Potent CCR5 Inhibition by Fully Recombinant RANTES Analogs Requires Neither Internalization Nor Signaling

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ABSTRACT

Background: Some RANTES analogs are potent inhibitors of R5 HIV-1 infection, but cost of production is likely to limit their use as microbicides if they have to be chemically synthesized, and signaling activity on CCR5 raises potential safety concerns. We have developed novel RANTES analogs that promise low cost recombinant production by fermentation. Molecules with two distinct modes of action have been evaluated for potency, durability of activity, and signaling activity.

Methods: Phage display libraries of RANTES molecules with diversity engineered into the N-terminal region were screened for interaction with CCR5 presented on live cells. Selected molecules isolated from these libraries were tested for their ability to block infection of PBMC cultures by primary R5 HIV-1 isolates, and to modulate CCR5 expression on activated CD4⁺ human T cells. Signaling activity was measured as induction of Ca^{2+} flux in HeLa-CCR5 cells or primary T cells. The durability of CCR5 modulation and inhibition of infection were determined after removal of the RANTES analog. CCR5 occupancy was measured by differential staining with monoclonal antibodies PA12 (N-terminus specific, binding not impaired by RANTES) and 2D7 (specific for the 2nd extracellular loop, binding blocked by RANTES). The new molecules were compared to the most potent chemically synthesized analog, PSC-RANTES.

Results: Three new molecules, 6P4-, 5P12-, and 5P14-RANTES, were found to be at least as potent as PSC-RANTES at preventing R5 HIV-1 infection of PBMC, with IC_{50} values in the pM range. 6P4-RANTES, like PSC-RANTES, caused prolonged internalization of CCR5 and induced Ca^{2+} flux. By contrast, 5P12- and 5P14-RANTES caused no or modest CCR5 internalization, respectively, and neither induced detectable Ca^{2+} flux. 5P14-RANTES blocked 2D7 but not PA12 staining for up to 96 hr following a 1 hr. pulse exposure, and 5P12-, 5P14- and 6P4-RANTES blocked HIV-1 infection to a greater extent than PSC-RANTES for at least 24 hr after inhibitor removal. Both 6P4- and 5P12-RANTES were evaluated for protective activity in the rhesus macaque vaginal challenge model (see abstract 560 by R. Veazey *et al.*).

Conclusions: Novel, potent CCR5 inhibitors have been developed that promise reduced costs of production. The safety profile of two of them appears to be improved, since we do not detect signaling via CCR5. We are continuing the development of these inhibitors as microbicides.

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METHODS

Inhibitors - The CCR5 inhibitors PSC-RANTES, 6P4-RANTES, 5P12-RANTES, or 5P12-RANTES were used to block infection of PHA + IL-2-activated human CD4 T cells or MT-2 cells engineered to express different levels of wildtype or mutated CCR5 (Pastore *et al.*, 2003). The novel CCR5 inhibitors were selected by phage display as previously described (Hartley *et al.*, 2003). Fifty% inhibitory concentrations (IC_{50}) were calculated using curve-fitting software in Prism 4 (GraphPad Software, San Diego, CA, USA).

Calcium flux -Signaling of primary, activated human T cells was assayed by real-time recording of Ca^{+2} flux by staining with the Ca-sensitive fluorescent probe Fluo-4 and recording fluorescence prior to (15 sec) and during (45 sec) exposure to chemokines or their analogs.

CCR5 staining - Surface expression of CCR5 was determined by staining with one of two monoclonal antibodies, PA12 directed against the CCR5 N-terminal (insensitive to RANTES binding; kindly provided by Progenics Pharmaceuticals, Tarrytown, NY, USA), or 2D7 directed against the second extracellular loop (staining blocked by RANTES binding). Inhibition of staining was calculated by percent reduction in geometric mean fluorescence intensity compared to no inhibitor controls.

CITATIONS FOR METHODS

Pastore, C. *et al*., 2003. Two mechanisms for human immunodeficiency virus type 1 inhibition by N-terminal modifications of RANTES. Antimicrob Agents Chemother **47**:509-17

Hartley, O. *et al.*, 2003. Human immunodeficiency virus type 1 entry inhibitors selected on living cells from a library of phage chemokines. J. Virol. **77**:6637-44.

Lederman, M.M. *et al.*, 2004. Prevention of vaginal SHIV transmission in rhesus macaques through inhibition of CCR5. Science **306**:485-7.

RESULTS

Native RANTES is a poor inhibitor of HIV-1 infection, and a series of N-terminal modifications have led to dramatic improvements in inhibitor potency for preventing R5 HIV-1 infection. Beginning with AOP-RANTES, successive chemical modifications led to NNY-RANTES and then PSC-RANTES, the most potent CCR5 inhibitor available (Hartley *et al*, 2003; Lederman *et al*, 2004). The potent activity of PSC-RANTES depends on agonist signaling and prolonged CCR5 internalization. The goal of this program was to develop potent CCR5 inhibitors that lacked agonist activity yet retained potency equivalent to PSC-RANTES. A related practical goal was to produce inhibitors capable of large scale production by bacterial fermentation.

Three optimized candidates were developed by repeated rounds of selection in the phage display system. The properties of these molecules are summarized in Table 1.

I. 5P12-RANTES	II. 6P4-RANTES	III. 5P14-RANTES
No signaling activity No CCR5 internalization	Signaling activity CCR5 internalization (like PSC-RANTES)	No signaling activity CCR5 internalization

These three N-terminal modification of RANTES were compared to PSC-RANTES for their capacity to block infection of activated human PBMC by well-characterized R5 HIV-1 isolates. The results of typical experiments are shown in Figure 1.

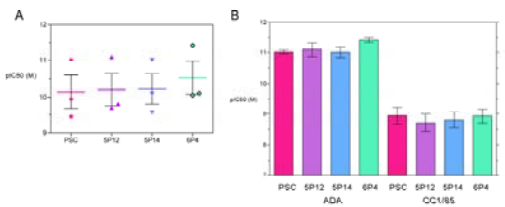


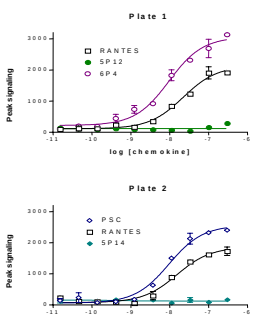
Fig. 1 - Inhibition of R5 HIV-1 infection by novel modified RANTES compounds.

A. pIC50 values for 3 individual PBMC donors; pIC50 is the negative log of the IC50 value (e.g., 9 = 10^{-9} M = 1 nM). These 3 donors were chosen to represent a range of CCR5 expression. The challenge virus was HIV-1 ADA.
B. pIC50 values for 2 representative R5 HIV-1 isolates; ADA and CC1/85. These 2 HIV-1 isolates span the observed range of sensitivity to CCR5 inhibitors observed in primary patient isolates.

These results confirm that each of the new inhibitor molecules is as potent as PSC-RANTES when tested against different viruses and different PBMC donors. As has been demonstrated in earlier studies, sensitivity to CCR5 inhibition varies between different donors and different R5 HIV-1 isolates.

Fig. 2 - Absence of calcium signaling with two new molecules

PHA and IL-2 activated PBMC were exposed to a range of concentrations of native RANTES or PSC-, 5P12-6P4-, or 5P14-RANTES. Peak Ca^{+2} influx data ± SD are shown.



These results show that both PSC- and 6P4-RANTES behave as super-agonists in this assay, while 5P12- and 5P14-RANTES lack detectable agonist activity.

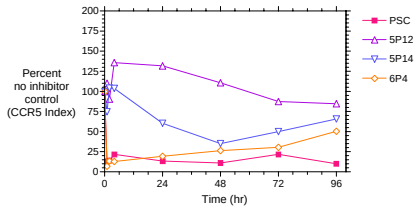
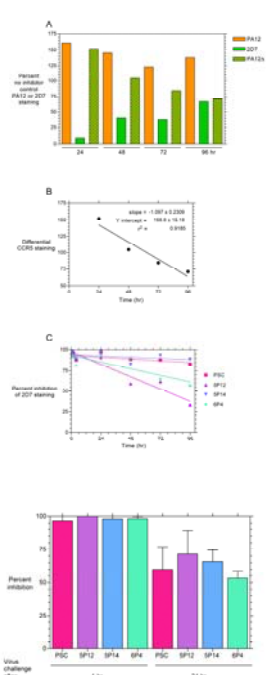


Fig. 3 - CCR5 staining of human CD4 T cells following a 1 hr. exposure To PSC-, 5P12-, 5P14-, or 6P4-RANTES.
CCR5 index is the geometric mean fluorescence intensity x percentage of cells above background staining.

The CCR5 staining data in Fig. 3 confirm that short exposure to both PSC- and 6P4-RANTES cause prolonged internalization of CCR5, as previously reported for PSC-RANTES. However, exposure to 5P12-RANTES causes no CCR5 internalization. There is a small but reproducible increase in CCR5 staining after 5P12-RANTES exposure that may reflect changes in the N-terminal PA12 epitope exposure or receptor aggregation. Exposure to 5P14-RANTES causes a slower and more modest CCR5 internalization. These data establish that potent anti-HIV-1 activity does not require receptor internalization.

Fig. 4 - Differential CCR5 staining to detect receptor occupancy.

A. CCR5 staining with PA12 or 2D7 mAb to detect total CCR5 (PA12+) and CCR5 with no RANTES bound (2D7+). The difference (PA12Δ) represents ligand-occupied CCR5. Data show differential staining after 1 hr. exposure to 5P12-RANTES.
B. Regression analysis of 5P12-occupied CCR5 showing a half-life of ~50 hr.
C. Similar analysis of CCR5 occupancy after a 1 hr. exposure to PSC-, 6P4-, 5P14- or 5P12-RANTES.



The data in Fig. 4 indicate that a substantial fraction of cell surface-exposed CCR5 remains occupied by all modified RANTES molecules. The recovery of unliganded CCR5 following brief exposure to 5P12-RANTES, the molecule that fails to induce CCR5 internalization, is consistent with prior estimates of CCR5 turnover.

Fig. 5 - Inhibition after delayed virus challenge.

Activated human CD4 T cells were exposed to the indicated inhibitor for 1 hr, the inhibitors removed, and virus added immediately or after a 24 hr delay.

The results in Fig. 5 show >50% inhibition of virus replication with all modified RANTES molecules when virus is added 24 hr after inhibitor removal.

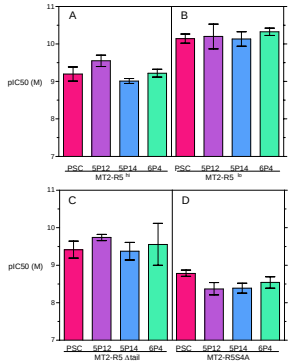


Fig. 6 - Inhibitory potency on cells lines expressing native CCR5 or internalization-defective mutations of CCR5.
HIV-1 ADA was used to infect MT-2 cell lines expressing endogenous CD4 and lentiviral vector-derived CCR5 (Pastore *et al.*, 2003). A. Wildtype (wt) CCR5 expressed at high levels (GMFI=800). B. wt CCR5 expressed at low levels (GMFI = 40). C. CCR5 (1-302), with the C-terminal cytoplasmic domain deleted; no CCR5 internalization and low expression (GMFI = 38). D. CCR5 S4A, with the 4 C-terminal serine phosphorylation sites mutated to alanine; very slow internalization and high expression (GMFI = 860).

The data in Fig. 6 show that each of the new CCR5 inhibitors shows equal potency to PSC-RANTES and confirm that CCR5 expression levels impact the level of inhibition. Moreover, all of the molecules show comparable activity in the absence of CCR5 internalization. This result demonstrates that receptor occupancy and receptor internalization are two mechanisms of activity for these molecules, and these mechanisms are separable. 5P12-RANTES does not cause CCR5 internalization and is as potent as 5P14- and 6P4-RANTES that cause slow and rapid CCR5 internalization, respectively. Conversely, 6P4- and PSC-RANTES cause profound internalization of native CCR5, but are equally potent inhibitors on mutated CCR5 that cannot be internalized.

CONCLUSIONS

- Three new optimized CCR5 inhibitors capable of microbial synthesis have been developed.
- Each is as potent as the previous lead compound PSC-RANTES, a molecule requiring some chemical synthesis.
- Two of the molecules, 5P12- and 5P14-RANTES, show no detectable Ca^{+2} signaling on primary human T cells, suggesting an improved safety profile for human application.
- Two of these new CCR5 inhibitors are capable of preventing vaginal transmission of SHIV162P4 in the macaque model [see R. Veazey *et al.*, abstract 560, session 91.]
- These molecules are attractive candidates for microbicides to prevent transmission of R5 HIV-1, with reduced cost of production and better safety profiles than earlier molecules in this series.