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Peptide Triazole Inactivators of HIV-1 Utilize a Conserved Two-Cavity Binding Site at the Junction of the Inner and Outer Domains of Env gp120

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Abstract

We used coordinated mutagenesis, synthetic design, and flexible docking to investigate the structural mechanism of Env gp120 encounter by peptide triazole (PT) inactivators of HIV-1. Prior results demonstrated that the PT class of inhibitors suppresses binding at both CD4 and coreceptor sites on Env and triggers gp120 shedding, leading to cell-independent irreversible virus inactivation. Despite these enticing anti-HIV-1 phenotypes, structural understanding of the PT–gp120 binding mechanism has been incomplete. Here we found that PT engages two inhibitor ring moieties at the junction between the inner and outer domains of the gp120 protein. The results demonstrate how combined occupancy of two gp120 cavities can coordinately suppress both receptor and coreceptor binding and conformationally entrap the protein in a destabilized state. The two-cavity model has common features with small molecule gp120 inhibitor binding sites and provides a guide for further design of peptidomimetic HIV-1 inactivators based on the PT pharmacophore.

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1. Introduction

Despite the availability of combination drug regimens to treat HIV-1, neither a cure nor a vaccine has been found.^{1,2} At the same time, the virus envelope glycoprotein remains a potentially attractive surface-exposed target for inhibiting HIV-1 entry as both a preventive and therapeutic strategy.³ HIV-1 Env protein forms trimeric heterodimers of surface gp120 and transmembrane gp41 protomers and engages cell surface CD4 and coreceptor CCR5 or CXCR4 in a virological synapse required for cell–virus membrane fusion and infection.^{4,5} The Env protein/ receptor protein interactions and consequent conformational rearrangements that are essential to induce cell entry provide multiple targets for antagonism and intervention of disease progression.⁶ Nonetheless, the development of broadly effective Env protein inhibitors has encountered obstacles due to structural flexibility and mutagenic permissiveness of this target and consequent potential for conformational masking and drug resistance.^{7–9} Inhibitors that could disrupt the essential binding and conformational rearrangement program built into the Env protein trimer could represent an effective means to inactivate the virus and block cell entry.

Peptide triazoles (PTs) have previously been found to bind specifically to HIV-1 Env gp120, inhibit cell infection by both R5- and X4-tropic viruses, and exhibit a high degree of breadth against different HIV-1 subtypes.¹⁰ Strikingly, in addition to being potent cell infection inhibitors, this class of compounds induces shedding of gp120, thus inactivating the virus before cell encounter.¹¹ In addition, peptide triazole thiols (PTTs), PTs containing a free sulfhydryl group (SH) on Cys at the C-terminus, cause envelope disruption and p24 capsid protein release from the virus lumen.^{11,12} Both gp120 shedding and lytic inactivation by PTs appear to occur by taking advantage of the intrinsic metastability of the Env protein that the virus normally uses to promote virus–cell fusion.

At the molecular level, PT binding inhibits gp120 interactions with both CD4 and the coreceptor surrogate 17b.^{13–15} Dual receptor site antagonism has been interpreted to be a consequence of gp120 conformational perturbation, which in turn may explain gp120 shedding and lytic deformation of the virus induced by PT and PTT inhibitors. The phenotype of broadly effective HIV-1 inactivation makes PTs appealing as a lead class for

HIV-1 antagonists. Nonetheless, the nature of these compounds as peptides presents a substantial barrier to antagonist development due to the potential of proteolytic degradation and consequent short half-life. An improved structural understanding of the PT–gp120 encounter complex would help define approaches to design more protease-resistant variants.

In the present study, we sought to advance the structural definition of PT–gp120 binding using the sequence-minimized PT denoted peptide 1, a six-residue peptide containing the Ile-X- Trp, (IXW) pharmacophore, where X is a ferrocenyl-triazolePro amino acid residue.¹⁶ We combined recombinant mutational variation of YU-2 gp120, synthetic variation of peptide 1, and flexible docking simulations of PT–gp120 complexes to define structural elements that are critical for function. This work has led to the conclusion that PTs utilize two distinct and neighboring hydrophobic cavities in gp120 to enable Env binding. The two-cavity model provides a guide for optimizing gp120 contacts with peptidomimetic derivatives of the inhibitor and at the same time an improved explanation of the structural mechanism the PTs use to effect gp120 conformational entrapment resulting in disruption of Env trimers and consequent virus inactivation.

2. Results

HIV-1 gp120 Mutational Variations

Previous single-site mutagenesis and molecular modeling studies in the context of the crystallographic structure of HIV-1 gp120 have helped define a combined binding and conformational change model for peptide triazole interaction and consequent function.^{17–20} The previous findings argued that the hydrophobic Ile-X-Trp pharmacophore of PT interacts with residues lining the highly conserved CD4 Phe43 site to cause conformational alterations in gp120 that suppress formation of the bridging sheet used for coreceptor binding. The conformational effect induced by PT in gp120 correlates with the observation that all PT variants examined so far cause gp120 shedding from the virus envelope spike.¹¹ PTs have been found previously to exert their conformational impact through interactions with distinct hotspots in gp120.²⁰ Nonetheless, prior modeling had implicated an interaction subsite involving inner domain residues,¹⁸ while the previous mutagenic analysis²⁰ had not identified this site. In addition, the prior modeling suggested possible hydrogen bonding of PT pharmacophore components, while prior mutagenesis did not offer a test for such stabilizing noncovalent interactions. To obtain a more unified understanding of PT interaction, we here expanded investigation of the gp120 residues involved by mutations in monomeric Env gp120 HIV-1 YU-2 protein. As shown in Figure 1, residues examined included two neighboring regions defined as Site 1, the Phe43 cavity in the outer domain of gp120 (Thr257 and Ser375), and Site 2, in the inner domain (Ile109, Trp112, Phe210, and, in the β 20/21 turn, Met426).

Soluble monomeric Env gp120 mutant proteins were expressed and purified (Methods, Figure S1, Supporting Information), and their binding to soluble CD4 and coreceptor surrogate 17b was validated by SPR. Increasing concentrations of different gp120 mutants (0 nM to 500 nM) were passed over medium density (1000 RU) CD4 and 17b surfaces, and sensorgrams were obtained as shown in Figure 2 for wt gp120 and representative mutants S375A, S375W, and W112A. Binding affinities were calculated using BIAeval 3.0 and

fitted to a 1:1 binding model; binding data measured for all mutants are given in Table 1. The results reveal that all mutants examined retain substantial CD4 binding activities, thus enabling competition of CD4 binding to be used to assess PT variant binding in this work. Importantly, in site 1, the cavity-filling mutation S375W increases the 17b binding affinity as compared to S375A and S375H mutant, consistent with the prior observation that this mutation stabilizes the CD4 bound state with consequent improved binding to CD4-induced antibodies such as 17b.²¹ For site 2 mutants, W112A and F210A have strikingly suppressed 17b binding affinities, and M426A has significantly perturbed 17b binding. The residues Trp112 and Phe210 are known to be involved in the formation of bridging sheet,^{19,22} a prerequisite for 17b binding. Nonetheless, as noted above, all of these gp120 mutants bind well to sCD4.

Interactions of Site 1 Mutants with Minimized Peptides 1–4

Most of our past investigations of the PT binding footprint in gp120 have used longer peptides such as 12p1, HNG-156, and KR-21 that contain the ferrocenyl triazole group for maximized gp120 binding affinity.^{13,20} Here, to focus the analysis on interactions of gp120 with the PT active core, we evaluated mutant binding with the sequence-minimized peptide Ile-Asn-Asn-Ile-X-Trp, where X is a ferrocenyl-triazolePro amino acid residue,¹⁶ denoted peptide 1. As shown in Figure S2, Supporting Information, this six-residue peptide triazole gp120 antagonist functions as an entry inhibitor of HIV-1. This minimized peptide contains a stereochemically specific hydrophobic Ile-triazolePro-Trp pharmacophore cluster, with a short N-terminal peptide extension, to provide groups for main-chain and side-chain interactions essential for gp120 binding and simultaneous suppression of envelope protein binding sites for both CD4 and coreceptor. Furthermore, since previous molecular modeling studies and peptide truncation studies have highlighted the importance of the Trp residue in the pharmacophore cluster for possible binding inside the Phe43 cavity, we investigated several Trp variants of peptide 1 in the present study (Figure 3 and Table 2). These variants were made to test possible modes of interaction of the Trp side chain with Ser375 and Thr257 in site 1.

We used SPR competition assays to measure PT binding, through effects of peptide 1 and its derivatives on interactions of monomeric wt gp120 YU-2 gp120 with chip-immobilized sCD4 and 17b. Mean inhibitory concentrations (IC₅₀) were calculated after fitting the data to a logistic function (Origin Lab) and are reported in Figure 3 left panel and Table 2. Compared to the strong inhibitory activity of peptide 1, replacing the peptide 1 Trp residue with Tyr in peptide 2 resulted in reduced but nonetheless observable competition of CD4 binding. Similar decreased function compared to peptide 1 was observed for 17b competition (data not shown). Other analogues, with more indole-like side chains, showed less loss of function than peptide 2. The PT analogues also showed similar degrees of function, compared to peptide 1, in assays of inhibition of cell infection with HIV-1 Bal.01 pseudo typed virus (Figure 3 right panel). The functionalities of PT analogues with wt gp120 by both competition SPR and cell infection inhibition set the stage for using these peptides to evaluate the effects of PT analogues with mutations in gp120.

With wt gp120 results as a background, we examined the effects of single-site gp120 mutations on binding to peptide 1 and its Trp variants using competition assays. To test multiple underlying interactions that might be involved in the interaction of PT with gp120 as predicted by molecular modeling studies, different side-chain substitutions were made in residues Ser375 and Thr257. These variations were made to evaluate the effects of Phe43 cavity filling, by S375W mutation, and the effect of altering the electrostatic and other noncovalent interactions of the cavity, by S375A, S375H, T257A, T257V, and T257R mutations. Representative sensorgrams are shown in Figure 4 and Figure S3, Supporting Information, while all SPR-based, dose–response inhibition results are shown in Figure S4, Supporting Information. As summarized in Table 3, we found that peptide 1 and most of the PT variants do not inhibit binding of sCD4 to mutant S375W. This was also observed (data not shown) for the nontriazole parent peptide 12p1¹³ (that also contains the Trp side chain in the pharmacophore). In contrast, competition was observed with the more minimally altered S375A mutant of gp120 (Table 3). For the S375H mutant, representing a mutation found in Clade AE strains of HIV-1, such as circulating recombinant form AE (CRF01_AE) and 93TH057, that are fully resistant to PT (data not shown), the soluble HIV-1 YU-2 S375H mutant was also resistant to peptide 1, peptide 3, and peptide 4. However, intriguingly, peptide 2 was able to partially overcome resistance to S375H (Figure 4 and Table 3). A similar escape of peptide 2 from gp120 mutational resistance was observed with the gp120 mutant T257R. As noted above, the latter was made to evaluate the effect of the electrostatic nature of Phe43 cavity on PT binding. The T257R mutation endowed gp120 with resistance to peptide 1 and most other PT variants tested. Overall, the above observations are consistent with the view that PTs are sensitive to both cavity filling (by S375W) and perturbation of the electrostatic nature of Phe43 cavity (by T257R) but that the impact of mutation in this site 1 region can be overcome, at least to a limited degree, by modification of the PT Trp position side chain as exemplified by peptide 2.

To test the prediction¹⁸ for a possible role of hydrogen bonding between the indole hydrogen of PT Trp (residue 6 in peptide 1) and residue Ser375 of gp120, the PT variant peptide 4 was synthesized in which the indole was replaced with N-methylated indole. Comparative isothermal titration calorimetry analyses of peptide 4 binding to wt gp120 vs gp120 alanine mutants S375A and T257A were used to investigate possible contribution of binding enthalpy due to hydrogen bond interaction.^{23–25} The ITC dose–responses obtained are shown in Figure S5, Supporting Information; comparative analysis of calculated thermodynamic parameters, namely binding constants (K_D), enthalpy (ΔH) and entropy change ($-\Delta T \Delta S$), and number of binding sites (n), are shown in Table 4. The thermodynamic analyses indicated that binding of gp120 to PT was in the 1:1 stoichiometric ratio for all cases measured. The decreased binding function of ~ 1.9 kcal/mol of peptide 4 compared to peptide 1 (Table 4) with wt gp120 however is consistent with a possible role for PT Trp indole hydrogen atom for gp120 binding. We compared interaction thermodynamics with several mutants to further assess the possibility. Peptide 1 and peptide 4 binding were evaluated for wt, T257A, and S375A gp120, since the mutations in the latter two cases would be expected to remove hydrogen bonding interaction possibilities compared to wt. The largest differences in ΔH were observed with the comparative pairs of (1) wt gp120 binding to peptide 1 vs peptide 4 and (2) peptide 1 binding to wt gp120 vs S375A. In

contrast, these same comparisons showed little change in $T \Delta S$. Hence, the affinity differences observed appeared more related to enthalpy (binding) than entropy (conformational) effects. While it is appealing to assign the enthalpy differences to loss of hydrogen bonding normally occurring in the wt gp120-peptide 1 interaction, the possible role of spatial effects cannot be discounted.

Functional Properties of HIV-1 gp120 Mutants of Site 2 (inner domain) of gp120

Since previous molecular modeling findings suggested the interaction of the aryl-triazole moiety of PT with residues at the interface of inner and outer domain encompassing the $\beta 20$ – $\beta 21$ loop, PT interactions were measured with gp120 containing mutations directed at this region, which we define as site 2. Alanine mutants W112A, I109A, F210A, and M426A in an HIV-1 YU-2 gp120 background were examined. Representative competitive SPR results for the effect of the mutation W112A on inhibition potential of PTs are shown in Figure 5 and competition potencies compared in Table 5. For peptide 1, little change was observed for F210A vs wt gp120. On the other hand, substantial decreased binding potency was observed with $\beta 20/21$ strand M426A and the inner domain $\alpha 1$ helix I109A, and strong resistance was observed with W112A as shown in Figure S6, Supporting Information, and Table 5. A similar pattern of mutational impact was found with peptide 3 potency. For peptide 2, W112A resistance was complete, while little decreased potency (vs peptide 1) was observed for M426A and I109A.

Flexible Docking of PT with gp120

The results obtained by coordinated mutational and synthetic variation analyses define residues in both sites 1 and 2 of Env gp120 important for PT binding. We sought to use computational simulation to assess PT binding modes possible in these combined sites and, in particular, to further test PT occupancy of the two cavities identified by mutagenic analysis. Prior modeling of PT–gp120 interaction had been carried out with seven-residue nonferrocene PTs to allow the use of MD simulation.¹⁸ In addition, the prior analyses used as the target protein the bridging sheet naïve crystal structure of gp120 (F105 bound, PDB code 3HI1¹⁷), based on the fact that PTs inhibit binding of the coreceptor binding surrogate 17b to gp120 putatively by preventing the folding of the bridging sheet.¹⁸ This model showed that PT can utilize this open-form conformation by insertion of the pharmacophore moieties (Ile-X-Trp, where X is the aryl-triazole-Pro), similar to the way that F105 antibody¹⁷ occupies the cavity by inserting two aromatic rings (phenyl and a hydroxyl phenyl rings). The prior PT–gp120 model showed that the phenyl-triazole can occupy a cavity between the bridging sheet $\beta 20/21$ loop of the outer domain and $\alpha 1$ helix of the inner domain. Since replacing the phenyl with ferrocenyl resulted in an improvement in gp120 inhibitory activities,²⁶ we sought in the current study to simulate interaction with peptide 1 and other six-residue ferrocenyl triazole peptides. However, starting with the same docked model as before¹⁸ resulted in no in silico PT binding, and all predicted poses had unfavorable positive binding energies (data not shown). Upon inspection of the protein surface of the model, we found that the residue Trp112 hindered the insertion of the bulky ferrocene ring yet allowed insertion of the smaller phenyl ring of nonferrocene peptides. We therefore used a flexible docking model,²⁷ where the gp120 Trp112 residue was allowed to move flexibly during the simulation, to avoid such clashes with the peptide and allow

evaluation of how the ferrocenyl moiety could engage the gp120 surface. We used this flexible docking approach to simulate interactions of the four active peptide triazoles evaluated above, peptides 1–4. During examination of the predicted PT binding modes, we took into consideration the hot spots that were identified to be critical for PT binding from mutational analyses²⁰ and above findings.

Exhaustive docking simulations identified similar binding poses, involving both cavities 1 and 2, for all four of the modeled peptide triazoles with comparable calculated binding energies. Table 6 summarizes the interacting residues observed in docking simulations and the calculated G_{binding} for each peptide. The binding energies were computed blindly, as there is no computational tool that enables the use of the actual binding data in this computation. While it is tempting to compare these predicted G_{binding} values to the binding potencies determined by SPR, it is important to emphasize that the binding activities of peptides 1–4 shown in Tables 2, 3, and 5 represent values obtained from competition experiments and do not represent direct binding affinities. The reason for the difference in

G_{binding} values for peptide 2 by docking vs IC_{50} by SPR may be related to the differences in the binding assessments used. Nonetheless, and most importantly, the simulations showed the ability of peptide triazoles to utilize two conserved sites in gp120 (Figure 6), namely the Phe43 cavity (site 1, shown in yellow) lined by Thr257, Ser375, and Phe382 side chains, and a second site (site 2, shown in orange), a pocket located in the inner domain of gp120 glycoproteins, and gated by the $\alpha 1$ helix Trp112 residue. The six-residue truncated peptide triazoles investigated here occupied site 1 by placing the aromatic side chain of the peptide residue 6 proximal to Thr257, Ser375, and Phe382. The orientation of residue 6 aromatic rings was stabilized by possible interactions with Ser375, Thr257, and/or Tyr384 (Table 6). The electrostatic nature of site 1, generated mainly by the OH groups of Ser256, Thr257, and Ser375 side chains, induced a preference for placement of the PT residue 6 side chains in this site rather than the bulky hydrophobic ferrocenyl moiety. Most of the lower energy poses were predicted to adopt this specific orientation of PT residue 6 side chains (Figure 6) in site 1. In some of the higher binding energy (positive G_{binding}) poses, the ferrocenyl ring occupied site 1 (data not shown); however, such poses were not considered during the pose examination filtration process due to the unfavorable energies observed.

The second site observed by modeling for PT interaction was the inner domain hydrophobic cavity gated by the Trp112 indole side chain. When the Trp112 indole was allowed to move flexibly during the search for stable binding poses, the inner domain hydrophobic cavity was accessed in most of the lower energy binding modes by the hydrophobic bulky ferrocene-triazole moieties of all of the PTs examined. In these modes, the Trp112 indole stabilized conformation through direct interactions so as to keep the ferrocene buried inside the hydrophobic cavity, resulting in stable low energy complexes with all of the docked peptides. Moreover, the buried ferrocenyl moiety could be stabilized by several hydrophobic interactions with residues such as Ile108, Ile109, and/or Leu111 (possible interactions listed in Table 6).

In the two-site poses derived and shown in Figure 6, the 1,4-regioisomer triazole (linear triazole) was found to be important to retain the ferrocenyl moiety inside the site 2 hydrophobic cavity relative to site 1. We also sought to include the 1,5-regioisomer triazole

(bent triazole) in this study. A ruthenium catalyst was used to access the 1,5-triazole during the click reaction of the alkyne with the azido proline of PT (Scheme 1). From SPR competition (data not shown), the 1,5-triazole peptide (peptide 6) was found to be inactive, with IC_{50} values greater than 20 μ M range, reflecting $\sim$ 200-fold weaker binding than peptide 1. Attempts to simulate this inactive peptide using flexible docking resulted in unstable high binding energy complexes with the ferrocene ring exposed to the solvent (data not shown).

Refinement of the Two-Cavity Model

We sought to obtain an evolved PT–gp120 two-site engagement model using a non-ferrocene PT to enable the use of more accurate docking algorithms and software packages that do not easily handle simulation with a metallocene moiety, and to evaluate similar behavior of other aryl groups on the triazole ring. To do this, we chose the peptide 1 analogue peptide 5, in which the ferrocenyl was replaced by a phenyl ethyl moiety. The phenyl ethyl analogue of PT has previously been found to retain substantial binding activity compared to ferrocenyl PT. Peptide 5 itself exhibited gp120-binding and cell infection inhibition activities similar to those of peptide 1, with SPR-derived IC_{50} values for competition of CD4 binding to gp120 and cell infection inhibition of 148 ± 12 nM and 4.3 ± 0.5 μ M, respectively.

Peptide 5 was docked, using the peptide docking algorithm Glide Peptide²⁸ developed by Schrödinger, to the same starting gp120 structure derived in the flexible docking experiments (above). The gp120 W112 side-chain indole was set as flexible during the simulations (constraint-free docking). Different binding modes were generated from this simulation. The most stable predicted binding mode (Figure 7, calculated $G_{\text{binding}} = -32.7$ kcal/mol) showed burial of the phenyl ethyl triazole moiety deeply in the site 2 inner domain cavity, whereas the peptide indole ring was found to occupy site 1 proximal to Thr257 and Ser375. The peptide 5–gp120 complex was energy-minimized and subjected to binding energy calculation to represent a more refined model of PT–gp120 interaction. The refined PT–gp120 model developed for peptide peptide 5 (Figure 7) showed the peptide in a more relaxed state than in the model developed with peptide 1 (Figure 6). In this PT–gp120 model, the Trp112 side chain was oriented perpendicular to the β 20/21 loop of the bridging sheet, providing access to the inner domain hidden cavity. Intriguingly, this specific configuration of Trp112 also has been observed in the F105 bound gp120 crystal structure.¹⁷ In the latter, the F105 inserts phenylalanyl and tyrosyl moieties¹⁷ inside the open conformation, orienting the phenylalanine phenyl ring toward Trp112. Comparing this Trp112 orientation with that of Trp112 in the CD4 bound state, the Trp112 in the latter case is more buried in the inner domain cavity to accommodate the folded β 20/21 loop Trp427 residue.^{19,29}

3. Discussion

The primary goal of this study was to expand our understanding of the nature of stabilizing interactions and likely binding poses in the encounter of the peptide triazole class of HIV-1 inactivators with the virus envelope protein gp120. We found that the activities of PTs in SPR-based assays are highly sensitive to alterations in two conserved and neighboring

hotspot sites on the gp120 glycoprotein. The first hotspot (site 1) comprises a pocket lined by Thr257/Ser375 residues of the gp120 at the interface between the outer and inner domains, whereas the second hotspot (site 2) is located mostly at the inner domain cavity gated by gp120 α 1 helix Trp112 side-chain indole. The electrostatic environment of site 1 is determined largely by the side chains of residues Thr256, Thr257, and Ser375 lining this cavity. Reflective of the importance of this environment, we found that the T257R mutation abolished activities of most of the PTs evaluated (Table 2). The exception was peptide 2, which contains a phenolic ring of residue 6 and retains some activity against T257R. This latter result argues that steric fit also is important in site 1, with the smaller ring size of the Tyr 6 variant enabling greater activity than the other PTs, the latter of which have bigger indole-like aromatic structures in residue 6 side chains. The varying sensitivity of gp120 mutants in site 1 to the different PT residue 6 variants argues that PT residue 6 side chains reside in proximity to the residues lining site 1. Moreover, several stabilizing interactions between site 1 residues and PT residue 6 side chains were predicted by modeling (Table 6), including Ser375, Thr257, and/or Phe382, Tyr384 (Figures 6, 7). The second hotspot, site 2, comprises a hydrophobic pocket that opens at the interface between the inner and outer domain and further extends into the inner domain. The site 2 pocket is uncovered when the Trp112 side chain moves and can be accessed by hydrophobic ligands. The gp120 mutant W112A exhibited greatly decreased PT binding activity except for peptide 3, which showed a significant though weak tolerance for W112A. The effect of W112A mutation was also observed with the nontriazole parent peptide 12p1 (data not shown), arguing that Trp112 can interact directly with other structural elements besides the triazole moiety. Nonetheless, the large-scale resistance of W112A to PTs suggests that the Trp112 side-chain indole is quite important for PT function at site 2. Modeling analysis suggests that the Trp112 indole side chain can stack not only with the PT Pro residue but also with the triazole moiety and possibly the ferrocene ring (Table 6, Figures 6 and 7). Concerning other aspects of site 2, substantially decreased PT binding potency was also observed with the β 20/21 turn mutant M426A and the α 1 helix mutant I109A. Molecular modeling supported these findings by showing the ability of Met426 and Ile109 to stack with the aryl-triazole on the Pro moiety of PTs. The inner domain α 1 helix Trp112/ Ile109 cluster, along with the β 20/21 turn Met426, plays a major role in restructuring the gp120 bridging sheet during the Env transformations occurring in viral infection.¹⁹ Engaging the site 2 region on the protein by PT aryl-triazolePro moieties can explain how PTs disrupt the bridging sheet formation required for coreceptor binding.

Several observations lead to the conclusion that two-cavity PT encounter almost certainly has a strong impact on gp120 conformation. That mutations in either site 1 or site 2 cause loss of PT binding argues that PT function requires access to both of these sites. PT-interacting residues lining site 1 also line the Phe43 cavity used for CD4 binding¹⁹ to gp120. However, unlike CD4, PTs do not stabilize or induce a CD4-bound gp120 conformational state, as deduced from inhibition of 17b antibody binding, as opposed to enhancement of 17b binding by CD4. While gp120 bridging sheet formation induced by CD4 binding leads to limited access to Trp112 by the folded β 20/21 turn,¹⁹ PT requires access to Trp112 and hence likely requires a more open conformation (unfolded bridging sheet) than the CD4 bound state. Such an open conformation has been observed in gp120 complexes with some

neutralizing antibodies such as F105¹⁷ as well as some recent crystallized³⁰ and modeled³¹ HIV-1 Env structures. In the F105-bound open structure¹⁷ and the modeled unliganded Env,³¹ some residues of gp120 become more solvent accessible than in the CD4-bound¹⁹ state. These residues include Thr257 and Ser375 from site 1 and Trp112 from site 2, the combination of residues that we found in the current work to be important for PT binding. In the F105-bound gp120 structure, an F105 Tyr phenol ring inserts inside site 1, stabilized by an interaction with Ser375, through the OH group, and by a π - π interaction with Phe382.¹⁷ We envision that PTs can utilize site 1 in a similar manner, in this case by the aromatic side chains of PT residue 6. From the above, we hypothesize that PT-gp120 engagement uses two rings from the PT pharmacophore Ile-X-Trp: (1) the aromatic side chain of residue 6 in the site 1 cavity close to the Ser375/Thr257 site 1 and (2) the aryl-triazoloproline in the inner domain site 2 cavity close to Trp112. It is worth noting that the inner domain cavity exists in most gp120 forms, including the recent HIV-1 Env trimer structures,³⁰ arguing that it could be utilized generally as a conserved target by gp120 inhibitors.³¹ One difference in gp120 site occupancy between F105 and PTs is the extra aryl-triazolo moiety present on the Pro residue on PTs that further penetrates the inner domain hydrophobic cavity, gated by the Trp112 side chain. Because we observed a large-scale loss of PT binding by W112A mutation, we evaluated binding of the W112A mutant gp120 to F105. We found that the W112A mutant can still bind to F105 with high affinity compared to the wild type gp120 (24 nM for the wild type gp120 versus 83 nM for W112A mutant), thus arguing that W112A can still adopt the open conformational state required for F105 and other ligands. Since F105 can bind to W112A, while PT does not bind to this mutant, it can be surmised that PTs likely utilize direct interactions with the accessible Trp112 in site 2 of the PT-gp120 complex.

The conclusion that PTs can recognize and/or induce an inactive open conformation of gp120 in Env trimeric spikes on the virus surface fits with a growing view³¹ that gp120 can adopt multiple conformations in unliganded Env spike. Of particular note, single molecule fluorescence resonance energy transfer (smFRET)³² has recently shown that the trimeric envelope is dynamic and can exist in three definable prefusion states, namely an unliganded ground state, an intermediate activated state, and fully activated state.³² We hypothesize that when PT interacts with unliganded gp120 at the interface between the outer and inner domains through sites 1 and 2, two effects occur that ultimately lead to gp120 shedding. First, PT interacts with the site 1 unformed CD4 binding site, inhibiting CD4 binding. In turn, PT interferes with the reorganization of the V1/V2, V3, and bridging sheet regions (β 2- β 3- β 21- β 20 strands)^{19,30} of the Env trimer through interactions with the inner domain site 2. Engagement of PT with trimer may occur through induction or stabilization of an as-yet uncharacterized transition state that does not proceed to the CD4/coreceptor bound^{31,32} intermediate activated state. The inner domain layer 2 α 1 helix has been shown to be pivotal in translating rearrangements in gp120 to gp41.³³⁻³⁵ Moreover, gp41 HR1 and fusion peptide proximal region (FPPR) are in close proximity to the CD4 binding regions in the trimeric structure.³⁰ Hypothetical direct interactions of PT with the accessible site 1 and the inner domain layer 2 α 1 helix Trp112 in site 2 could explain how peptide triazole can disrupt the interface between gp120 and gp41, leading to the shedding¹¹ of gp120 from the virion and consequent irreversible inactivation.

The action of PT dual antagonists to stabilize an open state of the gp120, in which the CD4 Phe43 cavity site 1 and the inner domain cavity site 2 can be accessed by the PT pharmacophore, can be compared with modes of action of other gp120 inhibitors. Small molecule CD4 mimics³⁶ occupy the Phe43 cavity in site 1, as shown in several crystal structures.^{29,37} CD4 mimics have been found to cause opening of the trimer apex by reorganizing V1/ V2, V3 and bridging sheet regions,³⁸ stabilizing the trimer in a CD4/coreceptor bound state.³² In contrast, BMS class compounds,³⁹ which have been reported to target an unliganded state of gp120,^{31,38} act differently and were found to prime the Env trimer to prefusion without opening the trimer crown.³⁸ This priming action of BMS compounds was attributed mainly to interaction with the inner domain layer 2 α 1 helix.³⁸ Parker et al.⁴⁰ reported a putative site on gp120 for BMS compounds, that is composed of a hydrophobic cavity in the inner domain gated by Trp112 and Phe382 and that could be accessible during the spontaneous conformational sampling of the Env trimer.⁴⁰ Interestingly, Langley et al.³¹ recently published a hypothetical mode of action for the BMS inhibitors by targeting a hydrophobic cleft in the unliganded Env gp120 where CD4 binding site is not formed (misfolded bridging sheet).³¹ In the current work, we also found that this exposed hydrophobic region appears to be targeted by the PT components. 18A is a recently discovered, broadly acting inhibitor of HIV-1 Env which also might target an unliganded state of the Env trimer and disrupt the quaternary structure at the trimer apex, by inhibiting the CD4-induced rearrangement of the V1/V2 region.⁴¹ The target site of 18A is not yet known. However, gp120 mutations in Ile109 and Met426 residues greatly affect susceptibility to 18A,⁴¹ arguing that it also might target a hydrophobic site between the β 21/20 loop and the inner domain. On the basis of those facts, we hypothesize that the binding site of PTs overlap with those of CD4 mimics³⁶ in the site 1 region and possibly with BMS compounds and 18A binding in the site 2 region.

Despite the conserved nature of the two-cavity interface used for PT encounter, viral resistance is a challenge for PTs similarly as for other inhibitors. Some gp120 site 1 mutants, such as S375W⁴² and S375H,⁷ are resistant to CD4 mimics and BMS compounds.⁷ For PTs, a similar resistance profile was observed (Tables 3 and 5). However, a hint that PTs could potentially overcome this resistance was observed in the current study by the measurable activity against S375H with the PT peptide 2 (Table 3). This could be attributed to the smaller residue 6 side-chain ring. Interestingly, peptide 2 also showed some activity against T257R. One possible explanation for this finding is that peptide 2 may be able to partially engage site 1 due to the smaller side-chain ring vs the indole in peptide 1. This permissiveness is suggested by the peptide 2 gp120 docking model (Figure 6), in which the tyrosyl side chain makes contacts with residues lining site 1 such as Glu370, Phe382, and Tyr384 (Table 6). This partial engagement would lead to loss of crucial contacts with some residues such as Thr275 and Ser375. Absence of contact with the latter residues in turn might be responsible for the insensitivity of peptide 2 toward gp120 mutants S375A, S375H, T257A, and T257R. At the same time, this lack of contact was accompanied by greater loss in the peptide gp120-antagonizing activity compared to peptides 1, 3, and 4. Therefore, caution will be needed to tune the activity and mutation resistance profile through changes in the residue 6 side chain. In site 2, the effect of site 1 occupancy by residue 6 side chain was also observed, with peptide 2 being insensitive to site 2 mutations except for W112A

(Table 5). The different positioning of the tyrosine moiety of peptide 2 may be accompanied by a slightly different positioning of the aryl ferrocene moiety in site 2. This different behavior of peptide 2 will encourage synthetic variations at residue 6 side chain. The task will be achieving sufficient contact with important residues to maintain the potency and at the same time improve the mutation resistance profile. Substitutions at the Phe ring of peptide 2 residue 6 side chain at the *ortho*, *meta*, and *para* positions with different chemical environments, including aliphatic and aromatic groups, will be a possible strategy to achieve that aim.

In site 2, peptide 3 showed the least loss (relative to wt gp120) in activity against W112A (Table 5). Peptide 3 showed high potency in inhibition infection activity (Table 2), which may reflect higher affinity to the target site. A possible explanation is that the peptide 3 benzothiophene moiety is more lipophilic than the other PT side chains (see ClogP values in Table 2). Such a factor may be responsible for additional or stronger hydrophobic interactions that overcome loss of the important Trp112 interaction. The findings made here of partial regain of function with peptide 2 and peptide 3 with site 1 and 2 mutations merit further investigations to search for mutation-resistant PTs.

The two-site PT–gp120 binding model derived in this work (Figure 7) opens up opportunities for developing more drug-like PT-derived inhibitors via structural minimization and optimization of interactions with the target sites. A major focus going forward is to develop a class of stable peptidomimetics. The findings presented in this work can help identify pathways for trimming or replacing the peptide side chains and/or main chain to maximize the interactions with the identified target protein hot spots. The findings also can guide designs for cyclizing PTs into more locked active conformations. The possible overlap of PT sites with those of other inhibitors also opens up the potential to identify chemical moieties that could be incorporated into PT compositions to optimize the interactions with the target sites on the gp120 protein.

4. Experimental Section

Reagents

Escherichia coli strain XL-10 gold and Stbl2 cells were products of Novagen Inc. (Madison, WI). Thermostable DNA polymerase (pfu ultra) was obtained from Stratagene Inc. (La Jolla, CA). Custom oligonucleotide primers were supplied by Integrated DNA Technologies (IDT). DNA plasmids encoding BaL.01 gp160 and NL4-3 R⁻ E⁻ Luc⁺ were obtained from the NIH AIDS Reagent Program, Division of AIDS, NIAID, and were a kind gift from Dr. J. Mascola and Dr. N. Landau, respectively. All other reagents used were of the highest analytical grade available.

Design and Construction of Various gp120 Mutants

Wild-type (wt) gp120 YU-2 construct with a V5 coding sequence N-terminal to the C-terminal His 6 tag in a pcDNA3.1 vector carrying the mammalian codon-optimized sequence for a CM5 secretion peptide was previously made in gp120 YU-2 construct (a gift from Drs. Navid Madani and Joseph Sodroski). This construct was used to prepare gp120 substitution mutants. Mutants of gp120 were created using Quick-change site-directed

mutagenesis reagents and methods (Stratagene). The primers used for mutagenesis were custom synthesized at Invitrogen or IDT DNA. The following forward primers and their reverse complements were used in the 5'-3' direction: I109A: cag atg cat gag gac atc gct agt ctg tgg W112A: gac atc atc agc cta gcg gac cag agc ctg aag, F210A: ccc aag gtg agc gca gag ccc atc ccc atc, T257A: ccc gtg gtg agc gcc cag ctg ctg ctg, T257R: ccc gtg gtg agc cgg cag ctg ctg ctg, T257 V: ccc gtg gtg agc gtg caa ctg ctg ctg aac, S375A: gag atc gtg acc cat gcg ttc aat tgc ggc, S375W: gag atc gtg acc cat tgg ttc aat tgc ggc, S375H: gag atc gtg acc cac cac ttc aat tgc ggc, M426A: cag atc atc aac gcg tgg cag gag gtg. Mutagenesis was confirmed by sequencing (Genewiz Inc.) using the BGHR primer or a custom-made primer 5'-tcccatccactactgcgcc-3' (IDT).

Expression and Purification of Wild-Type gp120 YU-2/gp120 YU-2 Mutants

The DNA for gp120 YU-2/gp120 YU-2 mutants in pcDNA3.1 vector for transient transfection was purified using a Qiagen MaxiPrep kit (Qiagen) and transfected into HEK 293F cells according to manufacturer's protocol (Invitrogen). Five days after transfection was initiated, cells were harvested and spun down, and the supernatant was filtered through 0.2 μ m filters. Purification was performed over a 17b antibody-coupled 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 addition of 1 M Tris pH 8.0. Identity of the eluted fractions was confirmed by SDS-PAGE and Western blotting using antibody D7324 (Aalto Bioreagents). Eluted protein was immediately buffer exchanged into PBS using spin-columns (Amicon Ultra Ultracell-30K, Millipore). Protein was filtered through 0.45 μ m syringe filters (Millex-LH, Millipore) and separated by size exclusion on a HiLoad 26/60 Superdex 200 HR prepac gel filtration column (GE). Purity of eluted fractions and monomeric state of gp120 were identified by SDS-PAGE/Western blotting with mAb D7324. Monomeric fractions were pooled, concentrated, frozen, and stored at -80 °C.

Soluble Four-Domain CD4 Production

Hexahistidine-tagged soluble four-domain CD4 (CD4) was produced by transient transfection into 293F cells using standard protocols (Gibco). The pcDNA3.1 vector carrying CD4 was a gift from Dr. Navid Madani. CD4 was separated from the expression medium by nickel affinity purification on HiTrap columns (GE) using an Akta FPLC System (GE). CD4 was further purified by size-exclusion on a Superdex 200 column (GE). Protein size and functionality were verified by SDS-PAGE and anti-gp120 ELISA, respectively. 17b IgG was purchased from Strategic Diagnostics Inc. (Newark, DE).

Peptide Synthesis

Peptides were synthesized with a microwave peptide synthesizer (CEM LibertyBlue), using standard Fmoc chemistry on Rink amide resin (Scheme 1). The Fmoc-protected amino acids (Chem-Impex International, INC) were activated using the DIC/ Oxymapure activation scheme. The Fmoc group was then removed using a solution of 20% piperidine/0.1 M HOBt in DMF for the following amino acid coupling. The terminal amino group (N-terminal) of the isoleucine residue was Boc-protected using a Boc-Ile during the peptide synthesis. Click

reaction of alkyne with the azido-proline moiety (residue 4 from the N-terminal) was carried out on resin using CuI catalysis (in acetonitrile/H₂O/diisopropylethyl amine/pyridine, rt, 12 h) to give the 1,4-triazole regioisomer. The 1,5-triazole regioisomer was synthesized using 2 mol % chloro(pentamethylcyclopentadienyl)bis-(triphenylphosphine)ruthenium(II) (Sigma-Aldrich) in 1,4-dioxane at rt for 10 h. Cleavage and global deprotection was carried out using a cocktail of trifluoroacetic acid/ethanedithiol/H₂O/thioanisole (4.75/ 0.1/0.1/0.05 v/v for 0.5 g of resin) for 2 h at rt. Cleaved peptide solution was concentrated under a gentle N₂ stream, added to precooled diethyl ether to precipitate to solid peptide pellets, and centrifuged. A diethyl ether washing cycle was repeated until the ether layer was no longer colored. Crude peptide pellets were dried by a gentle N₂ stream, dissolved, and purified (to 95% purity) using semiprep/prep C18 (or C4) RP-HPLC columns (ACN/H₂O/0.1% TFA) and finally lyophilized to yield solid peptides. Purity checks of PTs were carried out using analytical C18 RP-HPLC. PTs were validated (Table 1, Supporting Information) using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS).

Surface Plasmon Resonance (SPR) Assays

SPR experiments were performed on a Biacore 3000 optical biosensor (GE Healthcare). All experiments were carried out at 25 °C using standard PBS buffer pH = 7.4 with 0.005% surfactant Tween. A CM5 sensor chip was derivatized by standard 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide (EDC)/N-hydroxysuccinimide (NHS) chemistry, and coupling was performed through ligand amine groups. Antibody 2B6R (α -human IL5R) was immobilized for negative control surfaces. For competition experiments, soluble four-domain CD4 and 17b antibody were immobilized on the surface through standard EDC/NHS chemistry (above). For kinetic analyses, typically 2000–3000 RU of protein reagents was immobilized on SPR chips, and analytes were passed over the surface at 50–100 μ L/min. Surface regeneration was achieved by a 5 μ L injection of 10 mM HCl solution at 100 μ L/min. Analysis of peptide-mediated sCD4 and mAb 17b inhibition was achieved by injecting a fixed concentration of HIV-1 YU-2 gp120 (250 nM), with increasing peptide concentrations, over sCD4 (2000 RU) and mAb 17b (1000 RU) surfaces for 5 min association and 5 min dissociation at a flow rate of 50 μ L min⁻¹ in PBS. Regeneration of the surface was achieved by a single 10 s pulse of 1.3 M NaCl/35 mM NaOH and single 5 s pulse of 10 mM glycine, pH 1.5, for sCD4 and mAb 17b, respectively. All analyses were performed in triplicate.

Data analysis was performed using BIAevaluation v4.0 software (GE Healthcare). Signals from buffer injection and control surface binding were subtracted in all experiments to account for nonspecific binding.

For kinetic parameter determination, we used a method similar to that used by Morton et al.⁴³ The signal from the highest concentration of the analyte was used to calculate the off rate (k_d) using a simple 1:1 binding model. For each concentration, the association phase data were fitted to a simple 1:1 bimolecular interaction model using the BIAevaluation software. The resulting R_{eq} values were used to fit the corresponding steady-state model and calculate equilibrium dissociation constant (K_D) values. The evaluation method for SPR

inhibition data included a calculation of the inhibitor concentration at 50% of the maximal response (IC_{50}). The inhibition curve was converted into a calibration curve by the use of a fitting function. The fitting was done using the four-parameter eq 1 from BIAevaluation software,

$$\text{response} = R_{\text{high}} - \frac{(R_{\text{high}} - R_{\text{low}})}{1 + \left(\frac{\text{concn}}{A_1}\right)^{A_2}} \quad (1)$$

where R_{high} is the response value at high inhibitor concentrations, R_{low} is response at low inhibitor concentrations, concn is the concentration of inhibitor, and A_1 and A_2 are fitting parameters. At the IC_{50} , the following is true:

$$\text{response} = R_{\text{high}} - \frac{(R_{\text{high}} - R_{\text{low}})}{2} \quad (2)$$

Under this condition, $A_1 = \text{concn}$ and is therefore taken as the desired IC_{50} parameter.

Isothermal Titration Calorimetry

ITC experiments were performed with the VP-ITC titration calorimeter (MicroCal Inc.) fitted with a cell volume of ~ 1.4 mL. The titrations were performed by stepwise addition of peptide to wt gp120/gp120 mutants contained in the calorimetric cell at a constant temperature of 25°C . The volume per injection of peptide was $10\ \mu\text{L}$. The concentration of wt gp120/mutants was $\sim 2\ \mu\text{M}$, and the syringe contained the peptide at a concentration of $\sim 30\text{--}50\ \mu\text{M}$. The solutions contained within the calorimetric cells and injector syringes were prepared in $1 \times \text{PBS pH } 7.4$ and thoroughly degassed to avoid bubble formation in the calorimetric cell. The heats of reaction were measured and corrected for heats of ligand dilution determined by performing the ligand titration in the absence of protein. Based on the finding that the calorimetric titrations obtained had inflection points close to unity, the data were analyzed with MicroCal ORIGIN software using a single-site binding model.^{44,45} In this model, the total heat evolved (or absorbed) during the binding process at the end of the i th injection, $Q(i)$, is calculated using eq 3.

$$Q(i) = \frac{nP_t \Delta H V \left\{ 1 + \frac{X_t}{nP_t} + \frac{1}{nKP_t} - \left[\left(1 + \frac{X_t}{nP_t} + \frac{1}{nKP_t} \right)^2 - \frac{4X_t}{nP_t} \right]^{1/2} \right\}}{2} \quad (3)$$

where n is the number of binding sites, P_t is the total protein concentration, X_t is the total ligand concentration, V is the active cell volume, K is the binding constant, and H is the molar heat of ligand binding. The correction factor for the displaced volume dV_i and heat corresponding to the i th injection only, $Q(i)$, is equal to the difference between $Q(i)$ and $Q(i-1)$ and is given by eq 4, which enables measurement of $Q(i)$ value for each injection.

$$\Delta Q(i) = Q(i) + \frac{dV_i}{V_o} \left[\frac{Q(i) + Q(i-1)}{2} \right] - Q(i-1) \quad (4)$$

Experimental data were fitted to eqs 3 and 4 by nonlinear least-squares to derive values for n , K , and H . The individual heats plotted as a function of molar ratio further enabled measurement of T – S values using the relationships $G = RT \ln K_D$ and $G = H - TS$.

Pseudovirus Production and Antiviral Assay

Pseudoviruses were produced as described previously.^{16,46} Briefly, HEK 293T cells (3×10^6) were cotransfected with 4 μ g of BaL.01 gp160 plasmid and 8 μ g of NL4-3 R⁺ E⁺ Luc⁺ core DNA, using polyethylenimine (PEI) as a transfection vehicle. After 72 h, the supernatant containing virus was collected and filtered using a 0.45 μ m syringe filter (Corning) before being purified via gradient centrifugation on a 6–20% Iodixanol gradient (Optiprep, Sigma-Aldrich) spun on an Sw41 Ti rotor (Beckman Coulter) at 110 000g for 2 h at 4 °C. The bottom 5 mL was collected and diluted in serum-free medium before being aliquoted and frozen at –80 °C. All batches of virus were titrated for infectivity and p24 content immediately after production.

Pseudoviral infection assays were carried out as described previously.^{16,18} Briefly, HOS.T4.R5 cells were seeded the day before at 7000 cells, 100 μ L/well in 96-well plates. Virus stocks were diluted in growth media such that the final dilution gave 1×10^6 luminescence counts. Peptides to be tested were solubilized in 1 $\times$ PBS and serially diluted in 1.5 mL tubes. Virus was added to these tubes containing peptide 1:1 (v/v), and the tubes were mixed by repeated inversions. The samples were incubated at 37 °C for 30 min before addition to the cells. Medium was removed from the plates, and virus-containing medium was added. The plates were incubated for 24 h at 37 °C before the medium was changed. At 48 h after the virus was added to the plate, the medium was removed and the cells were lysed (Passive Lysis Buffer, Promega). Lysed cells were then mixed with Luciferin salt (Anaspec) in 0.1 M potassium phosphate buffer containing 0.1 M magnesium sulfate and the luminescence measured using a Wallac 1450 Microbeta Luminescence reader.

Inhibition of infection by PT was confirmed to occur at the entry stage by examining the effect of time of addition of inhibitor relative to mixing of virus and cells, analogous to the procedure used previously.⁴⁷ Briefly, pseudoviruses were preincubated with inhibitor before addition to cells ($t < 0$), added to cells at the same time as inhibitor ($t = 0$), or added to cells before addition of inhibitor ($t > 0$). A 15 μ M concentration of peptide ($10 \times \text{IC}_{50}$) was used to inhibit infection in all cases. Similar to the protocol above, medium was changed 24 h after the addition of virus. All time point samples, including the 24 h control, received fresh inhibitor with the medium change. The rest of the assay procedure was identical to that described above.

Molecular Modeling of the Peptide Triazole Binding to HIV-1 gp120

Peptide Preparation for Docking—Peptides were generated using VIDA 4.2.0 (Openeye Scientific Software, Santa Fe, NM, <http://www.eyesopen.com>). The ferrocene-containing peptides were then energy-minimized using the MM2 force field (ChemBio3D

Ultra 13.0) with RMS gradient of 0.01 and number of alterations of 10^4 . The minimized structures were then saved as pdb files for the docking simulations. Autodock tools graphical interface (MGtools 1.5.6rc3)^{48,49} was then used to prepare the peptides for docking.

Flexible Docking—In this modeling simulation work, we used the previously developed model of PT interactions with the monomeric gp120 protein. The model was simulated using the F105-bound crystal structure of gp120 (PDB code 3HI1¹⁷). The gp120 structure was extracted from the complex and was further energy-refined using Szybki 1.8.0.2 (Openeye Scientific Software, Santa Fe, NM). The option (-max_iter) in Szybki was set to 10^6 to ensure that the added hydrogen atoms were correctly optimized. The optimized protein structure was then prepared by Autodock tools graphical interface (MGtools 1.5.6rc3)^{48,49} where nonpolar hydrogens were merged, Kollman charges were added, and Gasteiger charges were calculated. Trp112 residue was set flexible²⁷ for the docking where the indole side chain can move during the docking simulation to uncover the hydrophobic pocket in the inner domain gated by this residue. The grid box for the docking search was set to $52 \times 52 \times 52$ points for the x , y , and z dimension with a spacing grid of 0.375 Å. X , Y , and Z grid centers were set to 55.784, 28.301, and -21.068, respectively. AutoGrid 4.2 algorithm^{48,49} was used to evaluate the binding energies between the peptides and the protein and to generate the energy maps for the docking run. For high accuracy mode docking of the ferrocene-containing peptides with the protein, the maximum number of evaluations (25×10^6) were used. Fifty runs were generated for each peptide by using Autodock 4.2 Lamarckian genetic algorithm^{48,49} for the searches. Cluster analysis was performed on docked results, with a root-mean-square tolerance of 2.0 Å. Visual inspections of the docked poses for all peptides were compared to each other and to the mutagenesis analysis results. Common poses for all the docked peptides were selected as representative binding modes. The calculated binding energy (G_{binding}) values for the peptide–protein complexes were recorded. The binding energy of peptide 3 was initially calculated to be -4.03 kcal/mol. However, in view of the SPR analysis finding (Tables 2 and 3) that peptide 3 is more active than peptide 4, the benzo[*b*]thiophene ring of peptide 3 was manually relaxed within the original complex, yielding a more negative binding energy (-5.28 kcal/mol, see footnote of Table 6 and Figure S7, Supporting Information). The complexes were then typed with the CHARMM force field with Discovery Studio 4.0 software (Accelrys Software Inc., San Diego, CA, 2013), to relax the obtained poses within the protein pockets, and visualized by VIDA 4.2.0 (Openeye Scientific Software, Santa Fe, NM).

Two-Cavity Model Refinement—The Schrodinger package (Schrödinger Suite 2014; Schrödinger, LLC) was used for the refinement. The gp120 protein was prepared with the protein preparation wizard⁵⁰ in Maestro 9.9. Peptide 5 was built and prepared in Maestro by the ligand preparation facility. Initially, Glide Induced Fit Docking⁵¹ was used to simulate peptide 5 with the prepared gp120 protein, with the W112 side chain assigned to be flexible, and with the use of Prime refinement and Glide-XP redocking mode. The search box size was set to dock ligand with length 45 Å. The output file of this docking was visualized, and all the predicted poses were subjected to binding energy calculations using Szybki 1.8.0.2 (Openeye Scientific Software, Santa Fe, NM). The most stable complex, which showed the

lowest binding energy, was selected for the following Glide-Peptide docking.²⁸ The gp120 protein structure was extracted from the previous complex and prepared by Glide Receptor Grid Generation to be used for Glide-Peptide²⁸ docking with the Maestro prepared peptide 5. After docking, predicted poses were ranked according to the Glide gscore, and the top ranked poses were then subjected to the binding energy calculations using Szybki 1.8.0.2 (Openeye Scientific Software, Santa Fe, NM). The most stable complex (lower binding energy in kcal/mol) was selected to represent the binding mode that was visualized by VIDA 4.2.0 (Openeye Scientific Software, Santa Fe, NM).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations Used

ACN	acetonitrile
Boc	<i>tert</i> -butyloxycarbonyl
CM5	carboxymethyl dextran
DIC	<i>N,N'</i> -diisopropylcarbodiimide
DMF	dimethylformamide
Env	HIV envelope gp120/gp41 complex
Fmoc	9-fluorenylmethoxycarbonyl
HAART	highly active antiretroviral therapy
HEK	human embryonic kidney
HPLC	high performance liquid chromatography
HOBT	hydroxybenzotriazole
ITC	isothermal titration calorimetry
IXW	IleferrocenyltriazolePro-Trp
Ni-NTA	nickel nitriloacetic acid
PBS	phosphate-buffered saline
PT	peptide triazole
PTT	peptide triazole thiol

SDS-PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SPR	surface plasmon resonance
tBu	<i>tert</i> -butyl
Trt	triphenylmethyl
TFA	trifluoroacetic acid
wt	wild type

Figure 1. Env gp120 residues studied in the context of the mAb F105-bound gp120 crystal structure. Crystal structure of HIV-1 core monomeric gp120_{YU-2} in the F105 bound conformation shows the open cavity between the inner and outer domain (generated from the PDB file 3HI1). Residues comprising site 1 (Phe43 cavity) and site 2 (gp120 inner domain cavity) included in this study are shown as CPK.

Figure 2.

Direct binding of wt gp120_{YU-2}/mutants to sCD4 and mAb 17b binding using SPR. Representative SPR sensograms show concentration-dependent binding of wt gp120, S375W, and W112A to immobilized CD4 (left panel) and 17b binding (right panel) on the CM5 sensor chip surface. Each curve represents the average response from two runs. Numbers on the right show the wt gp120/mutant concentrations in nM.

Figure 3.

Inhibition and antiviral potencies of peptide triazoles. (Left) Inhibition potencies of peptide 1 (■), peptide 2 (●), peptide 3 (▲), and peptide 4 (▼) for wt gp120_{YU-2} binding to sCD4 immobilized on CM5 sensor chip. Normalized binding responses (RUs) were measured at 5 s before the end of association and plotted. Binding signal obtained from binding of peptide alone was subtracted, and resulting signals were plotted versus peptide concentration. Fits obtained from applying a four-parameter sigmoidal logistic equation to the data points are overlaid. (Right) Antiviral potencies of peptides using single round cell infection assays. Recombinant HIV-1 BaL.01 virus was preincubated with serial dilutions of peptide 1 (■), peptide 2 (●), peptide 3 (▲), and peptide 4 (▼) for 30 min at 37 °C. The virus–inhibitor mixture was then added to HOS.CD4.CCR5 cells for 48 h. Infection was determined based on luciferase activity. IC₅₀ values were obtained by fitting data points to a simple sigmoidal inhibition model using the Origin software package to derive the best-fit lines. Data represent a minimum of three repeats.

Figure 4.

Inhibition of binding of gp120 site 1 mutants to immobilized sCD4 by SPR. Varying concentrations of peptide 1 (left panel) and peptide 2 (right panel) were mixed with a constant concentration of wt gp120, S375W, S375H, and T257R and injected over the covalently immobilized sCD4 on the CM5 sensor surface. Each curve represents the average response from two runs. Numbers on the right show the concentrations of peptides in μM .

Figure 5.

Inhibition of binding of gp120 site 2 mutant W112A to immobilized sCD4 by SPR. SPR sensograms are shown for binding of fixed concentrations of W112A (analyte) to CD4 on the SPR chip surface in the presence of increasing concentrations of peptide 1 (left) and peptide 2 (center). Numbers on the right show the concentrations of peptides in μM . (Right) Inhibition potential of peptide 1 (■), peptide 2 (●), and peptide 3 (▲) for W112A binding to sCD4 immobilized on CM5 sensor chip. Normalized binding responses (RUs) were measured at 5 s before the end of association and plotted. Binding signal obtained from binding of peptide alone was subtracted, and resulting signals were plotted versus peptide concentration. Fits obtained from applying a four-parameter sigmoidal logistic equation to the data points are overlaid. Results are an average of three data sets.

Figure 6. Selected binding modes of the four peptides 1–4. The peptide residue 6 side chains are located in the Phe43 site (site 1, shown in yellow), whereas the ferrocene moiety penetrates through the inner domain cavity (site 2, shown in orange). Autodock 4 was used for docking and calculation of the binding energy (G_{binding}). Modeling was performed on the crystal structure of F105-bound gp120 (pdb: 3HI1¹⁷) based on the previously¹⁸ developed PT model.

Figure 7.

Refined two-cavity model of PT binding to gp120. (A) 3D representation of the binding mode of peptide 5 in complex with gp120 (surface shown in gray). Hot spots Trp112, Thr257, and Asp474 are shown as CPK in red. Peptide 5 Trp residue rests in site 1, whereas the phenylethyltriazole moiety is buried in site 2. (B) 2D representation of the binding mode of peptide 5 showing possible interactions with residues in site 1 and site 2. H-bonds are shown as purple arrows and π - π interactions shown as green lines.

Scheme 1. Solid-Phase Synthesis of Peptide Triazoles 1–6^a

^aR is the alkyne derivative used for the click reaction. X is the peptide residue 6 side-chain aromatic ring.

Table 1
Relative Affinities of wt gp120_{YU-2}/Mutants to sCD4 and mAb 17b Measured by SPR^a

protein	CD4 binding (nM)	17b binding (nM)
wt gp120	4.9 ± 0.2	9.4 ± 0.5
S375A	6.2 ± 1.1	2.0 ± 1.5
S375W	3.7 ± 0.8	0.0022 ± 0.002
S375H	5.2 ± 1.3	5.4 ± 1.9
T257A	10.2 ± 2.2	11 ± 3.2
T257R	21 ± 2.5	23.3 ± 3.3
W112A	95 ± 6.2	>1000
F210A	230 ± 5.5	>500
M426A	59 ± 8.3	>500
I109A	6.8 ± 2.1	12.5 ± 2.5

^aDirect binding of wt gp120 and mutants of site 1 (Phe43 cavity) and site 2 (gp120 inner domain cavity) to surface-immobilized CD4 and mAb 17b. Binding constants (K_D) were obtained by fitting to a 1:1 Langmuir binding model.

Table 2
Structure and Inhibition Efficacies of Peptide Triazole Derivatives^a

Peptide	Structure	sCD4 (nM)	Antiviral (μ M)	ClogP of residue 6 side chain
1		102 $\pm$ 21	1.5 $\pm$ 0.8	2.14 $\pm$ 0.20
2		3000 $\pm$ 300	95.6 $\pm$ 5.2	1.48 $\pm$ 0.19
3		130 $\pm$ 12	1.5 $\pm$ 0.5	4.38 $\pm$ 0.25
4		398 $\pm$ 21	4.1 $\pm$ 0.5	2.54 $\pm$ 0.22

^asCD4 IC₅₀ values were determined using competition SPR. Antiviral EC₅₀ values were obtained through a single-round infection assay and expressed (Figure 3, right) as relative infection versus untreated (100%). All data represent a minimum of three experiments. ClogP was calculated separately for each aromatic side chain (shown in red) using (ACD/LABS Release 2012. Advanced Chemistry Development, Inc., Toronto, ON, Canada, www.acdlabs.com, 2014).

Table 3
Inhibition Potencies (IC₅₀, in μ M) of Peptides 1-4 for wt gp120_{YU-2}/Mutants for Binding to sCD4^a

	<u>Peptide 1</u>	<u>Peptide 2</u>	<u>Peptide 3</u>	<u>Peptide 4</u>
<u>Protein</u>
Wt gp120	0.10±0.02	3.0±0.3	0.13±0.01	0.39±0.01
T257A	0.55 ± 0.09	4.5±0.8	0.76±0.13	0.85±0.03
T257R	>100	5.36±2.1	>100	>100
T257V	0.69±0.31	3.7 ±0.3	0.84±0.22	0.85±0.01
S375A	0.57±0.02	1.22±0.7	0.61±0.01	1.0±0.2
S375W	>100	>100	>100	>100
S375H	>50	5.5±0.5	>50	>50

^aIC₅₀ values were determined using competition SPR by plotting binding response (RU) vs peptide concentration and fitting to a four-parameter sigmoidal model in Origin 7.0. Binding signal obtained from binding of peptide alone was subtracted, and resulting signals were plotted versus peptide concentration. Results are an average of three data sets.

Table 4
Thermodynamic Parameters for the Binding of Peptide 1 and Peptide 4 to wt gp120_{YU-2}/Mutants^a

protein	K_D (nM)		n		H (kcal/mol)		$-T \Delta S$ (kcal/mol)	
	peptide 1	peptide 4	peptide 1	peptide 4	peptide 1	peptide 4	peptide 1	peptide 4
wt gp120	45.4 ± 2	190 ± 10	1.15	1.12	-12 ± 0.9	-10.2 ± 0.2	2.2 ± 0.2	2.7 ± 0.3
T257A	300 ± 11	312 ± 18	1.18	1.15	-12.1 ± 0.8	-11 ± 0.9	4.2 ± 0.6	2.7 ± 0.2
S375A	271 ± 15	368 ± 20	0.95	1.13	-9.1 ± 0.7	-9.3 ± 0.8	2.2 ± 0.1	0.4 ± 0.1

^aThe heat evolved upon each injection of the peptide solution was obtained from the integral of the calorimetric signal. The individual heats were plotted as a function of the molar ratio, and nonlinear regression of the data provided the enthalpy change (ΔH) and the association constant ($K_A = K_D^{-1}$). The parameters were obtained by analysis of the data with MicroCal ORIGIN software using a single-site binding model.

Table 5
Inhibition Potencies (IC₅₀, in μ M) of Peptides 1–3 for wt gp120_{YU-2}/Site-2 Mutants for Binding to sCD4^a

protein	peptide 1	peptide 2	peptide 3
wt gp120	0.1 $\pm$ 0.04	3.0 $\pm$ 0.3	0.1 $\pm$ 0.01
W112A	16.8 $\pm$ 1.4	>50	5.8 $\pm$ 0.8
F210A	0.2 $\pm$ 0.01	4.1 $\pm$ 0.3	0.4 $\pm$ 0.02
M426A	4.6 $\pm$ 0.2	5.1 $\pm$ 0.2	3.60 $\pm$ 0.2
I109A	1.5 $\pm$ 0.1	3.1 $\pm$ 0.3	2.8 $\pm$ 0.2

^aIC₅₀ values were determined using competition SPR by plotting binding response (RU) vs peptide concentration and fitting to a four-parameter sigmoidal model in Origin 7.0. Binding signal obtained from binding of peptide alone was subtracted, and resulting signals were plotted versus peptide concentration. Results are an average of three data sets.

Table 6
Autodock Results of Peptides 1–4 with the gp120 Env Protein Using the Flex Trp112 Side-Chain Docking Protocol^a

peptide	interacting residues		G_{binding} (kcal/mol)
	residues in site 1	residues in site 2	
peptide 1	indole ring: H-bond (1.7 Å S375), π - σ (3.8 Å Thr257), 2 S- π (Met475)	proline: hydrophobic (Met426, Trp112) ferrocene: hydrophobic (Trp112, Ile423, Ile108, Leu111) triazole: hydrophobic (Ile108) other: peptide Ile1 (H-bond with His105 backbone 2.2 Å)	-5.38
peptide 2	phenol ring: H-bond (2.2 Å, Glu370), π - π (Phe382), π - π (Tyr384).	proline: contact with Trp112, Met426 triazole: hydrophobic (Ile108) ferrocene: hydrophobic (Val255, Leu111)	-3.42
peptide 3	benzothiophene ring: π - σ (3.8 Å Thr257), alkyl- π (Met475) other: backbone CO (H-bond, Met475 backbone)	proline: alkyl- π (Trp112) triazole: hydrophobic (Met475) ferrocene: hydrophobic (Ile423, Val255)	-4.03 ^b
peptide 4	indole ring: π - π (F382), S- π (M475), alkyl- π (Met475) other: peptide Asn3 (2 H-bonds, Gly473)	proline: hydrophobic (Trp112, Ile108) triazole: hydrophobic (Ile108, Ile109) ferrocene: hydrophobic (Ile109, Trp112, Ile423)	-4.96

^aDocking studies were performed to investigate the occupancy of the two-cavity site by the peptide fragments. Binding energies (G_{binding}) were recorded for selected binding poses.

^bThe binding energy of peptide 3 was blindly calculated to be $\sim$ -4.03 kcal/mol. However, in view of the SPR analysis finding (Table 2) that peptide 3 is more active than peptide 4, the benzo[b]thiophene ring of peptide 3 was manually relaxed within the original complex, yielding a recalculated binding energy of -5.28 kcal/mol (see Figure S7, Supporting Information, for corresponding binding pose). The G_{binding} values given here are taken as relative rather than absolute values.