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Design of HIV Coreceptor Derived Peptides That Inhibit Viral Entry at Submicromolar Concentrations

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Abstract

HIV/AIDS continues to pose an enormous burden on global health. Current HIV therapeutics include inhibitors that target the enzymes HIV protease, reverse transcriptase, and integrase, along with viral entry inhibitors that block the initial steps of HIV infection by preventing membrane fusion or virus–coreceptor interactions. With regard to the latter, peptides derived from the HIV coreceptor CCR5 were previously shown to modestly inhibit entry of CCR5-tropic HIV strains, with a peptide containing residues 178–191 of the second extracellular loop (peptide **2C**) showing the strongest inhibition. Here we use an iterative approach of amino acid scanning at positions shown to be important for binding the HIV envelope, and recombining favorable substitutions to greatly improve the potency of **2C**. The most potent candidate peptides gain neutralization breadth and inhibit CXCR4 and CXCR4/CCR5-using viruses, rather than CCR5-tropic strains only. We found that gains in potency in the absence of toxicity were highly dependent on amino acid position and residue type. Using virion capture assays we show that **2C** and the new peptides inhibit capture of CD4-bound HIV-1 particles by antibodies whose epitopes are located in or around variable loop 3 (V3) on gp120. Analysis of antibody binding data indicates that interactions between CCR5 ECL2-derived peptides and gp120 are localized around the base and stem of V3 more than the tip. In the absence of a high-resolution structure of gp120 bound to coreceptor CCR5, these findings may facilitate structural studies of CCR5 surrogates, design of peptidomimetics with increased potency, or use as functional probes for further study of HIV-1 gp120–coreceptor interactions.

Graphical Abstract

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Supporting Information

The Supporting Information is available free of charge on the [ACS Publications website](https://pubs.acs.org) at DOI: [10.1021/acs.molpharmaceut.7b00155](https://doi.org/10.1021/acs.molpharmaceut.7b00155). Tables summarizing the sequences, neutralization data, and hydrophobicity parameters for all peptides used in this study ([PDF](#))

The authors declare no competing financial interest.

Keywords

HIV entry; V3 loop; virion capture

INTRODUCTION

In the absence of an HIV vaccine or cure, HIV/AIDS continues to pose an enormous burden on global health. Advances in combination antiretroviral therapy (ART) have resulted in almost normal life expectancies for AIDS patients; however access to ART as well as compliance varies among socioeconomic groups. To date the majority of successful anti-HIV strategies have focused on three key viral enzymes—namely, reverse transcriptase, integrase, and protease—and the process of virus–cell or cell–cell fusion that ultimately leads to viral entry and infection. Viral envelope (Env) glycoproteins gp120 and gp41 mediate the entry process. To initiate entry, gp120 binds to the primary cellular receptor CD4, and subsequently engages coreceptors CCR5 and/or CXCR4. Formation of this ternary complex is believed to trigger the transmembrane protein gp41 for insertion into the host cell membrane, initiating membrane fusion. Among the 39 FDA-approved anti-HIV drugs, only 2 target the fusion process. These include the entry inhibitor maraviroc and the fusion inhibitor enfuvirtide. Maraviroc binds the CCR5 coreceptor, preventing its interaction with gp120, and is thus used to treat infections by CCR5-tropic (R5-tropic) viruses.¹ Enfuvirtide binds the transmembrane region of gp41 and blocks fusion between the viral and cellular membranes.^{2,3} Both drugs have side effects and generate resistance, making way for a continuing need for new HIV entry inhibitors.⁴

Peptides derived from HIV-1 receptors, coreceptors, or antibody-recognition loops have played prominent roles in developing entry inhibitors and molecular probes for studying the events that lead to membrane fusion.⁴ Previously, the C-terminal portion of the second extracellular loop (ECL2) of CCR5, comprising amino acids Cys-178 to Lys-191 and referred to as peptide 2C, was shown to inhibit HIV-1 entry at low tens of micromolar concentrations. **2C** inhibited both R5- and X4-tropic strains, and was suggested to bind in the conserved coreceptor binding site on gp120, separate from the binding site of a sulfated CCR5 N-terminal peptide (residues 2–18). The amino acids important for interacting with

gp120 included Tyr, Phe, Trp, and His and were identified using saturation transfer NMR techniques (Figure 1).⁵

Using the **2C** peptide as a starting point, in this study we sought to increase potency and/or improve breadth against diverse HIV-1 strains through systematic and iterative substitutions of amino acids. This approach yielded peptides with a hundred-fold improvement in potencies and submicromolar inhibitory constants. Moreover, the most potent peptides demonstrated expanded breadth inhibiting R5- and X4-tropic strains as well as a dual-tropic HIV strain, and showed no cytotoxicity at relevant concentrations. In cooperative infectivity assays wherein combinations of inhibitors were used, the lead peptide, which was most potent against the R5-tropic YU2 strain, was weakly synergistic with its predecessor **2C** and weakly antagonistic to a CCR5 N-terminal peptide. In virion-capture assays, both **2C** and the new-generation peptides blocked binding of antibodies with known epitopes on the base, stem, and tip of the V3 loop of gp120, albeit with different levels of efficacy. Taken together, these data suggest that the new-generation peptides have altered preferences to discrete regions on and around the V3 loop, yet maintain binding to the physiologically relevant CCR5-binding site of gp120 with increased potency and breadth toward diverse HIV-1 strains.

EXPERIMENTAL SECTION

Peptide Synthesis and Characterization.

Peptide synthesis was carried out on a Liberty Blue microwave peptide synthesizer (CEM Corp., Matthews, NC) using CLEAR amide and rink amide resins (Peptides International, Lexington, KY) and by Fmoc (*N*-(9-fluorenyl)-methoxycarbonyl) as protecting group. Fmoc protecting group was cleaved with 20% piperidine/DMF, and the free N-terminus was capped with acetic anhydride in each coupling. After the completion of sequence, the N-terminal amino group was acetylated. The peptides were cleaved from the resin by treatment with a deprotection cocktail containing TFA: -
H₂O:triisopropylsilane:2,2'-(ethylenedioxy)diethanethiol (92.5:2.5:2.5:2.5) for 2.5 h at room temperature, precipitated with ice cold diethyl ether, and centrifuged for 15 min at 0 °C. Crude precipitated peptides were suspended in H₂O and purified by RP-HPLC using a preparative symmetry shield RP18 column (Waters, Milford, MA) with 0.1% aqueous TFA and acetonitrile as eluents. Purified peptide solutions were lyophilized, and their purity (>95%) and compositions were verified by analytical HPLC and ESI-HRMS.

To evaluate the presence of secondary structure in solution, the CD data were recorded on a Jasco J-815 CD spectrometer using a quartz cell with an optical path of 1.0 mm on 75 μ M solutions of peptides in 50% trifluoroethanol/H₂O at 25 °C. Three scans were performed for each sample from 190 to 260 nm at a rate of 20 nm min⁻¹, with a 1 nm bandwidth, 0.5 s response, and resolution of 0.4 nm. The percentages of peptide secondary structure were estimated with the programs K2D2⁶ and K2D3.⁷

Peptide stability was tested on aqueous solutions (stocks prepared in ultrapure H₂O) diluted in DMEM medium (GIBCO), complemented with fetal bovine serum (BenchMark by GEMINI Bio-Products), and analyzed by analytical HPLC after 15–30 min, which

corresponds to the duration of the entry process. Additional time points for stability included 1 and 24 h of incubation at 37 °C, 5% CO₂.

HIV Neutralization and Cytotoxicity Assays.

Viral particles pseudotyped with HIV-1 envelopes (Env) were prepared as described.⁸ Briefly, 293T cells (American Type Culture Collection) were cotransfected with an SG3 Env backbone plasmid and the desired HIV-1 Env-expressing plasmid (NIH AIDS Reagent Program) using X-treme GENE HP DNA transfection reagent (Roche). The transfection culture was incubated at 37 °C, 5% CO₂. The medium was changed after 24 h, and at 48 h post-transfection the viral particles were harvested by passing the supernatant through a 0.45 μm filter and storing at –80 °C for later use.

Neutralization assays were performed as described.⁹ Briefly, 2-fold serial dilutions of inhibitors were prepared in 96-well plates followed by addition of the pseudotyped viral particles and TZM-bl cells, which express CD4, CXCR4, and CCR5 (NIH AIDS Reagent Program). The plates were incubated at 37 °C, 5% CO₂. At 24 h fresh medium was added. At 48 h postinfection the cells were lysed and luciferase activity was measured following the Bright-Glo assay protocol (Promega). Peptides were tested for cytotoxicity toward host cells (TZM-bl) and a colon tumor cell line (HCT116) following the MTT Cell Proliferation Assay protocol (ATCC Bioproducts).

Combination Experiments.

The new-generation peptide **40-2** was tested in combination with parent peptide **2C** or sulfated CCR5 N-terminal peptide **Nt** (residues 2–18). In each assay, dose response curves for constant ratios of peptides were obtained (Table 5). Combination effects were analyzed using the approach of Chou and Talalay.^{10,11} Briefly, the dose reduction index (DRI) of inhibitor *x* in combination with inhibitor *y* was determined from the formula $DRI_x = (IC_{50})_x / (IC_{50})_{x,y}$ where $(IC_{50})_x$ and $(IC_{50})_{x,y}$ are the IC₅₀ values of *x* alone and in combination with *y*, respectively. The combination effect of the two inhibitors is then calculated as the combination index (CI) from the formula $CI = (DRI_x)^{-1} + (DRI_y)^{-1} + (DRI_x)(DRI_y)^{-1}$, where the last term, which makes a small contribution to CI, accounts for the state where both inhibitors are bound.¹² CI values equal to 1, less than 1, or greater than 1 indicate the additive, synergistic, or antagonistic effects.

HIV-1 Virion Capture Assay.

The virion capture assay was performed as previously described.^{13,14} Briefly, the sequence of capture was as follows: sCD4–2D-treated virus stocks were first incubated with peptides and then mixed with antibody-armed magnetic beads to assess virion capture with various gp120-specific mAbs. Specifically, 200 μL of infectious HIV-1 BaL produced in primary human PBMC (30 ng/mL p24_{Gag}) was preincubated with sCD4–2D (5 μg/mL) for 15 min at room temperature and then incubated with each peptide (50 μM) for 15 min at room temperature. Protein G immunomagnetic Dynabeads (Life Technologies), at 600 μg (20 μL) per reaction, were armed with mAbs (412d, 447–52D, 268-D, 19b, 48d, PG135, PG16, D19, and B4e8) at 1 μg/reaction condition, washed with phosphate-buffered saline (PBS) containing 0.02% (w/v) bovine casein to remove all the unbound mAb, then mixed with

virus–peptide suspensions (0.2 mL, 6 ng of p24_{Gag} per reaction), and incubated for 1 h at room temperature. After incubation with virus, the beads were extensively washed to remove unbound virus particles and treated with 0.5% Triton X-100 to lyse the captured virions. The amount of captured p24 Gag protein was quantified by AlphaLISA (PerkinElmer).

RESULTS

Amino Acid Scanning at Positions Tyr 184 and Tyr 187 and Deletion of Cys178 Show Trends for Improving 2C Activity and Avoiding Toxicity.

The amino acids important for interaction of CCR5 ECL2-derived peptide **2C** with gp120, as determined previously by 1H STD NMR, include tyrosines (Tyr) 184 and 187, phenylalanines (Phe) 182 and 189, tryptophan (Trp) 190, and to a lesser extent histidine (His) 181 (Figure 1).⁵ As a first step toward optimizing the potency of **2C**, we constructed a library of 38 peptides in which amino acid scans were performed at positions Tyr 184 and Tyr 187. To prevent formation of disulfide bridged peptides or other adducts Cys 178 was not included on the N-terminus of the peptides in this initial library. All peptides were tested in single round neutralization assays against YU2 pseudotyped viral particles at three concentrations (Table 1). We observed a reduction in potency for peptide **12-1**. Because the sequence of **12-1** is identical to **2C** except for the absence of the N-terminal cysteine, this suggested that Cys 178 may contribute to the inhibitory activity of **2C**. Mutations where Tyr 184 was changed to Phe (**12-2**), Val (**12-3**), or Trp (**12-4**) and Tyr 187 was changed to Arg (**12-5**), Thr (**12-6**), or Val (**12-7**) resulted in modest gains in potency relative to the reference peptide **12-1**. Of specific interest were peptides **12-2** (2C- Cys178–184F) and **12-4** (2C- Cys178–184W); although these peptides lacked the N-terminal Cys, they prevented viral infection at concentrations comparable to **2C**, thus, indicating a net gain in potency. Mutations at positions 184 and 187 to the remaining 16 out of 19 naturally occurring amino acids did not yield significant improvements in potency (Table S1) and were not considered further.

Next, IC₅₀ values were determined against YU2 for those peptides that showed improved potency compared to the parent **12-1**. These included peptides with the noted single substitutions at residues 184 or 187 (see above), and systematic combinations of each to give doubly mutated peptides at positions 184 and 187. Cys 178 was reintroduced in select peptides to confirm its importance for potency, and cellular cytotoxicity was determined using an MTT cell proliferation assay (Table 2). Peptides with Val in position 184 (**12-3**) or Phe (**16-4**, **16-5**) or Val (**16-6**, **16-7**) in position 187 were cytotoxic or had low activity (**12-7**, **14-5**, **14-6**), and peptides containing other amino acid substitutions at position 187 also showed diminished activity (**12-5**, **12-6**, and **14-1** to **14-4**). Inclusion of the N-terminal cysteine improved potency by 7-fold (**2C** vs **12-1**), 8-fold (**12-4** vs **16-3**), and nearly 20-fold (**12-2** vs **16-2**, Table 3). Peptides that maintained Tyr 187 and Cys 178 and contained either Trp (**16-3**, Table 2) or Phe (**16-2**, Table 3) at position 184 were the most potent, with IC₅₀ values of 14.5 and 5.1 μ M, respectively, against YU2.

Systematic Combinations of Amino Acid Substitutions Lead to Peptides with Submicromolar IC₅₀s and No Cytotoxicity.

With the aim of further improving potency, we considered the chemical character of the side chains of residues shown to mediate binding to gp120 together with their positions in the peptide sequence (Figure 1). Knowing that hydrophobic residues Phe, Trp, and Tyr mediate binding to gp120, were important for inhibition, and are located at positions 181, 182, 189 and 190, we generated a second panel of peptides that systematically probed these variables. Using peptide **16-2** as a lead, we substituted residues 181, 182, 189, or 190 with Phe, Trp, or Tyr. The amino acid threonine was also included to explore the importance of hydrogen bonding potential. Peptides **18-1** to **18-16** were tested in neutralization assays, and peptide **18-2**, which contains a His to Tyr substitution at position 181, was found to be the most potent with an IC₅₀ value of 1.9 μ M (Table 3).

As a final iteration, substitutions 182Y, 189W, and 190Y, which yielded the most potent peptides in each subgroup (peptide **18-7**, IC₅₀ of 10.3 μ M; **18-10**, IC₅₀ of 3.2 μ M; and **18-13**, IC₅₀ of 14.1 μ M, respectively, Table S2.1), were systematically introduced into peptide **18-2**, and the potencies of 28 new peptides, containing from 3 to 5 amino acid substitutions, were tested (peptides **20-1** to **20-28**, Table S2). The most potent peptides, **20-2** and **20-5**, with IC₅₀ values against YU2 of 0.42 and 0.72 μ M, respectively, are shown in Table 3. In peptide **20-2** there is an additional Phe to Trp substitution in position 189 compared to peptide **18-2**. In peptide **20-5**, a substitution of Phe to Tyr in position 182 could be tolerated compared to peptide **20-2** while maintaining submicromolar potency. Both Phe and Trp in position 184 resulted in gains in potency in peptides **16-2** and **16-3**; therefore, we checked the effect of 184W in the context of 181Y and 189W. The resulting peptide **30-2** maintained submicromolar potency against YU2, with an IC₅₀ value of 0.96 μ M. In contrast, 184W in the context of 181Y, 182Y, and 189W caused a 2-fold loss in potency (peptide **30-5**, IC₅₀ of 2.1 μ M, Table S2.2). Interestingly, the N-terminal Cys 178 is important for potency of peptides **2C**, **16-2**, and **16-3**. For example, a replacement of Cys 178 with Tyr in **2C** leads to a 6-fold loss in potency (peptide **40-1**, IC₅₀ of 179 μ M, Table S2.2). This effect is similar to a complete removal of the N-terminal Cys in peptide **12-1**. However, a substitution of Cys 178 to Tyr in the background of peptide **20-2** yielded the most active peptide against YU2 identified in this study, with an IC₅₀ of 0.26 μ M. Reintroduction of Trp in position 184 in the background of peptide **40-2** with N-terminal Tyr (**50-2**, IC₅₀ of 2.1 μ M) or Ser (**60-2**, IC₅₀ of 2.7 μ M) caused a 10-fold loss in potency compared to the most potent peptide **40-2** (Table 3). Overall, a 100-fold improvement in potency against YU2 was achieved, compared to the starting point of peptide **2C**, with a number of peptides (**20-2**, **20-5**, **30-2**, and **40-2**) demonstrating activities at μ M or submicromolar concentrations (Figure 2).

Cellular cytotoxicity assays showed peptides **16-2** and **18-2** to be cytotoxic at concentrations above 65 μ M. However, other optimized peptides including **20-2**, **20-5**, **30-2**, and **40-2** are nontoxic at relevant concentrations, with LC₅₀ values 10-fold greater than those for the least potent peptides and many orders of magnitude higher than those for the most potent peptides (Table 3). To test whether these gains in potency and/or therapeutic indices were due to differences in stability under the conditions used in the neutralization assays, we used LC–

MS to measure peptide stability in PBS and cell growth medium complemented with serum. For the N-terminal Cys-containing peptides **2C** and **20-2**, glutathione (GST) adducts were detected starting around 30 min during the 24 h monitoring. We never detected GST adducts of **40-2** (having an N-terminal Tyr residue), and peptide degradation was negligible (Table S3). We interpreted these results to suggest that formation of GST adducts did not account for variations in activity because they formed around the time the membrane fusion process is complete,¹⁵ and peptides containing (**20-2**) or lacking (**40-2**) a Cys residue showed comparable potencies.

Optimized Peptides Show Increased Breadth and Potency against R5-, X4- and Dual-Tropic (R5/X4) HIV-1 Strains.

In addition to improved potency against the R5-tropic primary isolate YU2, the new-generation peptides were approximately 10-fold more potent than the starting peptide (**2C**) against other strains, including R5-tropic BaL26 and JRFL and X4-tropic HxB2 and NL4-3 (Table 3). The new-generation peptides **30-2** and **40-2** gained activity against the R5-tropic JRCSF and SF162, for which no activity was observed with previous generations of peptides (Table 4). Interestingly, the new-generation peptides were able to inhibit the dual-tropic strain 89.6 at low to submicromolar concentrations (peptide **20-2**, IC₅₀ of 13 μ M; **20-5**, IC₅₀ of 7 μ M; and **30-2** IC₅₀ of 0.57 μ M). Overall, **30-2** and **40-2** were the most active peptides of this series. Peptide **40-2** was the most active peptide against YU2, while **30-2**, in which the N-terminal Cys 178 is maintained, showed submicromolar activities against both the R5-tropic YU2 and the R5/X4-tropic 89.6. Also, peptides **30-2** and **40-2** had activities at single digit and low tens of μ M concentrations across multiple R5- and X4-tropic viruses (Tables 3 and 4). Interestingly, peptides **20-13** and **20-16**, which contain Trp at position 181 but otherwise share the same sequence as peptides **20-2** and **20-5**, were active against the R5-tropic strain YU2 and X4-tropic strain NL4-3, but are inactive against dual-tropic 89.6 (Table S2).

Effects of 40-2 in Combination with CCR5 Nt and 2C Suggest Alternative Modes of Binding within the Coreceptor Binding Site.

To probe the binding site of the new-generation peptides and to test the mode of inhibition compared to the natural peptide **2C**, we carried out infectivity assays using constant combinations of pairs of inhibitors on HIV-1 YU2 (Table 5). These included peptide **40-2** in combination with either **2C** or a synthetic sulfated CCR5 N-terminal peptide **Nt** (amino acids 2–18).¹⁶ The constant combination dose–response curves were analyzed according to the method of Chou and Talalay, where combination indices (CI) less than, equal to, or greater than 1 are indicative of synergistic, additive, or antagonistic effects, respectively.^{10,12} The CI of 1 for the **2C:Nt** combination indicated an additive effect, suggesting separate binding sites on gp120 for the **2C** and **Nt** peptides of CCR5, consistent with previous results.⁵ However, the CI for the **Nt:40-2** combination of 1.2–1.5 indicates a weakly antagonistic effect and suggests that the binding site of **40-2** is at or very near to the CCR5 N-terminus binding site. Consistent with this finding, the CI for the **2C:40-2** combination of 0.7–0.8 indicates a weakly synergistic effect. Taken together, these data suggest that the binding site of **40-2** on gp120 may differ slightly compared to the binding site of **2C**.

2C and the New-Generation Peptides Block HIV-1 Capture by V3 Loop-Directed Antibodies.

To further probe the binding site of the new-generation peptides versus the parent peptide, **2C**, we performed virion-capture assays using HIV-1 BaL viral particles and a panel of monoclonal antibodies (mAbs) that target the coreceptor binding site (Figure 3) or other gp120 epitopes.

Select mAbs were immobilized on magnetic beads, and incubated with HIV-1 virions in the presence of varying concentrations of peptides, or buffer as a control. Peptide-binding sites were inferred by their ability to block virion capture by specific mAbs, as quantified by the amount of p24 Gag viral protein present in captured and lysed virions. The coreceptor-binding site on gp120 is composed of the bridging sheet (β strands 20 and 21) and the base of the third variable loop (V3),¹⁷ and models have implicated the V3 loop itself in binding to the coreceptor CCR5.^{18,19} Thus, we used a panel of V3 loop-directed antibodies that bind to each of these regions to probe peptide binding. Antibody 412d recognizes a well-defined epitope at the base of V3,^{16,20} while 447–52D, D19, and B4e8 bind to the tip of V3.^{21–26} The epitope of 268D is discontinuous and overlaps widely with epitopes of 447–52D and D19.^{21,26,27} Antibody 19b has its epitope in the crown of the V3 tip, and extends by a couple of amino acids into the remainder of the tip.²⁸ Antibody 48d recognizes a discontinuous epitope in the bridging sheet, which in part extends to the V3 loop.^{17,29} Broadly neutralizing antibodies PG16 and PGT135 were used as controls (Figure S1) as PGT135 binds the intrinsic mannose patch of the gp120 glycan shield at Asn332, Asn392, and Asn386 glycosylation sites, adjacent to the V3 loop;³⁰ and PG16 recognizes glycopeptide epitopes in the V1–V2 region of gp120.³¹ Peptide **2C** and the new-generation peptides **18-2**, **20-2**, and **40-2** all inhibited virus capture by 412d (V3 base epitope), although parent **2C** was the most effective. **2C** was modestly effective at blocking virion capture by 447–52D, whose epitope centers around the crown of the V3 tip, while peptides **18-2**, **20-2**, and **40-2** blocked poorly. Interestingly neither **2C** nor **40-2** blocked capture by B4e8 or D19, additional V3-tip binding antibodies (Figure S1). Both **2C** and **40-2** effectively blocked virus capture by 268-D, 19b, and 48d, antibodies that bind to discontinuous regions of V3.⁴⁰ Overall, both **2C** and the new-generation peptides blocked virus capture by antibodies whose epitopes include the base and stem of V3, and to a lesser degree the V3 tip.

DISCUSSION

In the area of HIV entry, peptides and peptide conjugates have been important in elucidating the mechanisms of membrane fusion as well as in providing new leads for designing therapeutics. The FDA-approved peptide drug enfuvirtide comprises a region of the C-helices of gp41 and blocks membrane fusion by binding to the gp41 N-helical region.³² Constrained peptides that present CD4-derived sequences are extremely potent entry inhibitors and bind gp120 with nanomolar dissociation constants.³³ Peptides derived from complementarity-determining-region (CDR) loops of mAbs that bind to the coreceptor binding site inhibit HIV infection³⁴ and are being developed as inhibitors through incorporation into biologics.³⁵ Several previous studies demonstrated that peptides comprising the second extracellular loop of CCR5, and more specifically the C-terminal portion of ECL2 (**2C**), could also block HIV-1 entry, albeit with modest potency in the

midmicromolar range.^{5,36,37} Because residues that govern binding of ECL2 peptides to gp120 had been identified, we used that information to systematically improve potency by modifying the ECL2-gp120 binding interface. Amino acid scans of key positions, followed by iterative accumulations of mutations at additional positions important for gp120 binding (Figure 1), led to a 100-fold improvement in potency over the parent **2C** peptide against the primary HIV-1 isolate YU2, along with improved activity against additional strains, including inhibition for a dual-tropic HIV-1 strain.

Superficially, it could appear that merely increasing hydrophobicity of the peptides accounts for increased potency. Class I membrane fusion occurs when viral and host cell membranes are brought into contact, or fuse, allowing for transfer of genetic material. Though the precise details of the final steps of HIV entry and membrane fusion have yet to be elucidated, this process requires viral fusion peptides and membrane-proximal regions often juxtaposed to amphipathic helices. To consider HIV-1 coreceptor derived peptides for further development, it is useful to elucidate modes of binding and inhibition that may involve both virus and host cell proteins. On the basis of transferred NOE data, it was suggested that a full-length ECL2 peptide binds to gp120 and serum via nonspecific hydrophobic interactions. Wimley and colleagues have studied the properties of peptides derived from various viral fusion peptides or helices and suggested that amphipathic peptides containing aromatic and hydrophobic amino acids have the potential to act as broad-spectrum antivirals by binding to transient amphipathic peptide–lipid interfaces exposed during the fusion process.³⁸ This latter mechanism was of interest here due to the presence of hydrophobic, aromatic residues in the **2C** peptides, together with the suggestion that their binding to gp120 occurred nonspecifically. Though it remains possible that nonspecific mechanisms contribute to the fusion-blocking activity of the **2C** peptide analogues reported here, a number of corroborative findings support direct interactions with HIV-1 gp120 and suggest that binding takes place with some degree of specificity. For example, a scrambled **2C** peptide having the same hydrophobicity parameters (Table S4) as **2C** was inactive in HIV-1 neutralization assays, as was a retro-inverso version of **2C** (Table S2); and **2C** was inactive toward amphotropic pseudotyped murine leukemia virus and vesicular stomatitis virus, arguing against broad-spectrum antiviral activity due to hydrophobic interactions (Supporting Information).

Expanding on the notion of specificity, through iterative optimization of **2C** peptide libraries we found that indiscriminate incorporation of hydrophobic amino acids did not lead to improved activity. Rather, there were strict preferences for certain amino acids at specific positions within the peptide sequence. In particular, single amino acid changes could have large detrimental effects on potency, while other changes could lead to toxicity toward mammalian cells. To view the trends in activity associated with amino acid type and position, we plotted IC₅₀ values as a function of hydrophobicity (Figure 4). An overall increase in potency was observed with increased hydrophobicity. However, within any group of peptides possessing the same hydrophobicity value (x-axis) a large range of IC₅₀ values was observed (expansion, Figure 4b), suggesting the need for site-specific substitutions. Sequence alignments of the most potent peptides in each bin provided consensus substitutions that led to increased potency. These include His 181 to Tyr, Phe 182 to Tyr, Tyr

184 to Phe or Trp, and Phe 189 to Tyr or Trp. Two other positions implicated in binding to gp120 including Tyr 187 and Trp 190 could not be changed without inducing strong cellular cytotoxicity or deleterious reductions in efficacy.

HIV-1 requires interaction with a coreceptor to promote membrane fusion. For CCR5, the extracellular regions comprising the sulfated N-terminus and ECL2 are thought to mediate interactions with gp120. The binding site of **Nt** has been revealed experimentally by NMR and X-ray crystallography, while that of ECL2 is more speculative. Virus capture assays using a panel of antibodies showed that **2C** and **40-2** effectively blocked capture of CD4-bound viral particles by 412d, 268-D, and 19b, and to a lesser extent 48d. Peptides **2C** and **40-2** only modestly blocked binding to 447–52d, and neither inhibited virion capture by antibodies D19 and B4e8. These results suggest that both peptides interact with gp120 through specific interactions that center around the coreceptor binding site and base-to-stem of V3 more than the tip of V3. For example, while antibodies 268-D and 19b bind V3-derived peptides, they do not strictly require the conserved Gly-Pro-Gly-Arg motif located at the tip of V3.²¹ On the other hand, 447–52d, which was modestly inhibited by the peptides, and D19 and B4e8, which were unaffected by the peptides, strictly require the V3 tip for binding.²¹ The slight differences in effects by **2C**, **20-2**, and **40-2** on V3-binding antibodies could reflect differences in their binding footprints, affinities, or both. Structures of additional V3-directed antibodies in complex with trimeric gp140s could help explain these subtle differences.

Our finding that some peptides gain inhibitory activity toward X4-tropic and/or dual-tropic viruses cannot easily be explained with currently available structural information. X-ray crystal structures of CXCR4 and CCR5 have been solved in complex with either small molecule or peptide antagonists, or chemokine fragments.^{18,19,39} From those structures, models of gp120 V3 loops binding to either CXCR4 or CCR5 have been built. Analyses of the coreceptor–gp120 interactions proposed in those models focus on interactions between the V3 loop and the inner barrel of each coreceptor. Among the peptide libraries generated here, we found that Tyr 187 could not be changed without deleterious effects. The models suggest that an interaction involving Tyr 187 may be important for inhibition by **2C** peptides.¹⁸ In particular, in a CCR5-V3 loop model Tyr187 located on ECL2 of CCR5 is positioned to interact with Ser 306 of an R5 V3 loop.¹⁸ Though this interaction is not observed in the V3 loop of X4 models, polar or charged residues are located near to Tyr 187, providing a possible explanation for the importance of this residue.

In summary, we have generated new submicromolar peptide inhibitors of HIV-1 entry. Experimental data demonstrate that site-specific modifications, that do or do not increase hydrophobicity, lead to improved potency. The fact that **2C** peptide variants with a scrambled or retro-inverso sequence but identical hydrodynamic parameters were uniformly inactive in neutralization indicates that the positions and display of hydrophobic amino acids are important for function. The lack of extended secondary structure in these peptides, as determined by circular dichroism, suggests that the sequence locations are unrelated to secondary structure. The stability and nanomolar potency of several lead peptides provide a basis for further development, and may facilitate structural and biochemical studies of HIV-1 envelope glycoproteins in complex with a critical region of CCR5.

Supplementary Material

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Figure 1.

CCR5 and its second extracellular loop ECL2. (A) Ribbon drawing of the structure of CCR5 (PDB ID: 4MBS) and (B) close-up of the ECL2 region. The N- and C-terminal regions, colored blue and red respectively, are separated by Cys 178 shown in spheres. (C) Sequence of peptide **2C** corresponding to the C-terminal portion of ECL2. Amino acid residues previously discovered to be important for binding to gp120 are shown in red.

Figure 2.

Neutralization activity of new-generation peptides against HIV-1 YU2. Multiple IC₅₀ values obtained in separate neutralization assays are shown for each peptide in scatter dot plots, with the thick and the thin lines, parallel to the *x* axis, denoting the median and interquartile ranges, respectively. Numerical data of the most representative experiment for each peptide are provided in Tables 4 and S2.

Figure 3.

Effect of 2C peptides on virion capture by HIV-1 V3 loop-directed antibodies. Virion-capture assays were performed as described in the Experimental Section. (A) Effects of peptides 2C, 18-2, 20-2, and 40-2 on virion capture by the well-characterized mAbs 412d and 447-52d that bind the base and tip of the V3 loop and (B) effects of 2C and 40-2 on three other V3-recognizing mAbs. Decreased percent capture indicates inhibition of virus capture. Peptides that did not block antibody-mediated virion capture by at least 30% were considered inactive (dotted line at 70% capture relative to control). (C) Surface representation of HIV-1 gp120 in complex with CD4 and 412d (PDB ID: 2QAD) showing a close-up view of the V3 region. The V3 loop of gp120 is colored gray, the tip that includes the conserved –GPGR– motif is colored orange, the base of V3 is colored blue, and the footprint of 412d is colored red. The region of 412d containing two sulfotyrosine residues that bind the base of V3 is shown as a red ribbon, and the sulfotyrosine residues are shown as yellow sticks. The colors of the histograms correspond to the epitope on gp120. Bars with two colors (412d and 19b) indicate overlapping antibody epitopes.

Figure 4.

IC₅₀ values of peptides plotted as a function of hydrophobicity. Peptide sequences shown in the box on the right correspond to the boxed data in the main plot. This shows that peptides with the same hydrophobicity parameters can display potencies that differ by around 50-fold, suggesting that hydrophobicity alone does not explain potency.

Table 1.

Effect of Amino Acid Scanning at Positions Y184 and Y187

		% viral infection at three concns ^a		
ID	peptide	200 μ M	100 μ M	50 μ M
2C	2C	36	47	61
12-1	2C- Cys178	93	99	99
12-2	2C- Cys178-184F ^b	35	42	54
12-3	2C- Cys178-184V	75	90	100
12-4	2C- Cys178-184W	38	51	68
12-5	2C- Cys178-187R	68	83	102
12-6	2C- Cys178-187T	68	72	75
12-7	2C- Cys178-187V	51	76	96

^a Measured as described in single round neutralization assays with TZM-bl cells and HIV-1 YU2 pseudotyped viral particles.⁹ In these initial screening experiments assays were performed with single concentrations and experiments.

^b Double mutant variants of peptide 2C lack the N-terminal Cys and contain an amino acid substitution at position 184 or 187 as indicated. See Table S1 for a complete list of peptides studied.

Table 2.IC₅₀ Values (μ M) and Cytotoxicity for the New-Generation Peptides

Peptide	Sequence ^a			IC ₅₀ (μ M) YU2 (R5) ^b	TI ^c (μ M)
	178	184	187		
2C	CSSHFPYSQYQFWK			28 $\pm$ 7 ^d	>125–250
12-1	SSHFPYSQYQFWK			208 $\pm$ 11	>500–1000
12-2	F.....			96.7 $\pm$ 15	>500
12-3	V.....			nt ^f	>5
12-4	W.....			122 $\pm$ 19	>500
12-5	R....			2182 $\pm$ 194	nt
12-6	T....			1285 $\pm$ 159	nt
12-7	V....			753 $\pm$ 398	nt
14-1	F..R....			1170 $\pm$ 134	>500–1000
14-2	W..R....			726 $\pm$ 31	>500–1000
14-3	F..T....			611 $\pm$ 69	nt
14-4	W..T....			147 $\pm$ 26	ND ^e
14-5	F..V....			NA ^e	>500
14-6	W..V....			NA	>200
16-3	C....W.....			14.5 $\pm$ 2.1	nt
16-4	C.....F....			nt	>100
16-5	C....F..F....			nt	>50
16-6	C....F..V....			nt	>5
16-7	C....W..V....			nt	>75

^aAmino acid sequence numbered as in ECL2 of CCR5, Figure 1.^bCCR5-tropic HIV strain. Neutralization assays were performed as described previously.⁹^cToxicity index. Ratio of cytotoxicity to neutralization IC values.^d₅₀ Data from ref 5.^ent, not tested due to weak HIV inhibitory activity or high cellular toxicity.^fNA, not active at 500 μ M.

Table 3.

IC₅₀ Values (μM) against Multiple HIV-1 Strains and Cytotoxicity of the New-Generation Peptides^a

Peptide	Sequence	181	184	189	R5 ^b		R5/X4		X4		LC ₅₀ ^c (μM)	d _{TI} (μM)
		YU2	BaL26	89.6	HxB2	NL4-3						
2C ^e	CSSHFPYSQYQFWK	28.0 ± 7	65.0 ± 3.0	NA ^f	ND ^f	53.0 ± 2	190					>125–250
16-2	F.....	5.1 ± 0.6	ND	NA	167 ± 132	44.4 ± 13.3	80					>63–250
18-2	...Y..F.....	1.9 ± 0.6	27.4 ± 5.6	132 ± 111	72 ± 89	11.8 ± 2.6	80					>63–125
20-2	...Y.F...W..	0.42 ± 0.1	14.1 ± 2.9	13 ± 1.9	>50	7.6 ± 1.3	330					>250
20-5	...YYF...W..	0.72 ± 0.2	9.5 ± 2.0	7.2 ± 1.0	>100	5.9 ± 0.8	ND					ND
30-2	...Y.W...W..	0.96 ± 0.1	6.4 ± 0.9	0.6 ± 0.2	21.3 ± 5.2	3.8 ± 0.4	Non-toxic ^f					>500
40-2	Y.Y.F...W..	0.26 ± 0.1	21.2 ± 5.5	26.9 ± 6.1	40.3 ± 13.4	17.1 ± 3.8	550					>250
50-2	Y.Y.W...W..	2.1 ± 0.4	ND	ND	ND	ND	ND					ND
60-2	S.Y.W...W..	2.7 ± 0.5	ND	ND	ND	ND	ND					ND

^aData for select peptides. See Table S2 for additional peptides studied.

^bVirus tropism and HIV strain.

^cEffects on host cell line TZM-bl. Standard deviations averaged 20%.

^dToxicity index. Ratio of cytotoxicity to neutralization IC₅₀ values.

^eData from ref 5.

^fEither peptides noted were not active, NA, at 500 μM; the IC₅₀ values were not determined, ND, due to low potency and/or toxicity; or the peptides were nontoxic at 500 μM.

Table 4.

Heat Map of IC₅₀ Values (μ M) for New-Generation Peptides against a Broader Panel of HIV Env-Pseudotyped Strains

	R5 ^a					R5/X4 ^a	X4 ^a	
Peptide	YU2 ^b	BaL26 ^b	JRFL ^b	JRCSF ^b	SF162 ^b	89.6 ^b	HxB2 ^b	NL4-3 ^b
2C ^c	30	65	ND ^d	NA ^d	NA ^d	NA ^d	54	53
16-2	5	ND ^d	ND ^d	ND ^d	ND ^d	NA ^d	167	44
18-2	1.9	27	ND ^d	ND ^d	ND ^d	132	72	12
20-2	0.42	14	56	NA ^d	NA ^d	13	>50	8
20-5	0.72	9.5	75	NA ^d	NA ^d	7	>100	6
30-2	0.96	6	8	47	24	0.57	21	4
40-2	0.26	21	35	91	69	27	40	17

^aVirus tropism. R5, R5/X4, and X4 are CCR5-, dual-, and CXCR4-tropic strains, respectively. Neutralization assays were performed as described previously.⁹

^bHIV strain.

^cReference 5.

^dIC₅₀ (μ M) key: red, <1; orange, <15; yellow, 40; green, 100; white, >100 or ND, not determined; gray, NA, not active at 500 μ M.

Table 5.Activity of Combinations of CCR5 2C, 40–2, and CCR5 Nt (2–18)^a

combination ratio ^b	dose reduction index (DRI)		combination index (CI)
2C:Nt ^c	2C	Nt	
1:1	2.1 ± 0.4	3.9 ± 0.8	0.9 ± 0.2
1:2	2.6 ± 0.5	2.4 ± 0.5	1.0 ± 0.2
1:5	4.9 ± 1.0	1.9 ± 0.4	0.9 ± 0.3
2C:40–2	2C	40–2	
50:1	5.8 ± 2.0	2.3 ± 0.9	0.7 ± 0.4
100:1	3.4 ± 1.1	2.7 ± 1.0	0.8 ± 0.4
200:1	3.5 ± 0.6	2.6 ± 0.7	0.8 ± 0.3
Nt:40–2	Nt	40–2	
40:1	7.0 ± 1.8	1.0 ± 0.2	1.3 ± 0.6
200:1	2.0 ± 0.4	1.4 ± 0.3	1.5 ± 0.5
400:1	4.4 ± 1.3	1.3 ± 0.2	1.2 ± 0.5

^aAll experiments were performed as described previously using YU2 pseudotyped HIV virus. The DRI is the ratio of the IC₅₀ in the absence and presence of the second inhibitor. IC₅₀ values for each of the three inhibitors on their own, measured in parallel with the combination experiments, were as follows: **2C**, 70 ± 11 μM; **40–2**, 0.58 ± 0.2 μM; CCR5 **Nt**, 302 ± 27 μM. The CI represents the combined effect of two inhibitors in combination where CI values of <1, 1, and >1 correspond to synergistic, additive, and antagonistic effects, respectively.^{10,12} **Nt**, N terminus.

^bThe ratio of inhibitor concentrations approximates the ratio of the IC₅₀ values of the inhibitors alone.

^cData from ref 5.