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Highly potent HIV inhibition: engineering a key anti-HIV structure from PSC-RANTES into MIP-1β/CCL4

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The HIV coreceptor CCR5 is a validated target for both the prevention and therapy of HIV infection. PSC-RANTES, an N-terminally modified analogue of one of the natural chemokine ligands of CCR5 (RANTES/CCL5), is a potent inhibitor of HIV entry into target cells. Here, we set out to engineer the anti-HIV activity of PSC-RANTES into another natural CCR5 ligand (MIP-1β/CCL4), by grafting into it the key N-terminal pharmacophore region from PSC-RANTES. We were able to identify MIP-1β/CCL4 analogues that retain the receptor binding profile of MIP-1β/CCL4, but acquire the very high anti-HIV potency and characteristic inhibitory mechanism of PSC-RANTES. Unexpectedly, we discovered that in addition to N-terminal structures from PSC-RANTES, the side chain of Lys^{33} is also necessary for full anti-HIV potency.
Keywords: CCR5/HIV coreceptor/MIP-1β CCL4/pharmacophore grafting/PSC-RANTES

Introduction

HIV requires a two-step interaction with the host cell in order to gain entry and initiate infection. Interaction with CD4 leads to a conformational change in the HIV envelope complex allowing a subsequent interaction with a coreceptor (Berger *et al*., 1999). Although a number of different chemokine receptors have shown coreceptor activity *in vitro*, it is probable that only two, CCR5 and CXCR4, are of physiological relevance (Moore *et al*., 2004). While infected persons generally harbor both CCR5 and CXCR4-utilizing strains of HIV (R5 and X4 strains), only R5 strains are involved to a significant extent in person-to-person transmission of the disease, and homozygotes for an inactivating CCR5 allele show a remarkable degree of protection from HIV acquisition. Hence, targeting R5-tropic viruses is a promising HIV prevention strategy (Lederman *et al*., 2006) as well as an additional option for HIV therapy (Maeda *et al*., 2004).

While the native chemokine ligands of CCR5, MIP-1α/CCL3, MIP-1β/CCL4, RANTES/CCL5 and MCP-2/CCL8 (Cocchi *et al*., 1995; Blanpain *et al*., 1999) are capable of blocking entry of R5 tropic strains, several N-terminally modified variants of these proteins with enhanced inhibitory potency have been identified (Oravecz *et al*., 1997a; Simmons *et al*., 1997; Polo *et al*., 2000; Hartley *et al*., 2003, 2004). The more potent of these molecules apparently share a novel inhibitory mechanism involving the induction of prolonged intracellular sequestration of CCR5 (Hartley *et al*., 2005). PSC-RANTES (Hartley *et al*., 2004) is the most potent molecule of this type to have been identified so far, and has shown potential for use both in HIV therapy (Hartley *et al*., 2004) and as a topical HIV prevention agent (Lederman *et al*., 2004).

All chemokines for which structures have been determined share a characteristic tertiary structure that consists of a highly structured core domain from which extends a flexible N-terminal region [reviewed in (Fernandez *et al*., 2002)]. The current model for chemokine structure-activity involves a two-site 'message-address' docking mechanism (Siciliano *et al*., 1994; Wells *et al*., 1996), which has been characterized in detail for RANTES/CCL5 (Blanpain *et al*., 2002). The core region carries 'address' structures with which receptors are initially engaged with high affinity and specificity, and then the N-terminal region, carrying 'message' structures, induces the subsequent receptor activation [reviewed in (Fernandez *et al*., 2002)].

According to this model, the enhanced anti-HIV potency of N-terminally modified chemokine analogues like PSC-RANTES would relate to changes in 'message' activity, influencing receptor modulation, rather than 'address' structures involved in specificity and affinity of receptor binding. Indeed, PSC-RANTES exhibits CCR5 binding affinity that is not significantly different to that of native RANTES/CCL5, and its increased anti-HIV potency does appear to be correlated with an effector function: capacity to induce intracellular sequestration of CCR5 (Hartley *et al*., 2004).

We therefore established a working hypothesis in which it would be possible to graft the pharmacophore region of PSC-RANTES onto another chemokine, giving it the capacity to induce the 'message' function, i.e. capacity to induce intracellular sequestration of CCR5, without altering its 'address' function, i.e. receptor specificity and affinity. We chose to test this hypothesis using MIP-1β/CCL4, a chemokine which is generally recognized (Bacon *et al*., 2002) as having a narrower receptor binding profile (CCR5 alone) than RANTES/CCL5 (CCR5 plus CCR1 and CCR3), since engagement of the latter two receptors is neither a necessary nor a desirable property for HIV entry inhibitors.

Materials and methods

Chemical synthesis of chemokines

The chemokines and chemokine analogues used in this study were prepared by total chemical synthesis as previously

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described (Wilken *et al*., 1999; Hartley *et al*., 2004). Briefly, N-terminal and C-terminal fragments were synthesized using Boc chemistry, assembled using Native Chemical Ligation (Dawson *et al*., 1994) and refolded under conditions promoting the formation of disulfide bridges. The purity and integrity of each sample was verified by hplc and mass spectrometry.

R5-tropic envelope-dependent cell fusion assay

The anti-HIV potency of the chemokine analogues was determined using a cell fusion assay as described previously (Hartley *et al*., 2003, 2004).

Competition binding assays

Competition binding experiments on CCR5 were performed as described previously (Hartley *et al*., 2003, 2004). For CCR1 and CCR3, experiments were performed on membrane preparation made from cells expressing CCR1 or CCR3, using 0.2 nM ^{125}I-MIP-1β or ^{125}I-Eotaxin (Amersham) as labeled tracers, and variable concentrations of chemokines as unlabeled competitors. Samples were incubated for 60 min at 27°C, and then bound tracer was separated by filtration through GF/B filters presoaked in 0.5% BSA. Filters were counted in a γ-scintillation counter. Binding parameters were determined with Prism software (GraphPad) using non-linear regression applied to a single-site binding model.

CCR5 downmodulation assay

The capacity of chemokine analogues to induce CCR5 downmodulation was adapted from flow cytometry-based techniques (Hartley *et al*., 2003, 2004; Pastore *et al*., 2003) to enable measurements to be made in 96-well plates. CHO-CCR5 cells were seeded at a density of 80 000 cells/well. After overnight incubation, medium was removed and replaced with medium containing chemokine analogues at different concentrations and cells were incubated for 1 h at 37°C. Medium was then removed and cells were fixed with 4% paraformaldehyde and washed twice with PBS. Cells were then labeled with either phycoerythrin-conjugated anti-CCR5 antibody (clone 3A9, Pharmingen) or phycoerythrin-conjugated anti-CCR1 antibody (clone 53 504, R&D systems; negative control) in PBS supplemented with 1% BSA (PBS-1% BSA) on ice for 1 h. Plates were washed three times with PBS-1% BSA and fluorescence values for each well were determined using a FLEXstation fluorimeter (Molecular Devices). Results are expressed as % control level of surface CCR5: 100 × [mean fluorescence (chemokine added, anti-CCR5) − mean negative control fluorescence (anti-CCR1)]/[mean positive control fluorescence (no chemokine added, anti-CCR5) − mean fluorescence (anti-CCR1)].

Ca^{2+} flux assays

Ca^{2+} flux assays on CCR5-expressing cells were performed essentially as described previously (Hartley *et al*., 2003) using 96-well plates and a FLEXstation fluorimeter (Molecular Devices). Fluorescence measurements were carried out on HeLa-P5L cells (Simmons *et al*., 1997) loaded with Fluo-4 (Molecular Probes) according to the manufacturer's recommendations and maintained at 37°C. Baseline measurements were recorded during 15 s prior to chemokine addition, after which fluorescence peaks corresponding to intracellular Ca^{2+} fluxes were measured over a further 60 s. Signals were quantified using the 'area under curve' function in the SoftMax software (Molecular Devices), and expressed in relative fluorescence units. Measurements were performed in duplicate. Responses on CHO-K1 cells expressing either CCR1 or CCR3 were measured in an aequorin-based assay as previously described (Blanpain *et al*., 1999). Dose-response curves were fitted to the data using Prism software (GraphPad) using non-linear regression applied to a sigmoidal dose-response model.

Results

Engineering potent anti-HIV activity into MIP-1β/CCl4: first generation

We began by designing a set of PSC-RANTES-MIP-1β/CCL4 chimeras in which N-terminal fragments of increasing size were grafted from PSC-RANTES into MIP-1β/CCL4, replacing the residues at the corresponding position in the sequence (Table I). These proteins were assayed for their anti-HIV potency in an R5-tropic envelope-dependent cell fusion assay (Table I). The potencies of both unmodified RANTES/CCL5 and MIP-1β/CCL4 were too low to be accurately determined over the concentration range used, but in previous experiments using the same assay we have found these molecules to have IC_{50} values of in the low micromolar range (unpublished results). These values indicate potencies lower than those published in other studies that used viral replication assays on T-cell-derived cell lines (e.g. (Oravecz *et al*., 1997b)), and we stress that the cell fusion assay was used in this study to provide relative, rather than absolute potency values. In this respect, previous work has shown that it is both highly reproducible and predictive of results obtained in replication assays using primary cells and CCR5-tropic HIV-1 strains (Hartley *et al*., 2003, 2004). An IC_{50} value of 40 pM for PSC-RANTES, in agreement with previously published work (Hartley *et al*., 2004). Simply substituting the PSC-RANTES moiety (*n*-nonanoyl-hioprolyl-cyclohexylglycyl-) for the corresponding part of MIP-1β/CCL4 (Ch1-MIP-1β) did not lead to a significant gain in potency. On the other hand, molecules in which a few more residues of MIP-1β/CCL4 were replaced by the corresponding residues from the N-terminal region of PSC-RANTES (Ch2-MIP-1β through Ch6-MIP-1β) showed potencies that were significantly improved (IC_{50} values in the range 60–300 nM), although still modest compared to that of PSC-RANTES (40 pM). Moving the junction between the sequence derived from PSC-RANTES and that derived from MIP-1β/CCL4 to the middle of the primary structure (i.e. to the third conserved cysteine residue, Ch7-MIP-1β) led to a striking increase in potency (IC_{50} value of 100 pM, i.e. only 2.5-fold less potent than PSC-RANTES). Comparison between the potencies of Ch6-MIP-1β (in which the junction point was moved a little nearer the N-terminus) and Ch7-MIP-1β suggests that key structures for inhibitory activity are located between positions 26 and 33 of PSC-RANTES.

Engineering potent anti-HIV activity into MIP-1β/CCL4: second generation

Noting the proximity of the side chain of Lys^{33} to the N-terminal region of RANTES in the available 3D structures (Fig. 1), we postulated that the side chain of Lys^{33} might be

Table I. Primary sequence and anti-HIV activity of a group of PSC-RANTES/MIP-1β/CCL4 chimera proteins

Molecule	Sequence	IC_{50} nM
	1 10 20 30 40 50 60	
PSC-RANTES	ZXXSSDTTPCCFAYIARPLPRAHIKEYFYTSGKCSNPAVVFVTRKNRQVCANPEKKWVREYINSLEMS	0.04
MIP-1β	APMGSDPPTACCFSYTARKLPRNFVVDYYETSSLCSQPAVVFQTKRSKQVCADPSESWVQEYVYDLELN	>1000
Ch1-MIP-1β	ZXXSDPPTACCFSYTARKLPRNFVVDYYETSSLCSQPAVVFQTKRSKQVCADPSESWVQEYVYDLELN	>1000
Ch2-MIP-1β	ZXXSSDTTACCFSYTARKLPRNFVVDYYETSSLCSQPAVVFQTKRSKQVCADPSESWVQEYVYDLELN	60
Ch3-MIP-1β	ZXXSSDTTPCCFSYTARKLPRNFVVDYYETSSLCSQPAVVFQTKRSKQVCADPSESWVQEYVYDLELN	200
Ch4-MIP-1β	ZXXSSDTTPCCFAYIARKLPRNFVVDYYETSSLCSQPAVVFQTKRSKQVCADPSESWVQEYVYDLELN	500
Ch5-MIP-1β	ZXXSSDTTPCCFAYIARPLPRNFVVDYYETSSLCSQPAVVFQTKRSKQVCADPSESWVQEYVYDLELN	600
Ch6-MIP-1β	ZXXSSDTTPCCFAYIARPLPRAHIKDYYETSSLCSQPAVVFQTKRSKQVCADPSESWVQEYVYDLELN	300
Ch7-MIP-1β	ZXXSSDTTPCCFAYIARPLPRAHIKEYFYTSGKCSQPAVVFQTKRSKQVCADPSESWVQEYVYDLELN	0.1

ZXX indicates the *n*-nonanoyl-thioprolyl-cyclohexylglycyl moiety of PSC-RANTES. Sequences derived from RANTES/CCL5 are boxed in gray; sequences derived from MIP-1β/CCL4 are indicated in plain text; sequences shared by the two chemokines are boxed in black. Anti-HIV potency (IC_{50}) was determined in an R5-tropic envelope-dependent cell fusion assay.

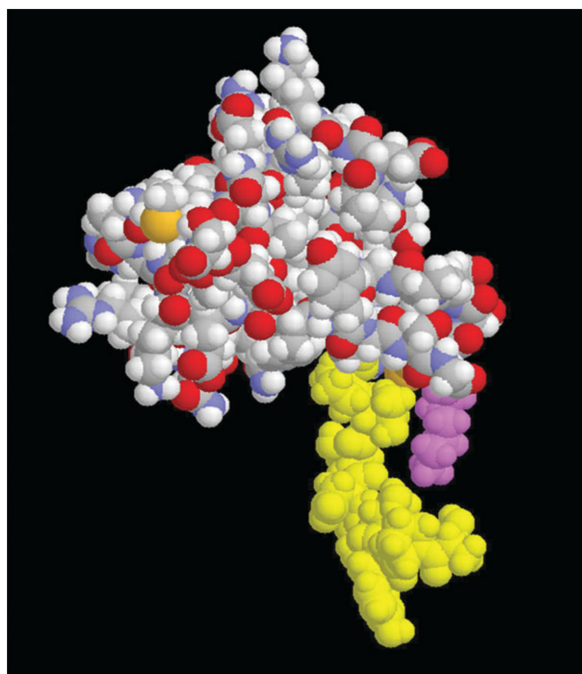

Fig. 1. Three-dimensional structure of the most likely configuration in solution of a RANTES monomer as determined by homonuclear NMR spectroscopy [1RTN (Skelton *et al*., 1995)], indicating the proximity of the side chain of Lys^{33} (indicated in mauve) to the N-terminal region (residues 1–9, indicated in yellow). Coloring elsewhere in the protein follows the CPK convention. This illustration was generated using RasMol (Sayle *et al*., 1995).

a key structure for potent anti-HIV activity, and this led us to design a second series of proteins to test the hypothesis (Table II). We substituted Lys^{33} of PSC-RANTES with leucine (PSC K33L), the residue at the corresponding position of MIP-1β/CCL4, which led to a loss in potency of approximately 1000-fold. Making the reverse switch, of leucine to lysine, at this position in the context of protein Ch2-MIP-1β from the first set of molecules, led to a 200-fold increase in potency (Ch8-MIP-1β; IC_{50} value of 300 pM, i.e. 8-fold less potent than PSC-RANTES). The leucine-to-lysine switch alone in MIP-1β/CCL4 did not lead to a detectable increase in potency (MIP-1β/CCL4L34K), however. Hence Lys^{33} is a key structure for the anti-HIV activity of PSC-RANTES, and is needed in combination with the structures derived from the N-terminal region of PSC-RANTES in order to confer maximal anti-HIV potency on MIP-1β/CCL4.

Structure-activity relationships at position 33

The key role played by the side chain at the position corresponding to Lys^{33} in RANTES/CCL5 on the anti-HIV activity of both PSC-RANTES and the MIP-1β/CCL4 analogues prompted us to investigate the manner in which structures at this position might influence the anti-HIV activity of the chemokines. The anti-HIV activity mechanism of both natural chemokines and chemokine analogues [reviewed in (Hartley *et al*., 2005)] has been linked to (i) steric blockade of cell surface CCR5 binding sites and (ii) induction of intracellular CCR5 sequestration, which has been argued to be dependent upon CCR5 agonist activity through the engagement of cellular processes linked to homologous receptor desensitization (Oppermann *et al*., 1999). We therefore chose to study the influence of the Lys^{33} structure on CCR5 binding affinity, capacity to induce CCR5 downmodulation and capacity to elicit CCR5 signaling.

CCR5 binding affinity. CCR5-binding affinity was compared in a competition binding assay on CHO-CCR5 cells using ^{125}I MIP-1β/CCL4 as a tracer (Fig. 2). In agreement with previously published work (Hartley *et al*., 2004), we found

Table II. Primary sequence and anti-HIV activity of a second group of PSC-RANTES/MIP-1β/CCL4 chimera proteins

Molecule	1 10 20 30 40 50 60	IC_{50} nM
PSC-RANTES	ZXXSSDTTPCCFAYIARPLPRAHIKEYFYTSGKCSNPAVVFVTRKNRQVCANPEKKWVREYINSLEMS	0.04
PSC (K33L)	ZXXSSDTTPCCFAYIARPLPRAHIKEYFYTSGLCSNPAVVFVTRKNRQVCANPEKKWVREYINSLEMS	40
MIP (L34K)	APMGSDPPTACCFSYTARKLPRNFVVDYYETSSKCSQPAVVFQTKRSKQVCADPSESWVQEYVYDLELN	>1000
Ch2-MIP-1β	ZXXSSDTTACCFSYTARKLPRNFVVDYYETSSLCSQPAVVFQTKRSKQVCADPSESWVQEYVYDLELN	60
Ch8-MIP-1β	ZXXSSDTTPCCFSYTARKLPRNFVVDYYETSSKCSQPAVVFQTKRSKQVCADPSESWVQEYVYDLELN	0.3

ZXX indicates the *n*-nonanoyl-thioprolyl-cyclohexylglycyl moiety of PSC-RANTES. Sequences derived from RANTES/CCL5 are boxed in gray; sequences derived from MIP-1β/CCL4 are indicated in plain text; sequences shared by the two chemokines are boxed in black.

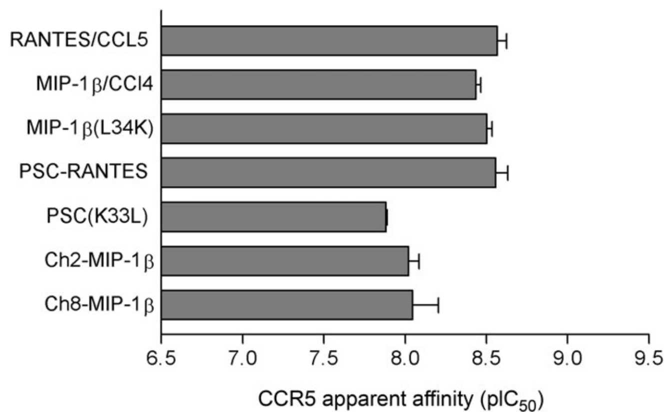

Fig. 2. CCR5 binding affinity of chemokines. Apparent binding affinities (pIC_{50}) were determined in a competition binding assay using CHO-CCR5 cells and radioiodinated MIP-1β/CCL4 as a tracer. Bars indicate mean pIC_{50} values from two independent experiments, error bars indicate SEM.

that RANTES and PSC-RANTES have comparable affinity for CCR5. While the K33L variant of PSC-RANTES (PSC K33L) has an apparent affinity approximately 5-fold lower than PSC-RANTES, substitution of lysine into either MIP-1β/CCL4 (MIP L34K) or the N-terminally modified analog, Ch2-MIP-1β (Ch8-MIP-1β), did not lead to a significant gain in affinity. Broadly similar results were obtained using ^{125}I RANTES/CCL5 as a tracer (data not shown). Hence Lys^{33} might make some contribution to receptor binding affinity, but not enough to account for either the 1000-fold loss in anti-HIV potency when it is replaced by leucine in PSC-RANTES (PSC K33L), or the 200-fold increase in potency when it is engineered into the MIP-1β/CCL4 analogue Ch2-MIP-1β (Ch8-MIP-1β). Finally, as was previously seen across a panel of RANTES analogues (Hartley *et al*., 2004), no correlation between anti-HIV potency and receptor (CCR5) binding affinity was seen across this group of chemokines.

CCR5 sequestration. Capacity to induce intracellular CCR5 sequestration was compared using a steady-state receptor downmodulation assay carried out on CHO-CCR5 cells. PSC-RANTES and Ch8-MIP-1β clearly exhibit a greater capacity to induce CCR5 downmodulation than the corresponding lysine-to-leucine analogues, PSC K33L and

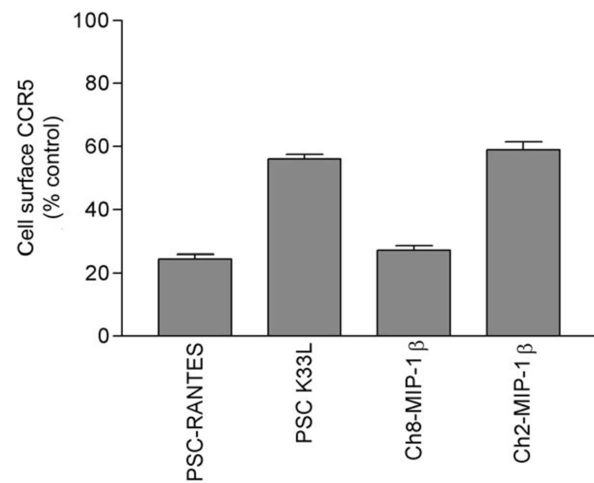

Fig. 3. Capacity of chemokine analogues to elicit CCR5 downmodulation on CHO-CCR5 cells. Cells seeded in multiwell plates were incubated with chemokines at 30 nM for 1 h with prior to quantification of cell surface CCR5. Bars represent mean cell surface CCR5 levels (% control) ± SEM ($n = 6$).

Ch2-MIP-1β (Fig. 3). As was previously seen across a panel of RANTES analogues (Hartley *et al*., 2004), the rank order of anti-HIV potency across this group of molecules (PSC-RANTES, Ch8-MIP-1β ≫ Ch2-MIP-1β, PSC K33L; Table II) correlates with their capacity to induce CCR5 downmodulation.

CCR5 signaling. CCR5 signaling capacity was tested in a Ca^{2+} flux assay in Hela-P5L cells (Fig. 4). In this assay, PSC-RANTES is clearly a CCR5 superagonist—it is both a more potent and a more efficacious agonist than native RANTES/CCL5 (Fig. 4A). This property has been observed for several other RANTES analogues with potent anti-HIV activity and the capacity to induce intracellular sequestration of CCR5 (Hartley *et al*., 2003). While inclusion of key N-terminal structures from PSC-RANTES is sufficient to create a superagonist from MIP-1β/CCL4 (Ch2-MIP-1β), a further increase in signaling activity is gained through inclusion of Lys^{33} (Ch8-MIP-1β). In the same way, substitution of Lys^{33} for leucine in PSC-RANTES leads to a reduction in signaling activity (Fig. 4B). The rank order of signaling activity of the analogues in this assay correlates

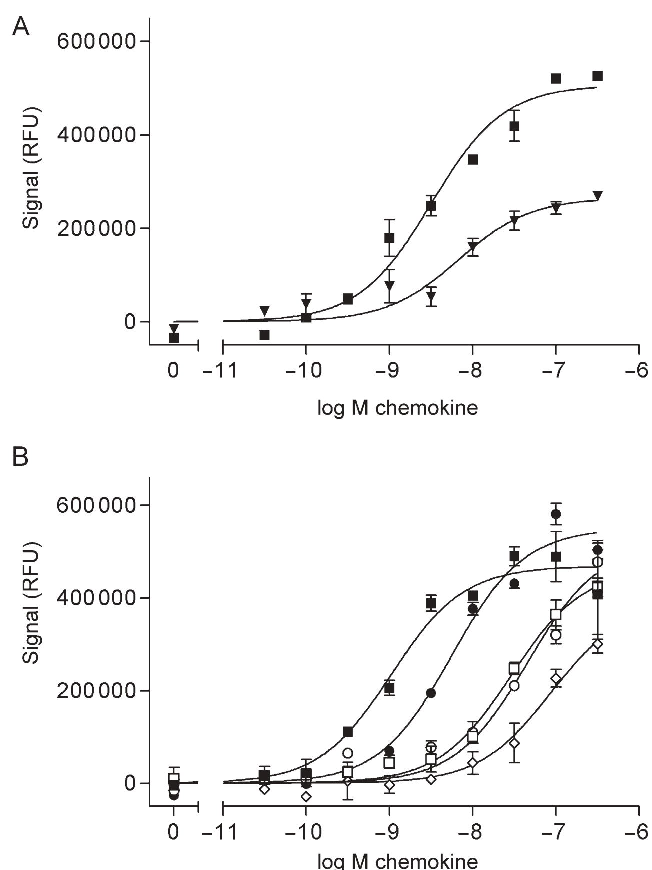

Fig. 4. Signaling activity on CCR5. The signaling capacity of chemokines was determined using a calcium flux assay on Hela-P4-CCR5 cells. Cells growing in multiwell plates were loaded with Fluo-4 and peak fluorescence measurements (relative fluorescence units) were made after addition of chemokines. Each data point was measured in duplicate (error bars indicate SEM). The displayed data are representative of at least three independent experiments. (**A**) Comparison of the signaling activity of PSC-RANTES (■) with that of the native ligand, RANTES/CCL5 (▼). (**B**) Lys33 and CCR5 signaling activity. Comparison of the signaling activity of PSC-RANTES (■) and native MIP-1β/CCL4 (◇) with that of the analogues PSC(K33L) (□), Ch2-MIP-1β (○) and Ch8-MIP-1β (●).

with the rank order of activity in the CCR5 downmodulation assay, with PSC-RANTES and Ch8-MIP-1β clearly stronger agonists than Ch2-MIP-1β and PSC K33L.

Receptor selectivity

According to our working hypothesis, analogues with modifications occurring in the 'message' region of the protein would retain the receptor-binding profile (a function carried by the 'address' structures) of the parent chemokines. Since RANTES/CCL5 is known to activate CCR1 and CCR3 in addition to CCR5, whereas MIP-1β/CCL4 is generally recognized as a CCR5-selective chemokine, we were able to test this hypothesis using binding assays and functional assays on CCR1 and CCR3.

Competition binding assays on CCR1 and CCR3. Binding affinities were determined in competition binding assays on CHO-K1 cells expressing either CCR1 or CCR3 and using either radio-iodinated MIP-1α/CCL3, RANTES/CCL5 or Eotaxin/CCL11 as tracers (Table III).

Table III. Binding affinity of chemokines on CCR1 and CCR3

Molecule	CCR1 IC_{50} (nM)		CCR3 IC_{50} (nM)
	^{125}I MIP-1α/CCL3	^{125}I RANTES/CCL5	^{125}I Eotaxin/CCL11
MIP-1α/CCL3	0.2 ± 0.1	0.3 ± 0.1	n.d.
MIP-1β/CCL4	11.0 ± 3.5	n.d.	n.d.
RANTES/CCL5	29.5 ± 12.4	8.1 ± 5.7	>100
Eotaxin/CCL11	n.d.	n.d.	0.4 ± 0.2
PSC-RANTES	4.3 ± 1.0	5.0 ± 1.9	>100
Ch2-MIP-1β	9.6 ± 3.2	n.d.	>100
Ch8-MIP-1β	2.5 ± 0.2	n.d.	>100

Competition binding assays were carried out on CHO-K1 cells expressing CCR1 or CCR3 using ^{125}I MIP-1α/CCL3, ^{125}I RANTES/CCL5 or ^{125}I Eotaxin/CCL11 as tracers. The data were normalized for non-specific binding (0%), determined in the presence of 300 nM unlabelled control competitor (MIP-1α/CCL3 for CCR1; Eotaxin/CCL11 for CCR3), and specific binding (100%) in the absence of competitor. IC_{50} values were determined by non-linear regression using the GraphPad Prism software applied to a single site model.

The binding affinities for the recognized natural ligands of CCR1, MIP-1α/CCL3 and RANTES/CCL5 were similar to previously published values (Neote *et al.*, 1993), and PSC-RANTES exhibited comparable CCR1 binding affinity to the parent chemokine, RANTES/CCL5.

In contrast to its generally accepted description as a CCR5-selective ligand [e.g. (Bacon *et al.*, 2002)], we found MIP-1β/CCL4 to be a high affinity CCR1 ligand. Its apparent affinity (IC_{50} value of 11.0 nM ± 3.5) lies between that of MIP-1α/CCL3 (IC_{50} value of 0.2 nM ± 0.1) and that of RANTES/CCL5 (IC_{50} value of 29.5 nM ± 12.4). We measured similarly high CCR1 binding affinities for both Ch2-MIP-1β and Ch8-MIP-1β (IC_{50} values of 9.6 ± 3.2 nM, and 2.5 ± 0.2 nM, respectively).

With regard to CCR3 binding affinity, while the apparent binding affinity that we obtained for Eotaxin/CCL11 (IC_{50} value of 0.4 ± 0.2) is comparable to previously published values (Daugherty *et al.*, 1996; Ponath *et al.*, 1996), we were unable to detect any binding activity for native RANTES/CCL5 when ^{125}I Eotaxin/CCL11 was used as a tracer (Table III). In this respect, our results were similar to those of Ponath *et al.*, who found RANTES/CCL5 gave barely detectable competition (IC_{50} > 100 nM) for CCR3 binding on both primary eosinophils and a transfected cell line. We were also unable to generate usable CCR3 binding data using ^{125}I RANTES/CCL5 as a tracer (data not shown). None of the other native chemokines and chemokine analogues that were tested showed any detectable competition in CCR3 binding assays. Given that we were unable to detect competition using RANTES/CCL5 as a competitor, we cannot exclude the possibility that some or all of these molecules bind to CCR3, but that binding activity is not detectable in this assay.

Signaling assays on CCR1 and CCR3. Signaling assays were carried out in CHO-K1 cells expressing either CCR1 or CCR3 (Fig. 5A and B). In agreement with previously published work (Neote *et al.*, 1993), we found both RANTES/CCL5 and MIP-1α/CCL3 to be potent CCR1 agonists (Fig. 5A). PSC-RANTES behaves as a very weak partial

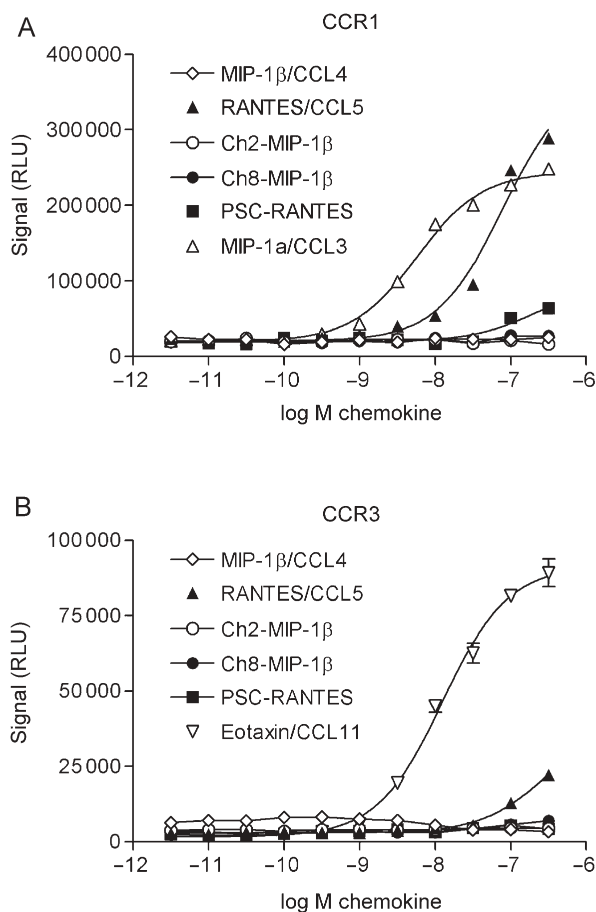

Fig. 5. Signaling activity on RANTES receptors. Functional responses were measured using the aequorin-based functional assay (Blanpain *et al*., 1999). Cells were incubated with a range of concentrations of chemokines as indicated in the legend, and luminescence (RLU) was recorded for 30 s. Each data point was measured in duplicate (error bars indicate SEM). The displayed data are representative of three independent experiments. (**A**) CCR1; (**B**) CCR3.

agonist on CCR1. In spite of its high binding affinity (Table III), MIP-1β/CCL4 has no detectable signaling activity on CCR1. Similarly, neither Ch2-MIP-1β nor Ch8-MIP-1β showed any detectable signaling activity on CCR1.

In the CCR3 signaling assay (Fig. 5B), we found Eotaxin/CCL11 to be a potent and efficacious agonist, with RANTES/CCL5 a less potent partial agonist. These results are in agreement with previously published studies (Daugherty *et al*., 1996; Ponath *et al*., 1996); the detection of signaling activity of RANTES/CCL5 in this assay indicates that our inability to detect binding of RANTES to CCR3 in the competition binding assay (Table III) was due to limitations of the assay system rather to problems with either the cell line or the ligands used. MIP-1β/CCL4 did not show any detectable CCR3 activation at any concentration tested, neither did PSC-RANTES or the MIP-1β/CCL4 analogues, Ch2-MIP-1β and Ch8-MIP-1β. Given our limited ability to detect CCR3 binding in the competition binding assay, there are two possible explanations for this result: either (i) the molecules do not engage CCR3 at all, or (ii) they engage the receptor without eliciting a signal.

Discussion

Generating a potent HIV inhibitor based on MIP-1β/CCL4

We set out with a working model under which it should have been possible to engineer potent anti-HIV activity into MIP-1β/CCL4 by grafting on the N-terminal pharmacophore from PSC-RANTES. While it was possible to attain modest improvements in potency by grafting in the PSC moiety (*n*-nonanoyl-thioprolyl-cyclohexylglycyl-) plus some adjacent N-terminal residues (Ch2-MIP-1β through Ch6-MIP-1β), it was necessary to include Lys^{33} (Ch8-MIP-1β), a residue that is located towards the middle of the primary sequence of RANTES/CCL5 and its derivatives, in order to reach levels of anti-HIV potency comparable to that of PSC-RANTES. Hence, it would appear that the key anti-HIV pharmacophore from PSC-RANTES includes not only the PSC moiety itself, but also Lys^{33} and one or more of residues 4–8 from the RANTES/CCL5 sequence. In spite of its separation from the N-terminal region in the primary sequence of RANTES/CCL5, the Lys^{33} side chain is in close proximity to the N-terminal region in 3-D structures of both RANTES/CCL5 (Fig. 1) and RANTES analogues for which structural data are available (not shown). A previous study has implicated structures in the 'N-loop' and '3_{10} turn' region of MIP-1β/CCL4 (corresponding to residues 12–23 in Table I) as important for interaction with CCR5 (Bondue *et al*., 2002). We did not observe major differences in anti-HIV activity for analogues in which part or all of this region was replaced by the corresponding region from RANTES/CCL5 (analogues Ch3-MIP-1β through Ch6-MIP-1β in Table I). This may be because the structure from the RANTES 'N-loop' and '3_{10} turn' region is capable of fulfilling the CCR5-binding function carried by the corresponding region from MIP-1β/CCL4. In this respect, we note that there are many identical residues in the primary sequence alignment for the two chemokines in this region (Table I).

We have previously shown that optimization of the anti-HIV activity of RANTES/CCL5 analogues including PSC-RANTES is not related to a gain in affinity for CCR5, instead it relates to increasing the capacity of the molecules to induce intracellular sequestration of CCR5 (Hartley *et al*., 2004). The results of this study, involving optimization of the anti-HIV activity of MIP-1β/CCL4 analogues by grafting in the anti-HIV pharmacophore from PSC-RANTES are also consistent with an inhibitory mechanism involving intracellular sequestration of CCR5.

A structure close to the core domain of PSC-RANTES which is crucial for anti-HIV potency

In this study, we demonstrate that Lys^{33} is a key constituent of the anti-HIV pharmacophore from PSC-RANTES: substitution of Lys^{33} for leucine, the corresponding amino acid from MIP-1β/CCL4, resulted in a 1000-fold reduction in potency, and making the corresponding leucine-to-lysine exchange at this position in the context of the most potent N-terminally modified variant of MIP-1β/CCL4 (Ch2-MIP-1β) led to a 200-fold gain in potency (Ch8-MIP-1β, Table II). The identification of a role for these additional positions, 4–8 and 33, has permitted us to take them into account in the further optimization of PSC-RANTES and related molecules (Gaertner *et al*., 2008).

Lys^{33} has been previously identified as a residue that plays a key role in the interaction of native RANTES/CCL5 with

CCR5 (Martin *et al.*, 2001). The proximity of its side chain to the N-terminal region of the protein suggests that it could readily participate in either intra- or intermolecular interactions that are key to receptor engagement, and it has been suggested that the position corresponding to Lys^{33} in some other chemokines might play an important role in receptor engagement [Eotaxin-1/CCL11, MCP-3/CCL7; (Fernandez *et al.*, 2000)]. However, structure-activity studies carried out on IL-8/CXCL8 (Clark-Lewis *et al.*, 1994) and SDF-1/CXCL12 (Ueda *et al.*, 1997) have shown that the residue in this position can be substituted for alanine without any impact on biological activity for certain other chemokines.

Lys^{33} and the 'two-site' model for the interaction between chemokines and their receptors

According to the 'two-site' model for the interaction between chemokines and their receptors, 'message' structures are generally located in the flexible N-terminal region of the proteins, with 'address' structures located further into the primary sequence, predominantly in the N-loop region (Fernandez *et al.*, 2002). Skelton *et al.* (Skelton *et al.*, 1995) used proton NMR to determine the 20 most likely structures of RANTES in solution. Comparison of these structures shows that the first three residues do indeed represent an extremely flexible part of the chain, while Lys^{33}, which is located neither in the N-terminal region nor in the N-loop region, is not in a particularly flexible part of the structure (Fig. 6A). It is possible that Lys^{33} interacts with the N-terminal sequence through a hydrogen bond between its ϵ-NH_3^+ and the main chain carbonyl group of Ser^4 (Fig. 6B). Ser^4 is already in a much less flexible region than the three residues that precede it. It is probably legitimate to extrapolate from the RANTES structure to that of PSC-RANTES, as both X-ray and NMR determinations of the structures of a number of N-terminally modified RANTES derivatives all show the same general features [AOP-RANTES, (Wilken *et al.*, 1999); Met-RANTES, (Hoover *et al.*, 2000)].

Lys^{33} does not appear to act as an 'address' structure. While there is some evidence of an effect on CCR5 binding affinity shown by the 10-fold loss in apparent affinity between PSC-RANTES and the modified analogue PSC K33L (Fig. 2), this is not sufficient to account for the striking effects on anti-HIV activity (Table II), and the analogous leucine–lysine exchange which provides Ch8-MIP-1β with a 200-fold improvement in anti-HIV potency over Ch2-MIP-1β (Table II) is not accompanied by any detectable change in CCR5 affinity (Fig. 2).

Instead, Lys^{33} appears to act as a 'message' structure, or to stabilize a favorable conformation of that structure. Inclusion of Lys^{33} significantly increases the capacity of the chemokines in this study to elicit effector functions—both CCR5 downmodulation (Fig. 3) and CCR5 signaling (Fig. 4B). There is some evidence to suggest that Lys^{33} may also function as a 'message' structure in native RANTES/CCL5: a previously published study (Martin *et al.*, 2001) showed that acetylation of Lys^{33} almost completely abrogates the capacity of RANTES to signal via CCR5.

MIP-1β/CCL4: a high affinity CCR1 ligand

Although MIP-1β/CCL4 is often cited in the literature as a CCR5-specific chemokine (Ward *et al.*, 1998; Murdoch *et al.*, 2000; Thelen *et al.*, 2001; Bacon *et al.*, 2002), we (this study) and others (Neote *et al.*, 1993; Ben-Baruch *et al.*, 1995; Sarau *et al.*, 1997; Chou *et al.*, 2002) have found it to be a high-affinity CCR1 ligand. We suspect that the frequent designation of MIP-1β/CCL4 as an exclusively CCR5-selective chemokine relates to its lack of signaling activity on CCR1: MIP-1β/CCL4 is only functionally selective for CCR5 (Figs 4B, 5A and B). Since MIP-1β/CCL4 has no signaling activity on CCR1 but has a high affinity for the receptor, the suggestion has been made that it may have a physiological role as a CCR1 antagonist (Chou *et al.*, 2002).

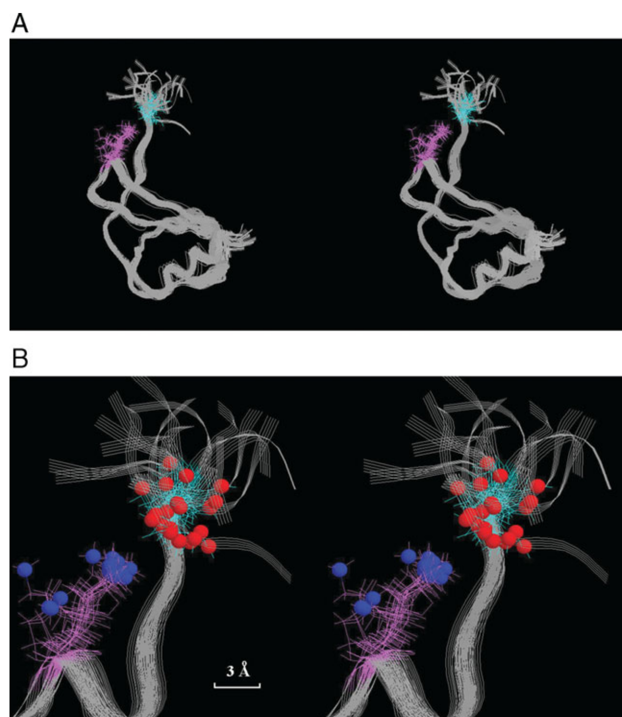

Fig. 6. (**A**) A stereo-view superimposition of the 20 most likely solution structures of RANTES as determined by homonuclear nmr spectroscopy (Skelton *et al.*, 1995). Only the configurations adopted by the main chain are shown (grey) together with the configurations adopted by the side chains of residue Ser-4 (cyan) and Lys-33 (mauve). Note the extreme mobility of the main-chain parts of residues 1–3 (shown at the top of the image). (**B**) A closer view of the potential interaction between the ϵ-NH_3^+ of Lys-33 and the oxygen of the backbone carbonyl of Ser-4. The positions of the centers of these two atoms in the 20 most likely structures are shown as small blue and red spheres, respectively. Other graphic conventions are as in A. This illustration was generated using RasMol (Sayle *et al.*, 1995).

Signaling activity of PSC-RANTES on RANTES receptors

In this study, we present the first data concerning the signaling activity of PSC-RANTES. As has previously been seen for other N-terminally modified RANTES analogues that induce intracellular CCR5 sequestration (Hartley *et al.*, 2003), PSC-RANTES is a CCR5 superagonist. On the other hand, while PSC-RANTES is a high affinity ligand of CCR1 (Table III), it is only a weak partial agonist, where native RANTES/CCL5 is a full agonist on this receptor (Fig. 5A), and unlike native RANTES/CCL5, PSC-RANTES has no detectable signaling activity on CCR3 (Fig. 5B). Reduction in signaling activity on CCR1 and CCR3 has been seen for several other N-terminally modified RANTES analogs with enhanced anti-HIV activity (Elsner *et al.*, 2000; Hartley *et al.*, 2003). It is possible that modifications that enhance

CCR5 engagement are not compatible with agonist activity on CCR1 and CCR3.

Ch8-MIP-1β: a new chemokine analogue with highly potent anti-HIV activity and narrowed receptor selectivity

Since engagement of receptors other than CCR5 is unnecessary for HIV inhibition, and could be undesirable, the goal of this study was to develop a new chemokine analogue with both (i) highly potent anti-HIV activity and (ii) receptor specificity narrowed to CCR5 alone. In Ch8-MIP-1β, we were able to identify a MIP-1β/CCL4 analogue with potency comparable to that of PSC-RANTES, and which retains the receptor binding profile of MIP-1β/CCL4. This means that Ch8-MIP-1β is not a CCR5-specific ligand, however; like native MIP-1β/CCL4, it is a high affinity CCR1 ligand (Table III) without functional consequences that we have been able to detect.

Conclusion

In previous work, we have described the technique of 'protein medicinal chemistry' as a way to identify and optimize key pharmacophore regions in proteins through the rational incorporation of non-natural, non-coded structures (Hartley *et al*., 2004). In this study, we have shown that it is possible to graft such a pharmacophore region from a 'donor' protein to a related 'recipient' protein, conferring on the recipient protein the optimized characteristics of the donor protein. In doing so, we discovered that the key pharmacophore from PSC-RANTES, a highly potent anti-HIV molecule (Hartley *et al*., 2004, Lederman *et al*., 2004), extends beyond the N-terminal PSC moiety to include one or more residues adjacent to it in the sequence, plus the side chain of Lys^{33}. This new information could be usefully applied to the further optimization of anti-HIV molecules based on chemokines.

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