 2). CD4 ligation proved to be a decisive determinant for DARPin binding to gp120 amongst S*election II* clones (Figure 3). None of the probed 1st generation clones bound unliganded, wild-type gp120: All N3C clones bound to CD4M47-liganded gp120 but not to CD4M47 alone (Figure 2 and data not shown). In contrast, the N2C binder **3m2_A12** only bound to sCD4-liganded gp120 but none of the other probed targets, even though this binder was selected against CD4M47-liganded gp120. The 2nd generation clone **5m3_D12** was the only *Selection II* DARPin which recognized non-CD4-triggered wild-type gp120, albeit with markedly lower efficacy than when triggered with either sCD4 or CD4M47. In addition **5m3_D12** bound V1V2 deleted gp120 with high affinity.

Selection III

Despite relatively high sequence diversity amongst the 13 N2C clones obtained from *Selection III,* the probed clones displayed largely overlapping binding patterns (Figure 3 and data not shown). Two N2C clones, **AKNF1_10** and **AKNF1_14**, were selected for further analysis. In accordance with their selection against V1V2-deleted gp120 this envelope mutant was

recognized with highest efficiency but the clones also proved to recognize wild-type gp120. CD4 ligation again had a differential effect on the binding capacity of these clones. While CD4M47 ligation had no influence, sCD4 abolished the capacity of these DARPins to interact with gp120 (Figure 3).

Defining binding domains of gp120-specific DARPins

In order to map the binding sites recognized by the gp120-specific DARPins in more detail we studied the capacity of representative members of each selection to bind to a panel of recombinant JR-FL gp120 proteins which included (i) full length wild-type protein, (ii) the CD4 binding site (CD4bs) mutant $gp120^{D368R}$ known to obliterate CD4 and CD4bs antibody binding (55-57), (iii) the coreceptor binding site mutant (CoR-bs) mutant $gp120^{I420R}$ known to eliminate binding of antibodies recognizing the CD4-induced (CD4i) coreceptor binding site (27, 58) as well as mutant gp120 proteins lacking, (iv) the V1 loop ($gp120^{\Delta V1}$), (v) the entire V1V2 domain ($gp120^{\Delta V1V2}$), and (vi) the V3 loop ($gp120^{\Delta V3}$) (Figure 4). Functionality of all gp120 proteins used in these studies was verified by assessing binding of gp120-specific antibodies and CD4 (Figure 4A). The observed binding patterns of the mAbs were in accordance with the respective epitopes of the antibodies with CD4bs-specific mAb b12 and CD4IgG2 lacking the capacity to bind the $gp120^{D368R}$ mutant protein, the V3-loop-specific mAbs failing to bind $gp120^{\Delta V3}$ and the CD4i mAbs 17b and 48d portraying enhanced capacity to bind $gp120^{\Delta V1V2}$ in the non-CD4-triggered conformation. Likewise, binding of CD4i mAbs to $gp120^{I420R}$ was obliterated and strongly reduced for $gp120^{\Delta V3}$, in line with the known contribution of the V3 loop and bridging sheet to the binding domain of these antibodies (27, 58, 59).

With the exception of DARPins from *Selection II,* which were selected against CD4-triggered gp120 and which require this conformation for effective binding, all other DARPins bound efficiently to the CD4bs mutant $gp120^{D368R}$ (Figure 4B). Most strikingly, only *Selection II* DARPin **5m3_D12** bound the CoR-bs mutant $gp120^{I420R}$, indicating that the structural arrest inferred by this mutant is not tolerated by most of the selected DARPins. The V1 loop deletion, in contrast, was tolerated by all groups. Deletion of the entire V1V2 domain had a differential effect.

399 *Selection I* DARPin **H3_1_B3** failed to bind gp120$^{\Delta V1V2}$. Binding of the three *Selection II* DARPins
400 **3m3_A8**, **3m3_B9** and **3m3_F12** was markedly reduced in absence of the V1V2 domain, while
401 the remaining DARPins bound equally well in presence and absence of the V1V2 region. With a
402 single exception (**3m2_A12**) deletion of the V3 loop led to complete or near complete loss of
403 DARPin binding. Thus, both the gp120^{I420R} mutation and V3 loop deletion inferred structural
404 rearrangements in monomeric gp120 which affected recognition by DARPins but were largely
405 tolerated by gp120-specific Abs.

406

407 **Inhibition of HIV entry by gp120-specific DARPins**

408 We next evaluated the efficacy of the DARPins to inhibit entry of envelope-pseudotyped HIV
409 into TZM-bl cells (46, 60), probing a panel of Subtype B tier 1 (including highly neutralization-
410 sensitive V1V2-deleted viruses) and tier 2 viruses (Figure 5). We previously determined the
411 inhibitory capacity of mAbs b12 and 2G12 against the same virus panel (21, 46) and these data
412 are shown in comparison. The majority of DARPin clones portrayed a moderate to potent
413 inhibition of the highly neutralization-sensitive viruses lacking the V1V2 domain and the tier 1
414 virus isolates NL4-3 and SF162 but lacked activity against Tier 2 viruses. Notably, only one clone,
415 **5m3_D12,** portrayed some breadth against tier 2 viruses. The same DARPin also blocked *in vitro*
416 infection of activated macaque PBMCs with SHIV162P3 (data not shown). None of the selected
417 DARPin clones affected the entry of virions carrying the unrelated retroviral murine leukemia
418 virus (MuLV) envelope, confirming that the observed inhibition by DARPins is indeed HIV-
419 specific. Interestingly, DARPins **H3_1B3**, **3m3_B9** and **3m3_F12** blocked wt NL4-3 and wt SF162
420 but lost in neutralizing activity in absence of the V1V2 loop. The latter is in line with our
421 observation that these clones had a strongly reduced capacity to bind gp120$^{\Delta V1V2}$, indicating
422 that they bind to domains on gp120 encompassing the V1V2 region. Similarly, **3m3_A8** showed
423 decreased activity against SF162 but not NL4-3 in the absence of the V1V2 domain. DARPins
424 **AKNF1_10** and **AKNF1_14,** selected against V1V2-deleted JR-FL gp120, neutralized tier 1
425 isolates and V1V2-deleted viruses with high potency (low nanomolar to sub-nanomolar range)
426 but failed to block wild-type tier 2 isolates with one exception, (6535.3 neutralization by
427 **AKNF1_14).** Thus, as can be expected from the panning against V1V2-deleted gp120, DARPins

AKNF1_10 and **AKNF1_14** are limited in their action by V1V2 shielding. They can only access their binding domain when the envelope trimer has adopted an open, tier 1 like conformation for which V1V2 shielding is less efficient or when the V1V2 shielding is artificially removed, but not when the trimer is in the closed conformation adopted by tier 2 viruses. Despite the fact that all DARPins in *Selection I* and *II* were derived from selection against wild-type JR-FL gp120, only DARPin **5m3_D12** blocked entry of wild-type JR-FL efficiently. Since DARPin **5m3_D12** also showed the highest reactivity against tier-2 viruses probed in our panel, we focused for the remaining analysis on this clone.

DARPin 5m3_D12 recognizes the V3 loop

To obtain further information on the epitope recognized by **5m3_D12**, we performed competition binding experiments using a panel of gp120-directed mAbs specific for the gp120 core, C-terminus, CD4bs, CoRbs and the V3 loop and the glycan-dependent mAbs 2G12 and PGT128 (Figure 6). **5m3_D12** binding to gp120 was only competed off by mAbs directed to the V3 tip (447-52D, 1-79). The fact that none of the other DARPins was affected by these mAbs and the failure of **5m3_D12** to bind to V3 loop deleted gp120 (Figure 4) provided strong evidence that **5m3_D12** binds the V3 loop directly. MAb PGT128 which recognizes a glycan dependent motif at the V3 stem region had in contrast to the tip specific mAbs only a marginal influence on **5m3_D12** binding**.** Of note, PGT128 had a more substantial effect on all other DARPins in our panel suggesting that PGT128 binding induces conformational rearrangements that afflict a variety of domains within gp120. In line with the CoRbs mutant analysis and the selection against a CD4 liganded target, the CoRbs epitope directed mAb 17b (and to a lesser extent mAb 48d) affected binding of all DARPins from Selection II. Of note, **5m3_D12** which preferentially binds to CD4 and CD4-M47 triggered gp120, failed to bind to gp120 in presence of mAbs b12 and b6 but recognized gp120 in presence of VRC01 suggesting that the latter mAb induces conformational changes required for DARPin recognition similar to CD4 (Figure 6).

DARPin 5m3_D12 recognizes the V3 loop in a conformation-dependent manner

To define whether **5m3_D12** is indeed specific for the V3 loop we performed direct binding studies and competition binding experiments, using both linear peptides and a panel of V3 loop mimetics, based on structures in the Protein Data Bank (PDB) of V3 loop peptides bound to the mAbs F425-B4e8 (29), 2219 (61), 537-10D (62) and 447-52D (63) (Figure 7A). The peptides in each complex adopt β-hairpin conformations, but differences arise in the orientations of side chains on each face of the hairpin (the register of the hairpin). In the complex with 2219, the I307 and F317 side chains point to the same side of the hairpin and comprise a cross-strand hydrogen-bonding (HB) pair, whereas in the complex with F425-B4e8, I307 and Y318 form the HB pair, and with 537-10D the bond is between H308 and F317 (Figure 7A). In designing the mimetics, the V3 loop sequences were transplanted onto a D-Pro-L-Pro template in order to stabilize the backbone hairpin conformation and fix the hairpin register (64). The pair of residues directly attached to the template should orient their side chains onto the same face of the hairpin, and occupy a HB position. In this way, the four cyclic peptide mimetics referred to here as **HF**, **IY**, **IF** and **HY** (Figure 7A) were designed, which should structurally mimic the V3 peptides complexed with the respective mAbs. The **IY** mimetic was reported earlier (31), whereas the other mimetics studied here were produced and characterized in the same way. NMR structures of the mimetics were determined in aqueous solution, which confirmed the expected hairpin structures (Figures 7A, S2, S3 and Tables S1-S5). All V3 peptides comprised the conserved GPG motif at the tip of the loop as well as parts of the loop stem.

We performed competition binding studies of **5m3_D12** binding to plate-immobilized JR-FL gp120 triggered by CD4M47 in the presence and absence of linear V3 loop peptides and V3 mimetics from strains JR-FL and MN (Figure 7B). Interestingly, **5m3_D12** portrayed a very clear preference for structurally arrested mimetics of the IY register, as these competed effectively with gp120 for DARPin binding, whereas the linear peptides based on the same sequences did not. The mimetic IY (MN^{mut}) which contains a proline to alanine substitution in the conserved GPG motif showed reduced competition (Figure 7B). In contrast to IY mimetics, mimetics with HY and IF register did not compete off **5m3_D12** binding to gp120. The mimetics with HF register and the cyclic SS, a V3 mimetic cyclized by a disulphide bond, showed weak to

moderate competition. Overall, the competition experiments highlighted a strong dependence of **5m3_D12** on a specific V3 conformation, which is in sharp contrast to V3-loop-directed antibodies (31). Interestingly, the mAbs 447-52D (epitope model for the HY mimetic) and F425-B4e8 (epitope model for the IY mimetics) reacted equally well with the linear V3 peptide and their modeled epitope mimetic, but showed a reduced efficacy in binding mimetics with a different register (Figure 7C). We verified these findings by performing direct V3 peptide binding experiments using plate-immobilized biotinylated peptides. DARPin **5m3_D12**, but none of the DARPins from other groups (Figure 8 and data not shown), bound to the mimetic with IY register based on the V3 sequence of the MN virus strain. Binding to the linear V3 peptide from the same strain by the DARPin was approximately hundredfold weaker. In contrast, the V3-specific mAbs 1-79 (25), 19b (65) and 447-52D bound both the linear and structurally arrested peptides at comparative levels with 1-79 even displaying a slight preference for the linear peptide (Figure 8).

Most interestingly, DARPin **5m3_D12** is able to neutralize not only V1V2-deleted JR-FL but also wild-type JR-FL pseudotyped virus, albeit with 2 logs lower potency (Figure 8). This contrasts with V3-directed mAbs 1-79, 19b and 447-52D which are more than 5 logs less potent against wild-type JR-FL, compared to V1V2-deleted JR-FL (Figure 8). Since the V3 loop is potently shielded against antibody attack by the V1V2 domain, most V3 loop antibodies defined to date cannot, or only with low efficacy, neutralize HIV (21). In the absence of V1V2 shielding, however, V3 loop antibodies display remarkable potency and cross reactivity (21).

DARPin **5m3_D12** is thus able to partially circumvent V1V2 shielding. Intriguingly, we observed a notable capacity of DARPin **5m3_D12** in blocking divergent strains of wild-type HIV (Figure 5). However, while our data suggest that **5m3_D12** is able to partially pass by the V1V2 shielding on genetically divergent isolates, the activity of **5m3_D12** against viruses lacking the V1V2 domain was still markedly enhanced, indicating that access of **5m3_D12** is also to some extent restricted by the V1V2 shield.

We next compared the neutralization breadth of **5m3_D12** to V3-specific mAbs 447-52D (28) and 1-79 against subtype B viruses (Figure 9). Both mAbs recognize an epitope in the V3 tip that is expected to at least partially overlap with the **5m3_D12** epitope (Figure 6). DARPin **5m3_D12**

neutralized a subset of strains with considerable potency (Figure 9, symbols in red), but showed no efficacy against the residual strains. Intriguingly, the majority of the strains which were sensitive to **5m3_D12** were also neutralized by mAb 1-79 but not mAb 447-52D, indicating also distinct differences amongst V3 loop antibodies in bypassing V1V2 shielding.

To obtain insights into the binding properties of **5m3_D12** in the context of the native envelope spike during virus entry, we next performed competition inhibition experiments using V3 peptides and mimetics. Neutralization activity of DARPin **5m3_D12** against SF162 and V1V2-deleted JR-FL pseudotyped virus was diminished by addition of V3 IY mimetics in a dose-dependent fashion, whereas the corresponding linear peptides and the mimetic with HF3 register showed no effect (Figure 10). This contrasted with the pattern observed for mAb 1-79, where neutralization activity was more strongly affected by addition of linear V3 peptides compared to V3 mimetics of the IY register. A V3 mimetic with HF register had an intermediate effect. In summary, these experiments underlined that DARPin **5m3_D12** recognizes the V3 loop in a structurally constrained manner.

Discussion

HIV has evolved an array of schemes, foremost heavy glycosylation and conformational masking, to shield vulnerable sites on the envelope trimer from immune recognition and attack by neutralizing antibodies to persevere functionality of the entry complex (8, 10). Engineered entry inhibitors at large suffer from the same limitations as antibodies in accessing relevant domains on the envelope trimer. In the present study we investigated the potential of the DARPin technology in selecting novel gp120-reactive entry inhibitors. Both antibodies and the smaller sized DARPins harbor the ability to recognize their targets with high affinity and specificity, while differing entirely in structure (19). We reasoned that the distinct binding properties of DARPins may allow the selection of novel gp120-reactive molecules with specificities in epitope recognition and inhibitory activity that differ from those found amongst neutralizing antibodies. Antibodies and the N2C (15 kDa) and N3C (18 kDa) type of DARPins employed in the present study cover a comparably sized binding footprint. DARPins recognize

the target by using their surface of α-helices and their row of β-hairpins, resulting in a groove-like binding surface, and thus they prefer to bind to the surface of a globular protein domain, or at least structurally well defined loops (16-18, 66-68). Antibodies can do this, too, but they can also bind to an unstructured terminal peptide or long loop, which adapts to a pocket or groove between the antibody variable domains. Conversely, the complementary determining regions (CDRs) of the antibody, especially a long CDR-H3, can bind within a pocket of the target or at the side of a domain (69). The preference of DARPins for recognizing structural components could be advantageous in selecting HIV envelope-directed inhibitors, particularly, as recent findings of potent broadly active neutralizing antibodies revealed a high prevalence of antibodies recognizing conformational, inter- and intra-protomer loop-spanning binding domains (70, 71). DARPins also have many favorable biophysical properties, such as exceptional stability and high-yield prokaryotic production, which, if broad and potent HIV envelope-specific DARPin inhibitors are found, renders this type of molecule a promising candidate for use as topical microbicide.

In the present study we selected HIV entry-blocking clones from DARPin libraries via ribosome display in which gp120 was presented as panning target in four modifications aiming to investigate the influence of gp120 conformational flexibility and glycosylation on the efficacy of DARPin selection. We found that DARPins have a relatively limited capacity to recognize the wild-type, conformationally flexible gp120 envelope glycoprotein. Of the four gp120 modifications probed, only gp120 liganded with the CD4M47 miniprotein (and thereby structurally arrested) yielded a broader range of DARPin binders recognizing different epitopes. Common to all selected gp120-reactive DARPins was a relatively strong dependence on specific gp120 conformations. This was particularly evident in the mutant gp120 mapping we performed. The CoRbs mutant gp120^{I420R} and the V3 loop deletion variant were, with one exception, each not recognized by the selected DARPins, irrespective of which selection they came from, while gp120-specific antibodies recognized the same mutant proteins unless their epitope was known to be directly affected by the inferred mutations (Figure 4).

Preferences regarding specific gp120 conformations were also apparent amongst DARPins selected against CD4M47-liganded gp120. Only the V3-loop-specific N3C clone **5m3_D12** was

able to bind to non-liganded gp120. All other DARPins derived from this selection series require CD4 ligation (by either sCD4 or the CD4 mini-protein CD4M47) in order to bind to gp120.

The V3-specific DARPin **5m3_D12** was the only DARPin we selected with a notable activity to block Subtype B tier 2 virus infection. Even though this activity was limited to a relatively small number of isolates this is noteworthy, considering that V3-loop-specific antibodies commonly fail to inhibit these types of isolates, as they cannot bypass V1V2 shielding (21). Thus, although **5m3_D12** is to some extent restricted by V1V2 shielding, it interacts with the V3 loop of certain subtype B tier 2 isolates in a way that allows bypassing of the V1V2 shield and effective inhibition of entry. Investigations of the breadth of **5m3_D12** against viruses from other subtypes are currently underway.

In stark contrast to the interaction of V3 loop antibodies with their epitope on V3 **5m3_D12** depends however on a specific conformation of the V3 loop domain in order to recognize the loop and to block infection. The preference of DARPin **5m3_D12** for a mimetic structure with the IY register (31) suggest that the IY β-hairpin structure is close to the predominant conformation the gp120 V3 loop adopts after CD4 binding. Both epitope binding and neutralization activity of **5m3_D12** crucially relied on this V3 loop conformation. Overall, our findings highlight that the preference of DARPins to recognize specific structures may be utilized in further selection strategies to their advantage. Mimetics as employed here bear promise as targets for selections as they could allow to more efficiently steer DARPin selection towards a specific site.

In summary, our study highlights the potential of the DARPin technology in retrieving HIV envelope-reactive binders with unique properties, which harbor entry inhibitory capacity. In particular, the conformation dependence of the DARPin-target interaction may prove of advantage for selecting potent entry inhibitors with novel specificities, including alternatives to quaternary antibodies (72, 73). Once identified, HIV specific DARPin binders with inhibitory activity open multiple avenues for improving their potency. Besides refined affinity maturation, multivalent constructs (of DARPins with one or more specificities) to crosslink subunits within a trimer or neighboring trimers bear promise in boosting efficacy (74).

With the increasing understanding of the architecture of the viral spike (21, 75, 76), possibilities to generate stable soluble trimers which closely resemble the native spike (77, 78) and the means to generate structurally arrested peptide mimetics of gp120 micro-domains (31), a number of tools have become available which bear promise to tailor future DARPin selections to specific domains of interest. As discussed above, based on our current data the latter holds particular promise to improve envelope-specific DARPin identification and to harness the distinctive binding properties of DARPins for HIV inhibitor development.

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Appendix

Table A1. Alignment of V3 sequences of virus isolates probed in Figures 5 and 9. (Amino acid positions numbering according to HXB2).

Table A1		V3 loop sequence
		300 310 320 330
	HIV strain	....|....|....|....|....|....|....|
Sensitive to 5M3_D12	JR-FL	CTRPNNNTRKSIHIGPGRAFYTTGEIIGDIRQAHC
	RHPA4259.7	...H........N........A..K..........
	NAB1pre-cl_39x	....S.......T........A.........K...
	NAB2pre-cl_3	...L.....R..N......W.....V.....K.N.
	NAB10pre-cl_2	............R....S............K...
	NAB12pre-cl_7	............P........A..D..........
Resistant to 5M3_D12	6535.3	............NL.......A..D..........
	AC10.0.29	.I........G............D..........
	CAAN5342.A2	........S...T........A..R......K...
	PVO.4	............S........A..D..........
	QH0692.42	....G..............A..D..........
	REJO4541.67	..............A......A.........K.Y.
	SC422661	..........G.T.....V...-...V.....V..
	THRO4156.18	........S....M...G..FA..R......K.Y.
	TRO.11	.........R..........A..D..........
	WITO4160.33	....G....R..N........A..A......K...
	NAB3pre-cl_43	....................A..A...N......
	NAB4pre-cl_1	.........R..P........A.-D..........
	NAB5pre-cl_1	....S....R..T........A..D......K...
	ZA110_10.14	....S....R.......K....-.G..........

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Figure legends

Figure 1: Selection strategies employed for the generation of gp120-specific DARPins

A) Schematic view of a ribosome display DARPin selection round. B) The four employed DARPin selection strategies and numbers of obtained gp120-specific binders are shown. * 3 clones in *Selection III* and 1 clone in *Selection IV* with borderline reactivity for gp120 were detected which had, however, too low an affinity ($\geq$10µM) for further analysis.

Figure 2: Phylogenetic analysis of gp120-specific DARPin clones derived by different selection approaches.

Sequence relationships of clones obtained from four different selection strategies are shown as phylogenetic trees derived using the Maximum Likelihood method based on the JTT matrix-based model. Data are based on protein sequence alignments of N2C and N3C binders from each respective selection. 1st generation binders are shown in black and 2nd generation binders

in blue. Binders shown with inverse colored boxes were chosen to be analyzed in detail. The trees are drawn to scale, with branch lengths measured in the number of substitutions per site.

Figure 3: DARPin binding: Analysis of specificity to gp120

Overview of binding strength of individual, purified DARPin clones obtained from different selection strategies to gp120 and derivatives as assessed by ELISA. Shown is the half-maximal-binding concentration for each of the gp120-specific DARPin binders, as estimated by ELISA to recombinant gp120 (JR-FL) wild type (green), in complex with CD4 (blue) or CD4M47 (red) or V1V2-loop-deleted $gp120^{JR\text{-}FL}$ (orange). No bar is depicted when no binding to a respective construct was detected up to a concentration of 4 μM. Error bars indicate the standard error of the mean (SEM).

Figure 4: DARPin mapping: Reactivity with mutant gp120 proteins

Binding of gp120-specific mAbs (A) and DARPins (B) to the indicated JR-FL-derived gp120 proteins by ELISA. The ELISA binding signal of DARPins and mAbs to wt gp120 at saturating concentration is set to 100% and mutant gp120 binding is depicted relative to this value. For clones that only bind gp120 in a liganded form, the CD4 ligand was used in each ELISA. Since the CD4 ligands do not bind $gp120^{D368R}$, this construct could not be employed in the liganded form. Heat maps indicate 100% binding like wt in green and no binding in red with a linear gradient through yellow at 50%. (n/a, not applicable; n/d, not done).

Figure 5: HIV Entry inhibition by gp120-specific DARPins

DARPin inhibition of a panel of 21 env pseudotyped viruses of tier 1 (wt and V1V2-deleted), tier 2 and MuLV as control was probed in the TZM-bl assay. The 50% inhibitory concentration (IC_{50}) values are depicted. V1V2-deleted virus envelopes are shown in red, wt tier 1 envelopes in blue, wt tier 2 envelopes in black and the MuLV control in purple. For comparison, inhibition data of the mAbs IgG1-b12 and 2G12 from Rusert et al (21, 46) are depicted. DARPins were tested to a maximal concentration of 5 μM and mAbs to 666 nM.

Figure 6: DARPin mapping: Reactivity with gp120 in the presence of gp120-specific mAbs

Binding of DARPins to wt JR-FL gp120 (CD4-triggered or not, as indicated) in ELISA in the presence of the depicted gp120-specific mAbs was monitored. “None” indicates binding to gp120 in the absence of competitor mAb and is set to 100%. Binding signal in the presence of the competing mAbs is expressed relative to this value. For clones that only bind gp120 when liganded, binding in the presence of the CD4 ligand is set to 100%. Ligands were not employed if they target the same epitope as the used competitor. Heat map indicates 100% binding in absence of competitor in green and complete inhibition (0% binding) binding in red with a linear gradient through yellow at 50%. (n/a, not applicable; n/d, not done).

Figure 7: DARPin 5m3_D12 interacts preferentially with structural mimetics of the V3 loop

(A) Top row: Backbone ribbon representations of linear V3 peptides bound to the mAbs 537-10D, F425-B4e8, 2219 and 447-52D (from PDB files 3GHE, 2QSC, 2B0S and 2ESX). The C(ß) atom of selected side chains are shown with a ball, and the residues shown are color coded. Cross-strand hydrogen-bonded residues are indicated by light blue dotted lines. Middle row: The designed backbone cyclic V3 loop mimetics, with the HF, IY, IF and HY registers. Bottom row: Representative solution NMR structures determined for each mimetic, which confirm for each a stable ß-hairpin backbone conformation and the predicted hairpin registers. The D-Pro-L-Pro template is shown for each at the bottom of the structure in orange color. (B) Binding of DARPin **5m3_D12** to immobilized recombinant JR-FL gp120-liganded with CD4 mimetic CD4M47 was studied by ELISA in the presence of increasing concentrations of linear V3 peptides and V3 mimetics. Data are shown relative to **5m3_D12** binding without competitor peptides. (C) The same analysis as in (B) is shown for the mAbs 447-52D and F425-4e8.

Figure 8: DARPin 5m3_D12 interacts preferentially with structural mimetics of the V3 loop and inhibits entry of tier 2 virus JR-FL

Direct binding (left column) to plate-immobilized linear MN V3 peptide (black squares) and cyclic IY MN V3 mimetic (green dots) in ELISA and entry inhibition in TZM-bl cells (right column) of pseudoviruses JR-FL (blue dots) and JR-FLΔV1V2 (red squares) by DARPin **5m3_D12** (first row), mAb 1-79 (second row), mAb 19b (third row) and mAb 447-52D (fourth row).

Figure 9: Subtype B tier 2 inhibition by DARPin 5m3_D12 and V3-specific mAbs

Neutralization breadth against subtype B tier 2 viruses of DARPin **5m3_D12** and V3-specific monoclonal antibodies 1-79 and 447-52D was probed in the TZM-bl pseudotype virus inhibition assays. IC_{50} values are recorded. Isolates inhibited by DARPin **5m3_D12** are shown in red.

Figure 10: IY V3 loop mimetics efficiently compete with native V3 loop on intact viral spikes for binding to DARPin 5m3_D12

Inhibitory activity in the TZM-bl assay of DARPin **5m3_D12** (bottom row) and mAb 1-79 (top row) against pseudoviruses JR-FLΔV1V2 (left column) and SF162 (right column) was assessed in the presence and absence of competing linear V3 peptides and cyclic V3 mimetics.