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Enhancement of Anti-HIV-1 Activity by Hot Spot Evolution of RANTES-Derived Peptides

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SUMMARY

CCR5, the major HIV-1 coreceptor, is a primary target for HIV-1 entry inhibition strategies. CCL5/RANTES, a natural CCR5 ligand, is one of the most potent HIV-1 entry inhibitors and, therefore, an ideal candidate to derive HIV-1 blockers. Peptides spanning the RANTES N-loop/β1-strand region act as specific CCR5 antagonists, with their hydrophobic N- and C termini playing a crucial role in virus blockade. Here, hydrophobic surfaces were enhanced by tryptophan substitution of aromatic residues, highlighting position 27 as a critical hot spot for HIV-1 blockade. In a further molecular evolution step, C-terminal engraftment of RANTES 40′ loop produced a peptide with the highest solubility and anti-HIV-1 activity. These modified peptides represent leads for the development of effective HIV-1 inhibitors and microbicides.

INTRODUCTION

HIV-1 is the causative agent of the acquired immunodeficiency syndrome (AIDS) pandemic that has relentlessly afflicted several millions people over the past 3 decades. Despite the massive research commitment, current therapeutic options cannot eradicate the infection and remain financially prohibitive for most AIDS sufferers worldwide. The urgent need for novel therapeutics, including a long-sought vaccine, effective microbicides, and new systemic drugs, is therefore a major goal. New classes of drugs against HIV-1 have recently been developed, including inhibitors of virus entry into target cells. HIV-1 particles dock to target cells by interacting, via the envelope protein gp120, with two receptors, CD4 and a chemokine receptor, either CCR5 or CXCR4 (Lusso, 2006). Following these initial interactions, which include profound conformational changes, HIV-1 particles enter target cells by a gp41-mediated mechanism of virus-cell membrane fusion (Colman and Lawrence, 2003). Among the virus-entry inhibitors, T20, a peptide that impedes membrane fusion by intercalating into and locking gp41 upon its conformational change, represents a proof of principle for peptide-based anti-HIV-1 drugs (Kilby et al., 1998). Other entry inhibitors fall into the category of HIV-1 coreceptor antagonists. Most important, CCR5 is a major HIV-1 coreceptor that is almost exclusively used by HIV-1 strains responsible for initial transmission and prevalent throughout the asymptomatic phase of the infection (Lusso, 2006). Therefore, HIV-1 blockade at the CCR5 site represents an ideal therapeutic route, corroborated by the discovery that the CCR5 Δ32 deletion confers HIV-1 infection resistance (Huang et al., 1996). In order to avoid any proinflammatory activity, CCR5-targeting HIV-1 blockers should ideally not activate the receptor; hence, CCR5 antagonism is an important prerequisite. Maraviroc, a small chemical compound blocking HIV-1 acting as a CCR5 antagonist, is a successful example in this class of HIV-1 blockers (Gilliam et al., 2011). An intriguing alternative to the high-throughput screening of large chemical compound libraries is represented by the engineering of CCR5 natural chemokine ligands, as they are strong HIV-1 blockers (Cocchi et al., 1995; Lusso, 2006). Given the extensive knowledge on its structure and its potent anti-HIV-1 activity, CCL5/RANTES (Regulated upon Activation, Normal T cell Expressed, and Secreted) represents an ideal molecule to derive CCR5 antagonists with superior HIV-1 blocking capability (Vangelista et al., 2008). The CCL5/RANTES N terminus has been found to be the moiety responsible for CCR5 activation (Hartley and Offord, 2005). Several mutants and engineered variants of this region have been developed with interesting anti-HIV-1 features; however, most of these RANTES derivatives retained strong CCR5 agonistic activity (Vangelista et al., 2008). CCL5/RANTES mutants deprived of CCR5-activating propensity have been produced, such as C1C5-RANTES, whose anti-HIV-1 activity is similar to that of the wild-type protein (Polo et al., 2000; Vangelista et al., 2010), and 5P12-RANTES, a highly active anti-HIV-1 blocker (Gaertner et al., 2008). In parallel to full-length RANTES mutagenesis and chemical modification, short peptides have been derived that encompass the N-loop/β1-strand region of RANTES, revealing that this moiety accounts for CCR5 binding in a nonactivating mode (Nardese et al., 2001). Although RANTES full-length derivatives present higher anti-HIV-1 potency, increasing their potential clinical applicability, peptide derivatives should bear the advantages of a superior proteolysis resistance and a lower risk of eliciting a host antibody response.

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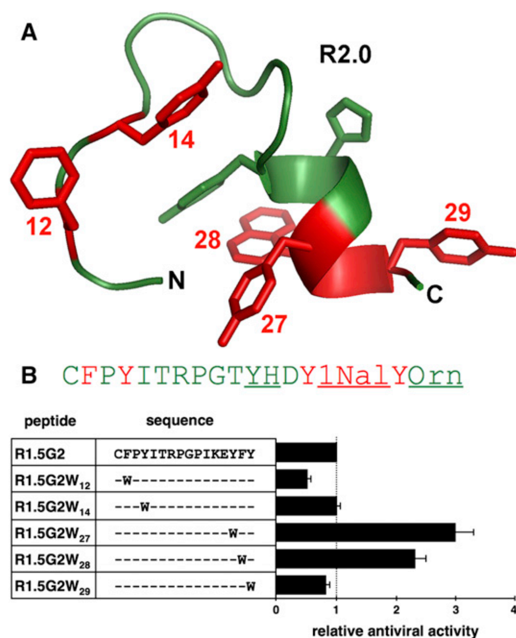

Figure 1. Tryptophan Scanning at Aromatic Residue Positions

(A) Three-dimensional representation of peptide R2.0, whose aromatic residues' side chains are shown, was generated using PyMOL. The NMR coordinates used were chosen arbitrarily among a large set. In red, the five original aromatic positions (12, 14, 27, 28, and 29) present in R11-29 and R1.5G2. N and C, denote N and C termini, respectively. The R2.0 amino acid sequence has the same color code of the NMR coordinates. Some aromatic residues, present in R2.0 but not in R11-29, and nonnatural amino acids are underlined.

(B) Inhibition of HIV-1_{BaL} envelope-mediated fusion by tryptophan-substituted R1.5G2 peptides. The antiviral activity for each peptide is shown relative to the IC_{50} calculated for R1.5G2 (468 nM), whose amino acid sequence is indicated.

In addition, the prototype peptide R11-29, spanning RANTES amino acids 11 to 29, has been produced in a retroinverted version that presented anti-HIV-1 activity similar to R11-29, disclosing an option for peptide variants with exceptional resistance to proteolysis (Nardese et al., 2001). R11-29 has subsequently been investigated by alanine substitution in hydrophobic regions present at the N and C termini, highlighting the importance of key hydrophobic residues (Vangelista et al., 2006). More recently, RANTES N-loop/β1-strand-derived peptides have been rationally designed to improve structuring and reduce the hydrophilic linker connecting the N- and C-terminal hydrophobic patches, achieving significant improvement in antiviral activity (Lusso et al., 2011).

In this report, the anti-HIV-1 activity of RANTES N-loop/β1-strand peptides has been significantly improved. Tryptophan substitution of aromatic residues highlighted position 27 as a hot spot for anti-HIV-1 activity. In addition, according to a previous mapping of CCL5/RANTES interaction with a CCR5 N-terminal peptide (Duma et al., 2007), a segment corresponding to the 40′ loop of the chemokine was added at the peptide C terminus, improving the resulting peptide's anti-HIV-1 activity and solubility. The potency of these new HIV-1 blockers has been tested in several cellular systems on a panel of R5 HIV-1 strains. The rationale for the possible implementation of these molecularly evolved peptides in the therapy or prevention of HIV-1 infection is also discussed.

RESULTS AND DISCUSSION

Evolution of RANTES-Derived Peptides: Anti-HIV-1 Activity Enhancement, Structure Stabilization, and Suitability for Recombinant Expression

The prototype RANTES-derived peptide R11-29 encompasses the N-loop/β1-strand region of the chemokine, which is involved in CCR5 docking but is not sufficient for receptor activation (Nardese et al., 2001). The N- and C-terminal hydrophobic patches of R11-29 were subsequently shown to be key elements for the anti-HIV-1 activity (Vangelista et al., 2006). Recently, R11-29 has been rationally refined through a sequence of steps that, in envelope-mediated fusion assays, lowered the 50% inhibitory concentration (IC_{50}) toward HIV-1_{BaL} approximately 30-fold, from ~3 μM of R11-29 to ~100 nM of peptide R2.0 (Figure 1A) (Lusso et al., 2011). In order to exert their maximal activity, these peptides need to be covalently dimeric (through a disulphide bond at the N-terminal C11), N-terminally acetylated, and C-terminally amidated. However, given the absence of detectable R2.0 monomer-monomer interactions by nuclear magnetic resonance (NMR) (Lusso et al., 2011), peptide structuring appears to occur exclusively within monomers; i.e., not through the aid of tertiary structure assembly. Hence, monomer moieties within covalent dimers appear to be relatively free and independent, a feature that has been further investigated herein. Efforts to increase anti-HIV-1 activity have been paralleled by attempts to engineer recombinant RANTES-derived peptides through: (1) the characterization of peptide monomers; (2) the elimination of the N-terminal acetylation and the exploration of alternative amino acids at the N terminus; (3) the conversion of the C-terminal amidation to a natural carboxyl group; and (4) the choice of natural instead of nonnatural amino acids. It is important to note that recombinant expression could allow the delivery of these peptides as live microbicides, as reported for full-length RANTES variants (Vangelista et al., 2010). Throughout their molecular refinement, our RANTES-derived peptides have been tested against CXCR4-tropic HIV-1 strains, on which they consistently showed no effect (data not shown).

Expansion of the Hydrophobic Surface by Tryptophan Substitution of Aromatic Residues

The hydrophobic N and C termini of RANTES N-loop/β1-strand-derived peptides are crucial for their HIV-1 blocking activity. Of special importance, alanine replacement or deletion of aromatic residues resulted in significant activity decrease (Vangelista et al., 2006). However, substitution of the original residue at position 28 (a phenylalanine) with 3-(1-naphthyl)-alanine (1Nal) led to an increase in anti-HIV-1 activity (Lusso et al., 2011). Therefore, the possibility to improve peptide antiviral activity by increasing the aromatic volume of hydrophobic residues inspired the tryptophan scanning procedure described herein. Many high-affinity protein-protein interactions involve hydrophobic residues, and often tryptophan is engaged into these interacting surfaces, supplying its large planar aromatic area to increase the interaction strength. In these cases, the thermodynamic

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cost of exposing a tryptophan residue to the solvent is balanced by the sequestration of the aromatic side chain from the solvent upon binding with the protein partner. A strong example of the aforementioned feature is provided by the high-affinity receptor for immunoglobulin E (IgE), where several solvent-exposed tryptophan residues are engaged in the binding to IgE (Garman et al., 2000). At the current stage of knowledge on the CCL5/RANTES-CCR5 interaction in terms of three-dimensional organization, antiviral activity improvements could not be assigned with certainty to a direct peptide interaction with CCR5 moieties. Thus, successful tryptophan substitutions could also account for an improvement in the stability of the peptide fold. In the two cases, the tryptophan involved would be either solvent exposed or packed within a minihydrophobic core. In a third possible scenario, a tryptophan residue could interact with the lipids of the cell membrane, as in the case of the HIV-1 gp41 epitope of the neutralizing monoclonal antibody 4E10 (Sun et al., 2008). Such tryptophan-lipid interaction could drive a local peptide enrichment at the cell membrane, facilitating the subsequent interaction with CCR5.

In order to investigate the effects of tryptophan substitutions, we stepped back from R2.0 (Figure 1A), the most active peptide reported to date, and used R1.5G2 as the optimal scaffold to test original aromatic positions for improvement. R1.5G2 contains a threonine residue at position 16, a substitution of the original alanine residue of RANTES, produced to increase water solubility of the resulting peptide (Lusso et al., 2011), a convenient feature for the prospected introduction of highly hydrophobic W residues. Moreover, five aromatic residues are present in R1.5G2, as in the R11-29 peptide representing the original CCL5/RANTES sequence. In a series of cell fusion inhibition assays (Figure 1B), tryptophan substitution of phenylalanine at position 12 (R1.5G2W_{12}) led to a marked decrease in antiviral activity (IC_{50}, 883 nM), while substitution of tyrosine 14 (R1.5G2W_{14}) resulted in an anti-HIV-1 activity identical to that of the original R1.5G2 peptide (IC_{50}, 468 nM). Substitution of tyrosine 29 (R1.5G2W_{29}) led to a decrease in anti-HIV-1 activity (IC_{50}, 616 nM). As expected, however, substitution of phenylalanine 28 with tryptophan (R1.5G2W_{28}) led to a significant increase of antiviral activity (IC_{50}, 207 nM). A favorable increase of hydrophobicity/aromatic surface in the amino acid side chain at position 28 has already been documented with the 1Nal substitution (Lusso et al., 2011), which should exert an effect similar to that of tryptophan. However, the use of tryptophan instead of 1Nal would allow a step toward the conversion of peptide production from the exclusive chemical synthesis to the option of recombinant expression systems. In search for the optimal aromatic residue to place at position 28, a tyrosine substitution was also produced in the R1.5G2 scaffold, leading to a 27–29 YYY motif. It is interesting that Y28 led to a significant increase in anti-HIV-1 activity (IC_{50} ~350 nM; data not shown) with respect to the original F28 in R1.5G2; however, the Y28 variant was less efficient in inhibiting HIV-1 as compared to the W28 variant. Remarkably, tryptophan substitution of tyrosine at position 27 (R1.5G2W_{27}) led to the highest increase in antiviral activity (IC_{50},149 nM), a striking result, and a milestone for the subsequent molecular analysis and evolution of RANTES-based peptides. In summary, the original aromatic residues in positions 12 and 29 (phenylalanine and tyrosine, respectively) appear to be the most favorable for the anti-HIV-1 activity of these peptides, confirming previous data (Vangelista et al., 2006). Tyrosine in position 14 could be exchanged for tryptophan with no difference in HIV-1 inhibition; hence, this substitution would only provide an unnecessary hydrophobicity load, likely resulting in reduced water solubility of the peptide. Most important, both peptides in which the original Y27 and F28 were separately substituted by W led to a substantial increase in antiviral activity.

A recent report on the three-dimensional organization of CCL5/RANTES oligomers highlighted the 27−29 stretch as one of the regions involved in the oligomerization surface (Wang et al., 2011). This evidence implies that residues 27–29 could be recruited for protein-protein interactions. The need for CCL5/RANTES to be in its monomeric form to efficiently bind CCR5 (Duma et al., 2007) implies the exposure of the 27–29 hydrophobic/aromatic residue stretch, likely making it available for CCR5 interaction.

Modifications toward Natural N and C Termini

An initial screening of peptides (based on R1.5G2 as scaffold) with N-terminal amino acid alternatives to acetylated C11, in which nonacetylated single residues (M, A, S, T, Y, and W) preceded C11, did not provide any encouraging result in terms of HIV-1 inhibitory activity (data not shown). The hydrophobic or polar amino acids tested were selected in an attempt to reach a balance between the need for a hydrophobic N-terminal moiety (important for anti-HIV-1 activity) and a sufficient solubility in water. Despite the failure of this approach, M and Y N-terminal elongations produced the best antiviral activities. Some of these peptides (A, M, and W) were also tested in C-terminally amidated versus carboxylated versions, yielding similar antiviral activities but higher water solubility when their C terminus was a natural carboxyl group (data not shown).

In light of this evidence and the results obtained from the tryptophan scanning of aromatic residues in R1.5G2, attempts were made to produce potent anti-HIV-1 peptides with a natural N terminus using the W27 version of R1.5G2 as a scaffold and the natural carboxyl group at the C terminus. Peptide variants were synthesized in which the N-terminal acetyl group was replaced by one to three additional amino acids preceding C11. According to the results from the initial screening, the new N termini selected included M and Y as single residues and combinations of Y- and M-containing double- and triple-amino-acid stretches. However, the anti-HIV-1 activity of these N-terminally modified peptides remained well below that of R1.5G2W_{27}, confirming that the N-terminal acetyl group is crucial for peptide activity (data not shown). The most interesting peptides in terms of antiviral activity and water solubility were those with Y, SY, and SPM preceding C11. It is interesting that these new peptides are fully natural and could be expressed as recombinant molecules. However, as the main focus of this work was HIV-1 blockade, the subsequent characterization steps were conducted in the context of N-terminally acetylated C11 peptides.

W versus 1Nal Comparison at Positions 27 and 28

Given the results obtained with W substitutions at positions 27 and 28 and considering the previously reported enhancement of activity obtained introducing a 1Nal residue at position

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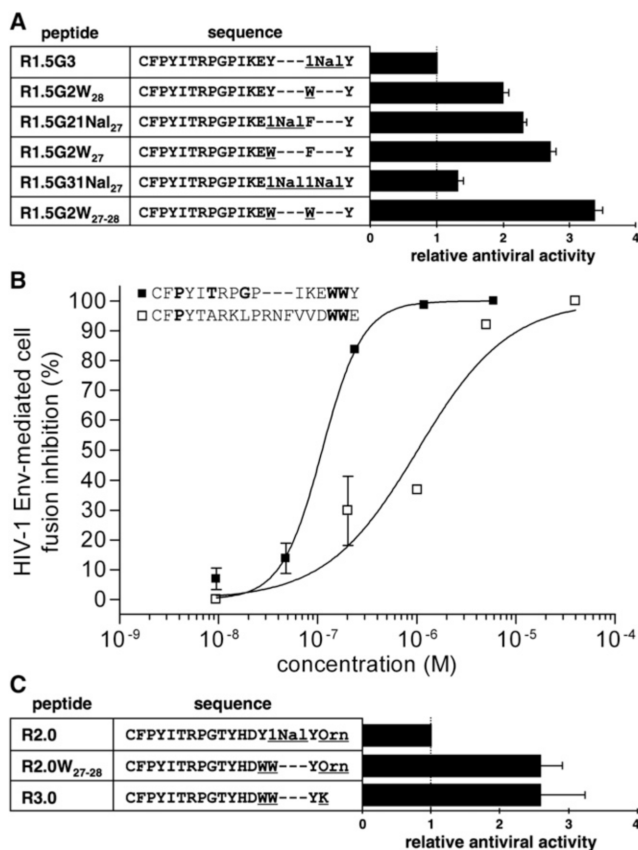

Figure 2. Tryptophan Residues in the β1-Strand Hydrophobic Patch Are Key for HIV-1 Blockade in CC Chemokine Peptide Scaffolds

(A) Comparison of 1Nal versus W residues in the inhibition of HIV-1_{BaL} envelope-mediated fusion. The antiviral activity for each peptide is shown relative to the IC_{50} calculated for R1.5G3 (403 nM). Amino acid sequences are indicated; 1Nal and W residues are underlined.

(B) Comparison of the MIP-1β-derived peptide M12-30PWW (open squares) with R1.5G2W_{27-28} (closed squares) for the inhibition of HIV-1_{BaL} envelope-mediated fusion. Amino acids modified from the original chemokine sequence are in bold, and dashes indicate a portion of the hydrophilic linker previously deleted with no loss of anti-HIV-1 activity (Lusso et al., 2011).

(C) Modification of RANTES-derived peptides from R2.0 to R3.0 and their inhibition of HIV-1_{BaL} envelope-mediated fusion. The antiviral activity for each peptide is shown relative to the IC_{50} calculated for R2.0 (104 nM). Amino acid sequences are indicated, and key residues are underlined.

28 (Lusso et al., 2011), the effect of W versus 1Nal at these positions was compared. A series of peptides was synthesized including double W and 1Nal at positions 27 and 28 (Figure 2A). As reported earlier for the W substitution, the 1Nal substitution of the original Y at position 27 (R1.5G21Nal_{27}) also provided a significant increase in anti-HIV-1 activity (IC_{50}, 175 nM), as compared to R1.5G3, which presents the 1Nal in position 28. The double 1Nal-containing peptide R1.5G31Nal_{27} (positions 27 and 28) yielded a slight increase in anti-HIV-1 activity (IC_{50}, 305 nM) compared to R1.5G3, yet remaining significantly less active than R1.5G21Nal_{27}. This discrepancy might be explained by the possibility that the close proximity of the two bulky nonnatural naphthyl groups may cause repulsions that in turn create distortions or instability in the optimal peptide fold and/or CCR5 interaction conformer. In all cases, the effect provided by W was superior to that derived from the 1Nal substitution. This evidence further supported the possibility to produce these peptides as recombinant molecules. As expected, the highest anti-HIV-1 activity (IC_{50}, 120 nM) was exerted by R1.5G2$W_{27–28}$, the peptide presenting both W27 and W28. Thus, the molecular evolution of these peptides was advanced keeping W residues at both positions 27 and 28.

Investigating MIP-1β-RANTES Similarities and Hot Spot Evolution in N-loop/β1-Strand Region Peptides

CCL5/RANTES and CCL4/MIP-1β share common physiological properties, including the ability to bind CCR5 and block HIV-1 entry, as well as a relatively high sequence similarity; hence, we compared the anti-HIV-1 activities of N-loop/β1-strand peptides derived from the two chemokines. MIP-1β is of special interest since it has been reported to bind solely to CCR5, while RANTES can bind and activate also CCR1 and CCR3. Therefore, a human MIP-1β-derived peptide, corresponding to the R11-29 amino acid region (named M12-30, referring to the MIP-1β amino acid numbering), was synthesized. A second peptide version, a M12-30 S14P variant (named M12-30P), and a third, including the S14P, Y28W and Y29W substitutions (named M12-30PWW), were also synthesized. In cell fusion inhibition assays, the anti-HIV-1 activities of M12-30 and M12-30P were comparable to that of R11-29 (~3 μM; data not shown), providing an interesting general proof of principle for the involvement of the N-loop/β1-strand in the anti-HIV-1 activity of CCR5-binding chemokines. Despite the amino acid sequence differences in the N-loop/β1-strand region of RANTES and MIP-1β, we predicted that the S14P substitution in M12-30 was likely to provide a structure stabilization similar to that reported for the A13P substitution in RANTES-derived peptides (Lusso et al., 2011). Likewise, we postulated that W substitution for Y28 and Y29 in MIP-1β (corresponding to Y27 and F28 in RANTES) would confer a higher antiviral activity. Indeed, M12-30PWW showed a higher HIV-1-blocking activity (IC_{50}, 1 μM), as compared to both M12-30 and M12-30P. Although the anti-HIV-1 activity enhancement observed in R1.5G2$W_{27–28}$ versus R11-29 is by far superior to that of M12-30PWW versus M12-30, the importance for an increase in the hydrophobic/aromatic surface in the side chain of these two positions was confirmed (Figure 2B). Once again, we confirmed the involvement of residues 27 and 28 (28 and 29 for MIP-1β) in the docking/binding of this group of chemokines to CCR5.

Insertion of W27 and W28 into the R2.0 Scaffold

Next, we switched back to the scaffold of R2.0, the peptide previously reported to possess the best anti-HIV-1 activity (Lusso et al., 2011). R2.0 is the result of a thoughtful study aimed at producing a peptide with a more structured linker connecting the two N- and C-terminal hydrophobic amino acid stretches, as compared to the original CCL5/RANTES sequence. Moreover, R2.0 has a C-terminal ornithine residue following Y29, an extension that improved significantly the peptide solubility. Solubility and activity were similar for a C-terminal carboxylated or carboxyamidated R2.0 (data not shown). Hence, a natural carboxyl group at the C terminus was introduced in all the peptides generated from this point onward.

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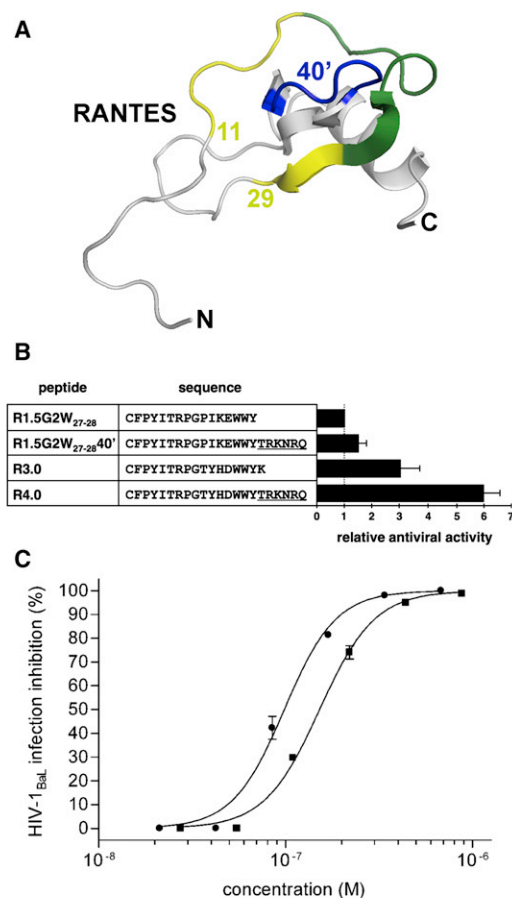


Figure 3. Engraftment of RANTES 40′ Loop Enhances Peptides' Anti-HIV-1 Activity

(A) Peptide regions are highlighted within full-length RANTES three-dimensional structure (PDB coordinates 1HRJ), represented using PyMOL. The 11–29-spanning N-loop/β1-strand is in green, with the two hydrophobic stretches in yellow, while the 40′ loop is in blue. N and C, N and C termini, respectively.

(B) 40′ loop elongation of R1.5G2W_{27-28} and R3.0 (generating R1.5G2W_{27-28}40′ and R4.0, respectively) enhances inhibition of HIV-1_{BaL} envelope-mediated fusion, shown in relation to the IC_{50} of R1.5G2W_{27-28}. Peptide sequences are indicated with the 40′ loop underlined.

(C) HIV-1_{BaL} acute infection inhibition by R3.0 (closed squares) and R4.0 (closed circles).

Peptide R2.0W_{27-28} (in which Y27 and 1Nal28 in R2.0 were both substituted with W residues) exhibited a significantly higher anti-HIV-1 activity (IC_{50}, 40 nM) than R2.0 (Figure 2C). Moreover, substitution of the C-terminal R2.0W_{27-28} ornithine with the closely related natural amino acid lysine (peptide R3.0) led to identical solubility and activity (IC_{50}, 40 nM) (Figure 2C), again pointing toward a possible implementation of these peptides for their expression in recombinant systems.

C-Terminal Engraftment of the RANTES 40′ Loop

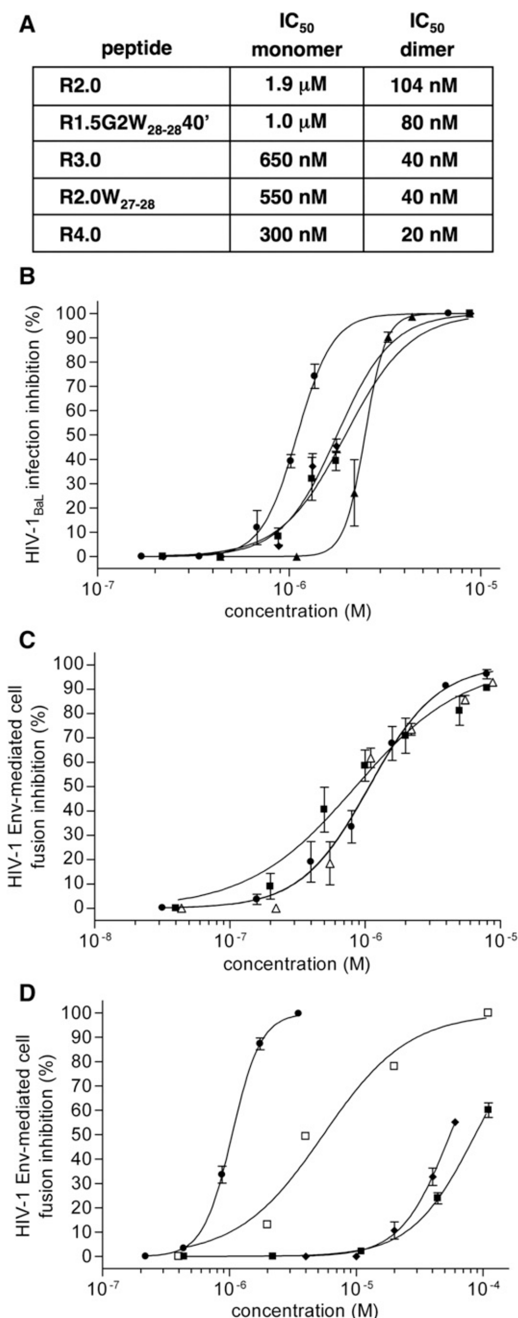
Previous mapping studies on CCL5/RANTES residues contributing to the interaction with a sulphated N-terminal CCR5 peptide documented the involvement of the 40′ loop region, suggesting its potential importance in the antiviral activity (Duma et al., 2007). This region contains a stretch of positively charged amino acids and is exposed to the solvent. Observing the three-dimensional structure of RANTES (Figure 3A), we found that the 40′ loop (indicated in blue) protrudes toward the solvent in a region equidistant from the two hydrophobic clusters identified within RANTES-derived peptides as the CCR5-interacting units (indicated in yellow), namely, the peptide's termini. Thus, we decided to investigate the importance of this region for the antiviral activity of our peptides, as well as its likely beneficial effect on peptide solubility. It is interesting that a threonine residue is present both at RANTES positions 30 and 43; hence, this residue was included following Y29, with amino acids 43–48 (the TRKNRQ stretch encompassing the RANTES 40′ loop) engrafted C-terminally into the peptide. The TRKNRQ stretch was added to R1.5G2W_{27-28} and R3.0, in order to compare its effects on peptides at different stage of molecular evolution. Indeed, the resulting peptides, namely R1.5G2W_{27-28}40′ and R4.0, displayed superior HIV-1 blocking activity (IC_{50}s, 80 nM and 20 nM, respectively), as compared to R1.5G2W_{27-28} and R3.0 (Figure 3B).

The most active peptides, R3.0 and R4.0, were tested also in experiments of acute HIV-1_{BaL} infection (Figure 3C) in which they exerted a hierarchical activity (IC_{50}s, 143 nM and 94 nM, respectively) similar to that observed in the cell fusion inhibition assays. According to their antiviral activity and sequence features, subsequent experiments were carried out in parallel with R3.0 and R4.0.

Monomeric Peptides Reveal a Positive Correlation between Structural Stability and Antiviral Activity

Predictably, the design of more stable RANTES-derived peptides was accompanied by a parallel increase in anti-HIV-1 activity. Given the fact that previous NMR analysis on disulphide-bonded dimeric peptides (R1.5G3 and R2.0) did not reveal significant tertiary interactions (Lusso et al., 2011), we reasoned that peptides with high structural stability could exert higher anti-HIV-1 activity also in their monomeric form, as compared to peptides of older generation (i.e., less structured). Several peptides were then analyzed in cell fusion inhibition experiments in their monomeric form (Figure 4A). Due to the reactive C11 sulfhydryl group present in the monomeric peptides, the exclusive presence of monomers was verified by high-pressure liquid chromatography (HPLC) under experimental conditions similar to those of the anti-HIV-1 assays, in order to rule out any activity interference contributed by the presence of spontaneously oxidized peptide dimers (data not shown). Indeed, peptide monomers followed an activity hierarchy identical to that of the respective dimers, with R3.0, R2.0W_{27-28}, and R4.0 showing remarkably higher antiviral potencies compared to peptides of the previous generation (Figure 4A). For each peptide, the gap in activity was approximately, yet consistently, corresponding to a 10-fold reduction for the monomer in respect to the dimer. Similar results were obtained when testing peptide monomers in acute HIV-1_{BaL} infection experiments (Figure 4B).

The consistent anti-HIV-1 activity increase (directly related to the increase in stability) observed with monomeric peptides justified a second attempt in the reshaping of the N terminus to allow recombinant expression of monomers. According to the initial results, Y, SY, and SPM residues were added at the



A

peptide	IC_{50} monomer	IC_{50} dimer
R2.0	1.9 μM	104 nM
R1.5G2W$_{28-28}$40'	1.0 μM	80 nM
R3.0	650 nM	40 nM
R2.0W$_{27-28}$	550 nM	40 nM
R4.0	300 nM	20 nM

Figure 4. Monomeric Peptides Present an HIV-1 Blocking Hierarchy Identical to that of Dimeric Counterparts

(A) Comparison of the monomer versus dimer HIV-1_{BaL} envelope-mediated fusion inhibition for the most relevant peptides.

(B) HIV-1_{BaL} acute infection inhibition by the monomeric form of R1.5G2W$_{27-28}$40′ (closed triangles; IC_{50}, 2.52 μM), R2.0W$_{27-28}$ (closed rhombi; IC_{50}, 1.77 μM), R3.0 (closed squares; IC_{50}, 2.06 μM) and R4.0 (closed circles; IC_{50}, 969 nM).

(C) HIV-1_{BaL} envelope-mediated fusion inhibition by dimers of SY-R3.0 (closed squares; IC_{50}, 852 nM), SY-R4.0 (closed circles; IC_{50}, 1.08 μM) and SY-R1.5G2W$_{27}$ (open triangles; IC_{50}, 1.06 μM).

(D) HIV-1_{BaL} envelope-mediated fusion inhibition by monomers of R3.0 (closed circles), R3.0 C11S (closed squares), SY-R3.0 (open squares), and SY-R3.0 C11S (closed rhombi).

N terminus of R4.0 and R3.0, thus eliminating the acetyl group. When tested in cell fusion inhibition assays, the dimeric versions of these peptides provided frustrating results, with anti-HIV-1 activities in the same range of the earlier peptide variants containing only the Y27W substitution in the R1.5G2 framework (Figure 4C). Once again, this likely reflected the importance of the acetyl group at the N terminus, whose loss appears to flatten the advantages obtained from the other hot spots. A C11S substitution variant of SY-R3.0 was also produced, hence generating solely peptide monomers. It is surprising that the SY-R3.0 C11S monomer was dramatically less active as HIV-1 inhibitor (IC_{50}, 55 μM) when compared to the SY-R3.0 monomer version (IC_{50}, 5.5 μM) (Figure 4D). As this result was rather unexpected, the C11S variant of R3.0 was also produced (retaining N-terminal acetylation), in order to exclude any effect due to the absence of the N-terminal acetyl group. Again, a dramatic reduction in HIV-1 blocking activity was observed (IC_{50}, 86 μM), confirming the peculiar role played by C11 even in its free thiol group state. Of note, and according to the reported three-dimensional organization of CCL5/RANTES oligomers (Wang et al., 2011), a possible explanation for the C11 effect might be provided with a reasoning similar to that made earlier for the 27–29 amino acid stretch. The cysteine 11 residue has in fact been reported to be involved in the RANTES dimerization surface and, partially, also in the oligomerization process (Wang et al., 2011). Hence, C11 not is only involved in a disulphide bonding with C50 (within full-length RANTES), and in a homodimerization disulphide bonding (in the peptides reported herein) but seems to participate also in the CCL5/RANTES-CCR5 interaction, as evinced from its fundamental importance in the anti-HIV-1 activity of our RANTES-derived peptides.

Broad Spectrum of HIV-1 Blocking Activity of RANTES-Derived Peptides

To assess the antiviral activity of our peptides toward primary viruses that can be encountered in different world regions, several HIV-1 R5 strains were tested in cell fusion inhibition experiments using PM1 cells chronically infected with the different strains as effectors and NIH 3T3 cells expressing human CD4 and CCR5 as targets. The fusion inhibitor T20 was tested in parallel for comparison. As expected, R4.0 showed the highest activity against most strains, and the hierarchy in the peptide molecular evolution was, overall, respected with R4.0 > R3.0 > R2.0 (Figure 5A; Lusso et al., 2011). Since several of the strains tested represent primary CCR5-dependent isolates potentially involved in viral transmission worldwide, the activity exerted by our peptides suggests that these could be implemented in a low-cost microbicide formulation for less developed countries.

RANTES-Derived Peptides Block HIV-1 Infection in Primary Human $CD4^+$ T Cells and Macrophages

Human $CD4^+$ T cells and macrophages are the major cellular targets for HIV-1 infection. Hence, R3.0 and R4.0 were tested in an acute infection assay using HIV-1_{BaL} on human monocyte-derived macrophages established from peripheral blood mononuclear cells (Figure 5B). The two peptides exerted similar potency, with IC_{50}s of 194 nM and 167 nM for R3.0 and R4.0, respectively, confirming their HIV-1 blocking efficiency also on this important cell target.

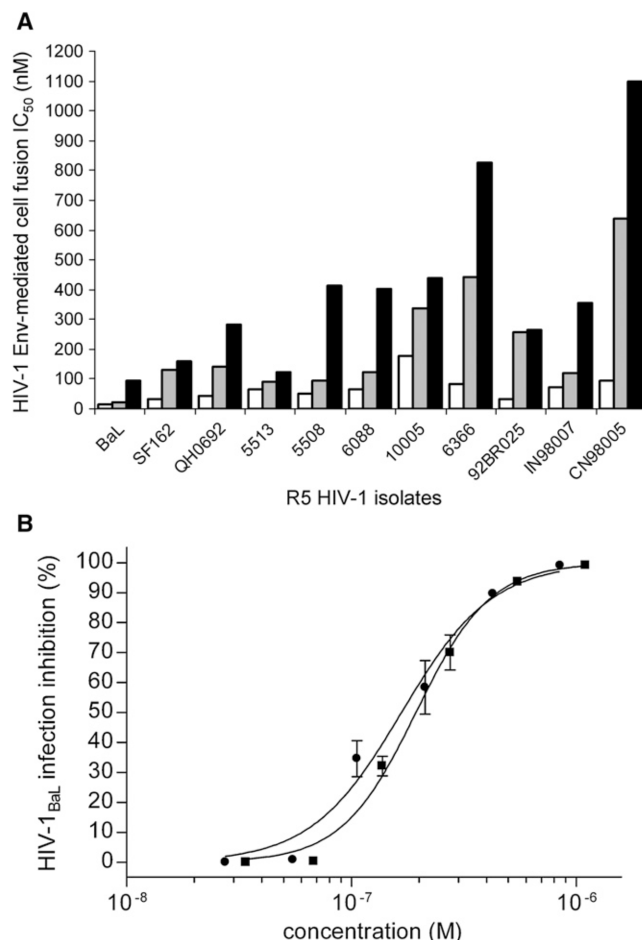


Figure 5. R3.0 and R4.0 Inhibit Several R5 HIV-1 Strains, as Well as $HIV\text{-}1_{BaL}$ Macrophage Infection

(A) R5 HIV-1 envelope-mediated fusion inhibition by T20 (white bars), R3.0 (black bars), and R4.0 (gray bars). Several R5 HIV-1 strains have been tested, including laboratory-adapted (BaL and SF162), primary clade B (QH0692, Trinidad and Tobago) and C (92BR025, Brazil; 98IN007, India; and 98CN005, China), and primary pediatric isolates (IT5513, IT5508, IT6088, IT10005, and IT6366, Italy).

(B) $HIV\text{-}1_{BaL}$ acute infection inhibition of human monocyte-derived macrophages by R3.0 (closed squares) and R4.0 (closed circles).

In order to test the new generation of RANTES-derived peptides in a more physiological context, a cell fusion inhibition assay has been developed in which purified human peripheral blood $CD4^+$ T lymphocytes were used as target cells, instead of NIH 3T3 cells. Considering the fact that resting human $CD4^+$ T cells express very low levels of CCR5, T lymphocytes were preactivated with high doses of interleukin-2 (IL-2; 500 U/ml) for 1 to 3 weeks, a treatment that is known to upregulate CCR5 surface expression (Bleul et al., 1997). As this T-cell-based setup is a novel cell fusion inhibition assay, two experimental variants were implemented to optimize and expand the potentials of the assay. A first variant (more physiological) is based on the IL-2-induced endogenous expression of CCR5, while the second variant takes advantage of the massive CCR5 expression obtained upon vaccinia virus-encoded exogenous CCR5. Effector cells were RK13 cells infected with a $HIV\text{-}1_{BaL}$ envelope glycoprotein-expressing vaccinia virus. R3.0 and R4.0 exerted strong anti-HIV-1 activity in both experimental variants, even though the highest IC_{50}s were obtained using the endogenous CCR5-based assay (18 nM for R3.0 and 10 nM for R4.0) (Figure 6A). In the vaccinia virus-encoded exogenous CCR5 assay, the antiviral activity resulted in an IC_{50} of 32 nM for R3.0 and 19 nM for R4.0 (Figure 6B).

Given the physiologic conditions of the primary $CD4^+$ T cell-based assay, the strong activity revealed by these peptides is very encouraging in the perspective of a potential translation into therapeutics. In order to confirm the results obtained with different primary R5 strains (Figure 5A), we selected the clade C $HIV\text{-}1_{92BR025}$ and used chronically infected PM1 cells as effector cells against IL-2-activated $CD4^+$ T lymphocytes as target cells; PM1 cells chronically infected by $HIV\text{-}1_{BaL}$ were used as control. This latest variant of the cell fusion inhibition assay is even more physiological, as it uses blood human T lymphocytes and a human T-lymphocyte-derived cell line. Inhibition of the two HIV-1 variants (BaL and 92BR025) by R3.0 and R4.0 was slightly higher than in the original assay using NIH 3T3 cells, confirming the effectiveness of these peptides in a physiologically relevant experimental model (Figures 6C and 6D).

CCR5 Antagonism and Receptor Specificity of RANTES-Derived Peptides in Chemotaxis Assays

CCR5 activation or antagonism by R3.0 and R4.0 were assessed using chemotaxis assays. Maraviroc, a well-known CCR5 antagonist was tested in parallel as a control, providing a direct comparison of two molecularly different CCR5 antagonist categories, peptides, and small chemical compounds. As illustrated in Figure 7A, R3.0 and R4.0 showed CCR5 antagonistic activity comparable to that of Maraviroc, potently blocking lymphocyte chemotaxis induced by CCL5/RANTES. When tested in the absence of CCL5/RANTES, neither the RANTES-derived peptides nor Maraviroc exhibited agonistic activity, as the resulting chemotaxis was lower than the basal level of nonstimulated lymphocytes (Figure 7B). Moreover, earlier peptide variants effectively blocked RANTES-induced phosphorylation of p38 MAP kinase, demonstrating an antagonistic effect on this G-protein-independent signaling pathway (Lusso et al., 2011).

Drug resistance remains a major concern in the therapy of HIV-1 infection. While resistance to Maraviroc has been well documented, experimental evidence suggests that resistance to full-length RANTES derivatives as CCR5-directed HIV-1 blockers may be limited (Nedellec et al., 2010, 2011). This advantage with respect to small chemical compounds is likely to be provided by the extensive surface of interaction between CCL5/RANTES and CCR5, a feature that would imply an insurmountable fitness cost for the virus in order to maintain CCR5 usage through extensive mutation. The alternative of a switch to CXCR4 usage appears to be remote, given the emergence of these strains only at late stages of HIV-1 infection, especially in prophylactic regimens, and considering the rarity of CXCR4 usage at primary infection. Although RANTES-derived peptides cannot cover the same extension in terms of CCR5 interaction surface, they are likely to be more restrictive than Maraviroc in terms of emergence of resistant strains. Indeed, our attempts

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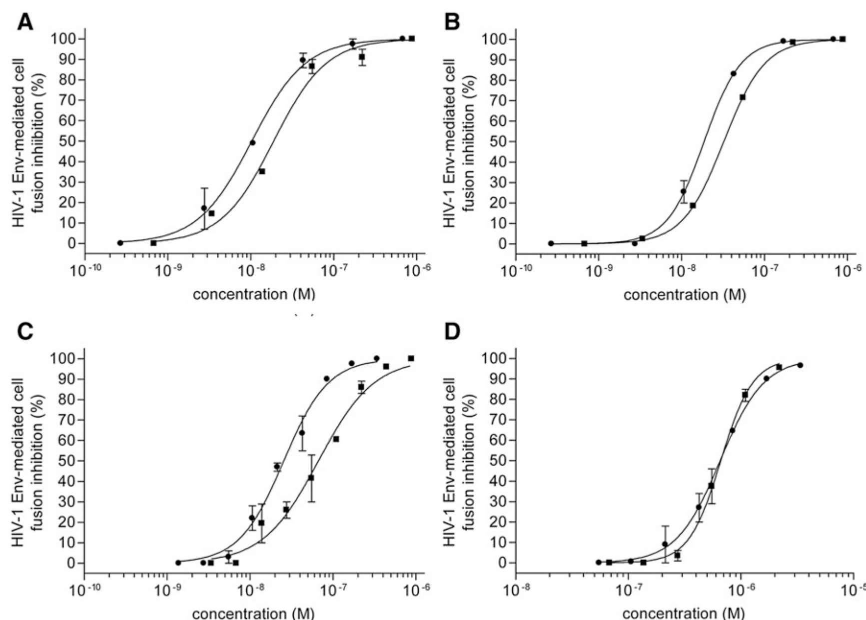

Figure 6. Anti-HIV-1 Activity of R3.0 and R4.0 Assessed on a Novel Human CD4+ T-Cell-Based Fusion Assay

(A) Antiviral activity of R3.0 (closed squares) and R4.0 (closed circles) was tested on human CD4+ T cells incubated with RK13 effector cells infected with a HIV-1_{BaL} envelope glycoprotein-expressing vaccinia virus.

(B) Experimental setup as in (A), with the additional infection of CD4+ T cells with a vaccinia virus encoding for CCR5.

(C) Experimental setup as in (A), replacing RK13 cells with HIV-1_{BaL} chronically infected PM1 cells. The IC_{50}s for R3.0 and R4.0 were 66 nM and 25 nM, respectively.

(D) Experimental setup as in (C), using HIV-$1_{92BR025}$ chronically infected PM1 cells. The IC_{50}s for R3.0 and R4.0 were 662 nM and 653 nM, respectively.

to generate HIV-1 strains resistant to early RANTES-derived peptide versions failed to produce HIV-1 strains that could be grown in the long term (data not shown).

Overall, the molecular evolution of RANTES-derived peptides reported herein, culminating in the generation of R3.0 and R4.0, has yielded promising new inhibitors with remarkable anti-HIV-1 and CCR5 antagonistic activities. Of note, a system has been successfully engineered to produce recombinant N-terminally acetylated proteins in *E. coli* (Johnson et al., 2010), opening an option for the expression of R3.0 and R4.0. These last-generation RANTES-derived peptides display a pharmacological potency comparable to that of Maraviroc, an anti-HIV-1 drug currently used in therapeutic regimens, and therefore represent new leads for the development of effective systemic or topical HIV-1 inhibitors.

SIGNIFICANCE

Efforts have been made to develop peptides originally derived from the N-loop/β1-strand region of human CCL5/RANTES that exert potent anti-HIV-1 activity. At present, very little is known about the structural details by which CCL5/RANTES and HIV-1 gp120 bind to CCR5 and interfere with each other. A significant advantage of peptides over full-length RANTES lies in their capability to block HIV-1 by binding to CCR5 as antagonists, i.e., in the absence of receptor activation. Clearly, the identification of the molecular portion that RANTES uses to interact with CCR5 and directly interfere with gp120 disclosed the possibility to enhance and evolve peptides as small folded units with safe anti-HIV-1 activity. These peptides also represent an ideal scaffold to investigate in more detail structural information on CCL5/RANTES-CCR5 binding, as well as computer-aided drug design. In addition, their relatively small size may be advantageous, as they could provide a better biodistribution and be less immunogenic. In this report, RANTES-derived peptides have been generated that brought a significant improvement in antiviral activity and evolved toward their suitability for the expression in recombinant systems. R3.0 and R4.0 are the most potent peptides developed and present IC_{50} values that closely match that of T20, an HIV-1 blocking peptide currently used in therapy. They also present pharmacological features that resemble those of Maraviroc, an anti-HIV-1 drug acting as CCR5 antagonist. Given the strong need for an efficacious prevention of the emergence of millions of new AIDS cases each year, RANTES-derived peptides represent an interesting option for their implementation in the form of topical microbicides.

EXPERIMENTAL PROCEDURES

Peptide Synthesis

Peptides were synthesized by standard solid-phase protocols using Fmoc chemistry and purified by reverse-phase HPLC (RP-HPLC) to >95% purity. Peptides were dimerized by oxidation of the N-terminal cysteine residues and N-terminally acetylated. Some of the peptides were C-terminally amidated. For stable dimerization, peptides were incubated overnight in 50% dimethyl sulfoxide (DMSO) (Sigma) in water; DMSO was removed by freeze drying, and the dimerized peptides were purified by RP-HPLC. The purity of the final stock was >97%; the Ellman test for free sulfhydryl groups was negative. For simplicity, amino acid residues in each peptide were numbered as in the full-length RANTES molecule.

HIV-1 Env-Mediated Cell Fusion Assay

Antiviral activity was evaluated using two assays: an HIV-1 envelope-mediated cell fusion assay and an acute HIV-1 infection assay, both based on the prototype CCR5-dependent (R5) isolate, HIV-1_{BaL}. The cell fusion assay was performed using a modification of the test (Nardese et al., 2001), based on a vaccinia virus technology, originally developed by Berger and coworkers (Nussbaum et al., 1994). In the modified assay, the effector cells were chronically infected PM1 cells (Lusso et al., 1995), whereas the target cells were NIH 3T3 mouse fibroblast cells stably expressing human CCR5 and human CD4. Sixteen hours before the test, effector cells were infected with a vaccinia vector expressing bacteriophage T7 RNA polymerase, while target cells were infected with a vaccinia vector expressing the *LacZ* reporter gene under the control of the T7 promoter, as described elsewhere (Nussbaum et al., 1994). All vaccinia virus infections were performed in Dulbecco's modified

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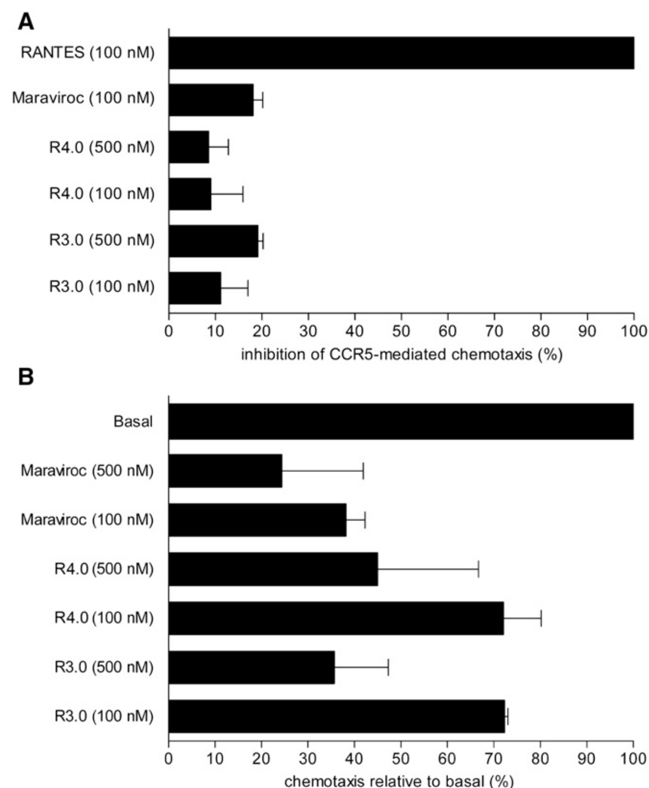

Figure 7. R3.0, R4.0 and Maraviroc Exert Similar Antichemotactic Activities

(A) Inhibition of RANTES-induced human lymphocyte chemotaxis by preincubation with R3.0, R4.0, or Maraviroc. CCR5-mediated chemotaxis is indicated as percentage of lymphocyte migration induced by RANTES. Basal chemotaxis of nonstimulated lymphocytes was set as 0.

(B) R3.0, R4.0, and Maraviroc CCR5 antagonism is assessed by the absence of lymphocyte migration. Chemotaxis is reported as percentage of nonstimulated lymphocyte migration (Basal).

Eagle's medium (DMEM) supplemented with 2.5% fetal bovine serum (FBS). The cells were then washed with DMEM 2.5%, and the effector cells were mixed for 2 hr with the target cells in the presence or absence of the inhibitors. Cell fusion was determined by measurement of β-galactosidase activity in nonionic detergent cell lysates as described (Nussbaum et al., 1994).

In addition to the assay described earlier, a second modified cell fusion inhibition assay was used. In this assay, primary $CD4^+$ T lymphocytes purified from human peripheral blood mononuclear cells (PBMC) were used as target cells instead of NIH 3T3 cells, and RK13 cells infected with a HIV-1_{BaL} envelope glycoprotein-expressing vaccinia virus were used as effector cells instead of PM1 cells. Briefly, PBMC were isolated by Lympholyte Cell Separation Media (Cedarlane Laboratories Limited) gradient centrifugation of buffy coat preparations from healthy blood donors. Subsequently, PBMC were stimulated with 500 U/ml recombinant human IL-2 (Chiron) in complete RPMI medium for 7 to 21 days to induce surface expression of CCR5. The day before the cell fusion inhibition assay, $CD4^+$ T cells were purified from PBMC by negative selection using Dynabeads goat antimouse immunoglobulin G (Dynal) and a cocktail of purified monoclonal antibodies against human CD19, CD16, CD56, CD8 (AL-Immunotools), and CD14 (Serotech). $CD4^+$ T cells were then infected with the vaccinia vector expressing the *LacZ* reporter gene under the control of T7 promoter. In a set of experiments, $CD4^+$ T cells were infected also with a vaccinia vector encoding for human CCR5. Infection was carried out in DMEM in the absence of FBS during the first 2 hours, then the cells were diluted using DMEM supplemented with 2.5% FBS. In parallel, RK13 effector cells were infected with a vaccinia vector expressing bacteriophage T7 RNA polymerase. The following day, $CD4^+$ T cells (target) were incubated with effector cells 4 hr in the absence or presence of inhibitors. After incubation, cells were lysed and cell fusion was determined as described earlier. As an alternative to RK13 cells, PM1 cells chronically infected with HIV-1_{BaL} and, in separate experiments, a primary R5 isolate of clade C (HIV-$1_{92BR025}$) were also used as effector cells.

HIV-1 Infection Assay

Acute HIV-1 infection was obtained by adding HIV-1_{BaL} stocks (50 median tissue-culture-infective doses [$TCID_{50}$] per well) to PM1 cells (2×10^4 per well) in complete RPMI medium. Experiments were performed in triplicate using 96-well round-bottom microtiter plates in the presence or absence of inhibitors. After incubation at 37°C for 16 hr, the wells were washed twice, and complete medium, with or without the inhibitors, was added. After 48 hr, 75% of the supernatant was removed for the HIV-1 p24 antigen measurement and replaced by an equal volume of medium containing the inhibitors. Virus replication was assayed at Day 4 postinfection by the p24 antigen ELISA. Supernatants were diluted in 1% Empigen BB detergent (Calbiochem) to disrupt virions and added to a 96-well ELISA plate coated with anti-HIV-1 p24 polyclonal antibodies (Aalto Bio Reagents Ltd.) and incubated 2 hr at room temperature. The plate was then washed three times in Tris-buffered saline (1.5 M NaCl, 250 mM Tris, pH 7.5) and an alkaline phosphatase-conjugated anti-HIV-1 p24 monoclonal antibody (Aalto Bio Reagents Ltd.) was added for 1 hr at room temperature. After washing three times with TROPIX buffer (10 mM $MgCl_2$, 200 mM Tris, pH 9.8), p24 was detected adding the luminescence substrate CSPD TROPIX (Applied Biosystems), and the signal was analyzed using a Mithras LB 940 luminometer (Berthold Technologies). Levels of p24 were calculated generating a standard curve with HIV-1 p24 antigen standards.

Human monocyte cultures were established from PBMC isolated from Ficoll-Hypaque (Pharmacia) density gradient centrifugation of buffy coat preparations obtained from healthy HIV-1 seronegative blood donors. PBMC (8×10^6/ml) were cultured in DMEM supplemented with 5% AB serum (Lonza BioWhittaker), 10% fetal calf serum (FCS), 2 mM glutamine, 50 mg/ml streptomycin, and 100 U/ml penicillin (Lonza BioWhittaker), and monocytes were allowed to adhere in T75 flasks for 2 hr at 37°C. Nonadherent cells were then removed by washing with medium. After 24 hr, adherent cells were recovered, seeded (1×10^5 per well) in 96-well flat-bottom plates in DMEM supplemented with 10% FCS and 5% AB serum, and allowed to differentiate into monocyte-derived macrophages (MDM) 7–10 days before infection. MDM were infected in quadruplicate with HIV-1_{BaL} (50 $TCID_{50}$ per well) in a total volume of 0.2 ml in the presence or absence of inhibitors. After overnight incubation, unbound virus was removed by extensive washing, fresh medium was added, and cultures were further incubated at 37°C. Supernatants were harvested at Day 4 for p24 antigen determination as described earlier.

Lymphocyte Chemotaxis Assay

PM1 is a unique $CD4^+$ $CCR5^+$ human T cell clone susceptible to a wide variety of primary HIV-1 isolates, including those exclusively using CCR5 as coreceptor (Lusso et al., 1995); thus, it was used for the chemotaxis assays in consistency with the antiviral assays. The chemotactic activity was assayed in duplicate 24-well Transwell chambers (pore size, 5 μm; Costar) as previously described (Lusso et al., 2011). To test agonistic activity, RANTES, RANTES-derived peptides, or Maraviroc were diluted in 600 μl RPMI containing 0.5% human serum albumin and added to the lower chamber; a total of 2×10^5 PM1 cells in 100 μl RPMI were added to the upper chamber. To test antagonistic activity, the peptides or Maraviroc were mixed with the cells prior to addition to the upper chamber. The number of cells migrated into the lower chamber in replicate wells was measured using a Gallios flow cytometer (Beckman Coulter). Specific cell migration was calculated by subtracting the number of cells migrated in the absence of the chemotactic factor (basal chemotaxis) from the number of cells migrated in the presence of the chemotactic factor; chemotaxis inhibition by RANTES peptides was calculated as the percentage of cells migrated toward RANTES in the absence of the peptide.

Statistical Analysis

Dose-response curves were fitted using GraphPad Prism version 5.04 (GraphPad Software) in order to calculate IC_{50} concentrations through

nonlinear regression analysis. All data are expressed as the means ± SD for two independent experiments performed in triplicate.

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