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Bioorganic & Medicinal Chemistry Letters 16 (2006) 93–95
Bioorganic & Medicinal Chemistry Letters

Analogues of N-terminal truncated synthetic peptide fragments derived from RANTES inhibit HIV-1 infectivity

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Received 24 August 2005; revised 13 September 2005; accepted 16 September 2005
Available online 19 October 2005

Abstract—A series of synthetic peptide fragments derived from RANTES were designed, synthesized, and evaluated to determine the effect of N-terminal truncation on the ability of the lead compound Ac[Ala^{10,11}]RANTES-(1–14)NH_2 to inhibit HIV-1 infectivity. Both the lead compound and the truncated analogue Ac[Ala^{10,11}]RANTES-(3–14)NH_2 were able to significantly inhibit HIV-1 infectivity. These results suggest that a small synthetic peptide may be able to mimic RANTES and have the ability to prevent transmission of HIV-1.

RANTES (regulated upon activation, normal T-cell expressed and secreted) is a potent CC-chemokine which activates leukocytes including T-lymphocytes and mediates chemotaxis in inflammatory processes.[1] RANTES, macrophage inflammatory protein (MIP)-1α, and (MIP)-1β hinder human immunodeficiency virus type-1 (HIV-1) infection by competing with the virus for binding to the CC-chemokine receptor 5 (CCR5), thereby inhibiting HIV-1 *env*-mediated membrane fusion.[2–4] Although the HIV-1 blocking proteins are CCR5 agonists, initiation of signal transduction is not required for co-receptor function by CCR5.[5] Mapping of RANTES using peptide fragments have shown that the most pronounced chemotactic activity is in the amino-terminal region of the protein[6] which is also one of the regions of RANTES associated with the ability to inhibit HIV-1 entry into host cells.[7] Limited structure–activity relationship studies on the anti-HIV activity of RANTES fragments has been reported.[7–10] Ac[Ala^{10}]RANTES-(1–10)NH_2 and Ac[Ala^{10,11}]RANTES-(5-14)NH_2 were first reported to have anti-HIV activity, with the former being reported as having an almost fourfold higher activity than the latter.[8] In screening RANTES analogues to determine optimal peptide length, we found considerable anti-HIV activity for the lead compound Ac[Ala^{10,11}]RANTES-(1-14)NH_2.[9] Our goal was to synthesize and evaluate N-terminal truncated analogues of this active peptide to better understand which residues were responsible for the loss of activity between Ac[Ala^{10}]RANTES-(1-10)NH_2 and Ac[Ala^{10,11}]RANTES-(5-14)NH_2. Blocking the charge at both termini of the peptide fragments appears to be important for anti-HIV activity as does having residues with hydrogen bonding capability in the N-terminus of the peptide.[10]

Figure 1 shows the structure of the lead compound Ac[Ala^{10,11}]RANTES-(1–14)NH_2, **AA114**. The analogues were designed to sequentially truncate the first four amino acid residues from the N-terminus. In all of the analogues synthesized for this study, the adjacent Cys^{10,11} residues were replaced by Ala to avoid unwanted disulfide bond formation.

Synthesis. The peptides were synthesized as previously described[9] on the Pioneer Peptide Synthesis System using the standard solid-phase Fmoc/HATU (*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluor-

Ac-Ser-Pro-Tyr-Ser-Ser-Asp-Thr-Thr-Pro-Ala-Ala-Phe-Ala-Tyr-NH_2

Figure 1. Amino acid sequence of Ac[Ala^{10,11}]RANTES-(1-14)NH_2, **AA114**.

Keywords: Chemokine; HIV; Peptide; RANTES; Solid-phase synthesis.
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doi:10.1016/j.bmcl.2005.09.044

ophosphate) chemical protocol with a fourfold excess of Fmoc-amino acid relative to the resin. The peptides were assembled on an Fmoc-PAL-PEG-PS [N^{α}-9-fluorenylmethoxycarbonyl-5-(4-aminomethyl-3,5-dimethoxyphen oxy) valeric acid–polyethylene glycol–polystyrene] resin using 20% piperidine in *N*,*N*-dimethylformamide (DMF) as the deblocking agent, and HATU and *N*,*N*-diisopropylethylamine (DIEA) as the coupling reagents. The N-terminus was acetylated with 1.0 M 1-acetylimidazole, and the side-chain protecting groups and peptide–resin bonds were then cleaved using 95% TFA with 5% water as a scavenger. The crude peptides were purified to generally greater than 98% homogeneity by semi-preparative reversed-phase high performance liquid chromatography (HPLC) using a Vydac C_{18} (218TP1022) column [300 Å, 10 μm, 22 × 250 mm] and characterized by analytical HPLC using a linear gradient consisting of 0–70% aqueous acetonitrile containing 0.1% TFA over 45 min at a flow rate of 1.5 mL/min; molecular weights determined using fast-atom bombardment mass spectrometry (FAB-MS).[11]

Anti-HIV-1 activity. The anti-HIV-1 activity of the compounds was examined using a $CD4^+$ PM1 cell line that is susceptible to a range of HIV-1 isolates[12] in a cell–cell infectivity screening assay.[13] The ability of these peptides to inhibit cell-to-cell infection by HIV-1 was screened by evaluating the efficacy of the peptides at both a high (10 μM) and low (10 nM) concentration, as previously described.[9] In brief, the peptide analogues (or controls) were incubated with uninfected PM1 cells, and then the cells were infected with the R5 viral strain HIV-1_{BaL}. The co-cultivation cells were added and incubated at 37 °C for 7 days. The media and peptide were replenished on day 5 and the extracellular release of the viral p24 core antigen was measured on day 7 post-infection. The ability of the synthetic peptide to inhibit HIV-1 infection was correlated to the reduction in the level of p24 antigen. Viral titers were calculated as 50% tissue culture infective dose ($TCID_{50}$) per milliliter of inoculum, using a computer ID_{50} package based on Fisher's statistical model.[14]

The anti-HIV-1 activity of the analogue was determined by comparing the reduction of p24 antigen production to those of cells treated with [Ala^{10}]RANTES-(1-10)NH_2 (as a negative control known to have no effect in inhibiting viral infectivity) and with the positive control RANTES which significantly inhibits the virus from infecting the co-cultivation cells. The qualitative results (Table 1) allow us to quickly ascertain whether the compounds are active (+) and inhibit HIV-1 infectivity like RANTES or whether they are inactive (−) like the negative control compound.

Both **AA114** and the N-terminal dipeptide truncated analogue **AA314** showed anti-HIV-1 activity at the high and low peptide concentrations. Although the efficacy of the peptides was comparable to that of intact RANTES, in this screening assay the potencies could not be directly compared because of the differences in the concentration used. **AA414** and **AA514** were moderately active and were able to inhibit viral infection at the high 10 μM concentration but were ineffective at the low concentration. **AA214** was completely ineffective at inhibiting viral infectivity even at the high concentration.

Table 1. Anti-HIV-1 activity screening of N-terminal truncated analogues of **AA114**

Analogues	Observed effect	
	10 μM	10 nM
Ac[$Ala^{10,11}$]RANTES-(1-14)NH_2, **AA114**	+	+
Ac[$Ala^{10,11}$]RANTES-(2-14)NH_2, **AA214**	−	−
Ac[$Ala^{10,11}$]RANTES-(3-14)NH_2, **AA314**	+	+
Ac[$Ala^{10,11}$]RANTES-(4-14)NH_2, **AA414**	+	−
Ac[$Ala^{10,11}$]RANTES-(5-14)NH_2, **AA514**	+	−
[Ala^{10}]RANTES-(1-10)NH_2	−	−
RANTES[a]	+	

[a] RANTES was tested at a concentration of 2 μg/mL (250 nM). The high concentration of RANTES was used to determine the maximum reduction in p24 antigen.

The deletion of Ser in position 1 from the lead peptide eliminates anti-HIV-1 activity as shown by **AA214**. We hypothesize that the loss of activity is due to the shifted location of the peptide within the binding site. The Pro residue at the N-terminus of the peptide eliminates activity perhaps due to the bulk of the pyrrolidine ring being unable to adequately fit into this region of the binding site, or the inability of Pro to participate in side-chain hydrogen bond formation like Ser. However, further truncation of the peptide by removal of both Ser^1 and Pro^2 (**AA314**) places Tyr as the N-terminal residue in the binding site. The phenol side chain of Tyr in **AA314** has the potential for favorable interaction in the binding site because it has both a hydrophilic nature and hydrogen bonding capability. This would explain the loss in activity between **AA114** and **AA214**, and the restored activity of **AA314**. Hence, the weak activity of **AA414** and **AA514** may also be explained by the non-optimal positioning of the Ser residues in the binding site. Thus, we hypothesize that amino acid residues capable of strong hydrogen bonding are important in positions 2 and 3 of the peptide fragment to have anti-HIV activity. Our hypothesis is supported by Ac[Ala^{10}]RANTES-(6-10)NH_2 which is also a highly active anti-HIV compound reported in the literature[10] that has Thr in positions 2 and 3. An alanine scan is currently being performed to confirm the importance of hydrophilicity and hydrogen bonding.

It is interesting to compare the difference between the synthetic peptides and the intact protein with respect to receptor interaction. In intact RANTES, Pro at position 2 is critical as Ala replacement abolishes both CCR5 binding and signaling.[15] Yet, in the short RANTES peptide fragment, Pro^2 can be completely eliminated without loss of anti-HIV activity. This suggests that there may be distinctly different interactions between proteins and peptide ligands at CCR5.

Activity in these N-terminal truncated series of analogues confirms that Cys in positions 10 and 11 are not critical for HIV-1 inhibition, as these residues can readily be replaced by Ala. This is consistent with the fact that the Cys residues play a structural role via disulfide bond formation in the intact protein. Findings from

this study reinforce the fact that small synthetic peptides can mimic protein function, as demonstrated by the ability of these synthetic peptides to successfully inhibit HIV-1 infectivity.

In a preliminary screen, the synthetic peptides Ac[Ala^{10,11}]RANTES-(1-14)NH_2 and the truncated fragment Ac[Ala^{10,11}]RANTES-(3-14)NH_2 both inhibit HIV-1 infection at a concentration of only 10 nM. Results from this study suggest that RANTES fragments with anti-HIV activity have amino acid residues capable of strong hydrogen bonding in positions 2 and 3. Our findings also reinforce the ability of small synthetic peptides to mimic the function of large proteins. Such peptides may play important roles in the development of lead compounds as anti-HIV-1 agents.

Acknowledgments

Funding for this work was provided in part through the American Association of Colleges of Pharmacy (AACP) New Investigator Program Award (S.V.) supported through the Burroughs Wellcome Fund and the American Foundation for Pharmaceutical Education. Additional funding was provided from the University of Connecticut, as well as the Graduate School and Research Foundation (UCRF) at the University of Connecticut. We acknowledge the work of Robert Praschunus and Michael Merges for running the infectivity assays and thank Dr. Robert Gallo for generously subsidizing the cost of those experiments.

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