Aminooxypentane Addition to the Chemokine Macrophage Inflammatory Protein-1αP Increases Receptor Affinities and HIV Inhibition*

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To enter its target cells, human immunodeficiency virus (HIV) must interact with CD4 and one of a family of chemokine receptors. CCR5 is widely used by the virus in this context, and its ligands can prevent HIV entry. Amino-terminal modified chemokine variants, in particular AOP-RANTES (aminooxypentane-linked regulated on activation normal T cell expressed and secreted), exhibit enhanced HIV entry inhibition. We have previously demonstrated that a non-allelic isoform of macrophage inflammatory protein (MIP)-1α, termed MIP-1αP, is the most active naturally occurring inhibitor of HIV entry known. Here we report the properties of a variant of MIP-1αP with an AOP group on the amino terminus. We show that, like RANTES, the addition of AOP to MIP-1αP enhances its interactions with CCR1 and CCR5, allows more effective internalization of CCR5, and increases the ligand’s potency as an inhibitor of HIV entry through CCR5. Importantly, AOP-MIP-1αP is about 10-fold more active than AOP-RANTES at inhibiting HIV entry, making it the most effective chemokine-based inhibitor of HIV entry through CCR5 described to date. Surprisingly, the enhanced receptor interactions of AOP-MIP-1αP do not translate into increased chemotaxis or coupling to calcium ion fluxes, suggesting that this protein should be viewed as a partial, rather than a full, agonist for CCR1 and CCR5.

Chemokines are a large family of proteins that play central roles in the chemotraction of leukocytes during immune surveillance, inflammation, and the establishment of immunity (reviewed in Refs. 1–4). As such, they have been implicated in the pathogenesis of many autoimmune and chronic inflammatory diseases. Their biological effects are mediated by a group of heptahelical G-protein-coupled receptors expressed on the surface of their target cells. Interactions between chemokines and their receptors are complex, but in many cases the amino-terminal domain of the chemokine plays an important part in regulating affinity and receptor activation (5–16). Thus, by modifying this region of the protein it is possible to generate variants that have enhanced receptor affinity and/or antagonistic activity. These variants have begun to reveal the nature of chemokine/receptor interactions and could be of use in the development of therapies targeting chemokine receptors. Indeed, such variants have been used experimentally to inhibit chemokine receptor activity in animal models of disease (17–20).

Some chemokine receptors are unusual in that they also are able to interact with proteins outside the chemokine family (21–25). Of particular interest is the gp120 protein in the envelope of the HIV virus, as this protein uses chemokine receptors, along with CD4, to mediate viral entry into target cells (reviewed in Ref. 25). In vitro, many chemokine receptors are able to act as HIV co-receptors, but in vivo two receptors, CCR5 and CXCR4, appear to be predominantly used. This is starkly demonstrated by the protective effects of polymorphisms in CCR5, its ligands, or the CXCR4 ligand stromal-derived factor-1, on the transmission of the virus and/or the development of AIDS (26–31).

The ligands for these receptors (MIP-1α, MIP-1β, monocyte chemoattractant protein-2 (MCP-2) and RANTES for CCR5; stromal-derived factor-1 for CXCR4) can inhibit HIV entry in vitro (25), thought to be due to steric interference and receptor internalization, and likely have a role in regulating HIV infection in vivo (32–36). Targeting these receptors may help in therapeutic regulation of HIV infection (25), and CCR5 in particular has been the focus of intensive research aimed at identifying small molecules or modified chemokines that can act as HIV entry inhibitors (10, 11, 37). Of particular relevance here, is an amino-terminal modified form of RANTES (aminooxypentane (AOP)-RANTES) that is markedly more potent than RANTES at inhibiting HIV entry through CCR5 (11). It has been proposed that this is due to decreased recycling of CCR5 following AOP-RANTES (compared with RANTES) binding and internalization, the consequence of which is more effective removal of CCR5 from the cell surface (38).

We have recently demonstrated that a non-allelic isoform of MIP-1α present in humans, MIP-1αP (or LD78β), is the most effective natural CCR5 agonist yet described, and moreover is

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1 The abbreviations used are: HIV, human immunodeficiency virus; AOP, aminooxypentane; RANTES, regulated on activation, normal T cell expressed and secreted; MIP, macrophage inflammatory protein; MCP, monocyte chemoattractant protein; PBS, phosphate-buffered saline; CHO, Chinese hamster ovary; HEK, human embryonic kidney; FACS, fluorescence-activated cell sorter.
the most potent natural inhibitor of CCR5-dependent HIV entry identified to date (39). Indeed, MIP-1αp shows equivalent anti-HIV activity to AOP-RANTES in these assays. Here, we have generated an AOP variant of MIP-1αp and examined its biological activity in comparison with MIP-1αp, RANTES, and AOP-RANTES. Our results show that AOP addition to MIP-1αp enhances its affinity for CCR1 and CCR5. Importantly, we also show that AOP-MIP-1αp is a more potent inhibitor of HIV entry through CCR5 than either MIP-1αp or AOP-RANTES. In fact, AOP-MIP-1αp is the most effective chemokine-based inhibitor of CCR5-mediated HIV entry described to date. Additionally, we present data that suggests that the enhanced affinity of AOP variants for CCR1 and CCR5 is not consistently reflected by enhanced signaling and chemotactic activity, and in fact AOP-MIP-1αp should be viewed as a partial receptor agonist.

**EXPERIMENTAL PROCEDURES**

**Chemokines—** AOP variants of RANTES and MIP-1αp were synthesized by Gryphon Bioscience (San Francisco, CA). MIP-1αp and murine MIP-1α (mutant PM2) were generated as described elsewhere (40, 41), and RANTES was purchased from Peprotech (London, UK). All chemokines were resuspended to 0.1 μg/ml in phosphate-buffered saline (PBS), aliquoted, and stored at −20 °C until use.

**Cells—** Cells were maintained in the following media supplemented with 10% fetal calf serum (Seromed, Berlin, Germany), 4 mM glutamine (Life Technologies, Inc., Paisley, United Kingdom) and antibiotics: CHO cells (Special Liquid Medium (Life Technologies, Inc.)), HEK293 cells (Dulbecco’s minimal essential medium (Sigma, Poole, Dorset, UK)), CEM cells (RPMI 1640 (Life Technologies, Inc.)), CEMx174 cells (RPMI 1640 plus 0.1 μM HEPES), THP1 cells (RPMI 1640 plus 20 μM β-mercaptoethanol). CHO and HEK293 receptor transfectants were grown in medium containing 1.6 and 0.8 mg/ml Geneticin (Life Technologies, Inc.), respectively. CCR5 expressing CEM or CEMx174 cells (CEM-CCR5 and CEMX174-CCR5) were grown in medium plus 0.5 μg/ml puromycin.

**Radioligand Displacement Assays—** Chemokines were labeled with 125I as described elsewhere (42) using IODO-GEN (Pierce, Rockford, IL). 300 nM stock solution of radiolabeled chemokine was stored at 4 °C. The derivation of CHO cells stably transfected with human CCR1, CCR5, and D6, and the displacement assays have been described previously (43). Briefly, 2 × 106 CHO cells were incubated overnight in each well of a 24-well plate, washed in PBS, then 250 μl of complete medium was added containing 20 nM HEPES, 0.5% sodium azide, radiolabeled chemokine, and PBS or a given concentration of unlabeled chemokine in PBS. Final concentrations of 20, 20, and 3 nM 125I-m-MIP-1α were used for CCR1, CCR5, and D6 transfectants, respectively. After 1.5–2 h at room temperature, the cells were washed three times with ice-cold PBS, lysed with 0.1% sodium dodecyl sulfate, transferred to a counting vial and counted for 1 min in a Beckman Gamma 5500B counter (Beckman, High Wycombe, UK). For CEM-CCR5 and THP1 cells, 2 × 106 cells were transferred to a 1.5-ml Eppendorf tube, washed, and incubated as above. For HEK293 transfectants, cells were harvested by trypsinization, washed, 2 × 106 cells transferred to a 1.5-ml Eppendorf tube and incubated as above. For binding assays done in suspension, cells were counted directly without lysis. In all experiments, each point was done in triplicate and each experiment was done at least twice.

**Ca2+ Fluxes—** Determination of intracellular Ca2+ was determined as detailed elsewhere (43). Briefly, cells loaded for 45 min with Fura-2-AM (Sigma) in SR buffer (136 mM NaCl, 4.8 mM KCl, 5 mM glucose, 20 mM HEPES (pH 7.4), 1 mM CaCl2, 0.025% bovine serum albumin) were incubated at 37 °C in a LS50 spectrophotometer (Instrument settings: 340 nm (λex), 500 nm (λem) (PerkinElmer Life Sciences, Norwalk, CT) for 2 min to warm the cells, fluorescent readings then started and chemokine (diluted in PBS) added after a further 30–60 s. Fluorescence emission was recorded for a further 1–2 min and additional chemokines added where appropriate.

**Chemotaxis Assays—** The ability of THP1 or CEM-CCR5 cells to migrate in response to chemokines was analyzed using a Transwell assay. Cells were loaded for 30 min at 37 °C in 1 μM Calcein-AM (Molecular Probes, Leiden, The Netherlands) and washed twice in chemotaxis buffer (RPMI 1640, 1% bovine serum albumin, 20 μM HEPES) by centrifugation at 1000 × g for 5 min. The cells were resuspended in buffer at 2 × 106/ml and 300 μl was added to 3-μm pore HTS Fluoroblok inserts (Becton Dickinson, Le Pont de Clai, France). The inserts were held in 24-well companion plates (Becton Dickinson) containing 700 μl of the appropriate chemokine diluted in chemotaxis buffer, and incubated for 1 h 45 min at 37 °C. The plates were read on a Fluoroscan fluorescent plate reader (BMG LabTechnologies, Offenburg, Germany).

**Characterization of AOP-MIP-1α**

**AOP Addition Enhances Interaction with CCR1 and CCR5—** To determine whether the addition of AOP to the NH2 terminus of MIP-1αp alters its interaction with chemokine receptors, we performed heterologous displacement studies using RANTES, MIP-1αp, and their AOP derivatives. CHO cells expressing known human receptors for MIP-1αp and RANTES (CCR1, CCR5, or D6) were incubated with a set concentration of radiolabeled chemokine (diluted in PBS) added after a further 30–60 s. Fluorescent plate reader (BMG LabTechnologies, Offenburg, Germany). The derivation of CHO cells stably transfected with human CCR1, CCR5, and D6, and the displacement assays have been described previously (43). Briefly, 2 × 106 CHO cells were incubated overnight in each well of a 24-well plate, washed in PBS, then 250 μl of complete medium was added containing 20 nM HEPES, 0.5% sodium azide, radiolabeled chemokine, and PBS or a given concentration of unlabeled chemokine in PBS. Final concentrations of 20, 20, and 3 nM 125I-m-MIP-1α were used for CCR1, CCR5, and D6 transfectants, respectively. After 1.5–2 h at room temperature, the cells were washed three times with ice-cold PBS, lysed with 0.1% sodium dodecyl sulfate, transferred to a counting vial and counted for 1 min in a Beckman Gamma 5500B counter (Beckman, High Wycombe, UK). For CEM-CCR5 and THP1 cells, 2 × 106 cells were transferred to a 1.5-ml Eppendorf tube, washed, and incubated as above. For HEK293 transfectants, cells were harvested by trypsinization, washed, 2 × 106 cells transferred to a 1.5-ml Eppendorf tube and incubated as above. For binding assays done in suspension, cells were counted directly without lysis. In all experiments, each point was done in triplicate and each experiment was done at least twice.

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effect on RANTES and MIP-1α on all three receptors, enhancing displacement of 125I-MIP-1α from CCR1 and -5, but reducing it from D6. Similar effects were seen with MIP-1α or AOP-MIP-1αP when these proteins themselves were radiolabeled and displaced from receptor by excess of the same, but unlabeled, ligand, i.e. homologous displacement assays (not shown).

**Fig. 1.** AOP variants of MIP-1αP and RANTES exhibit alterations in their ability to displace 125I-mMIP-1α from CCR1, CCR5, and D6. Displacement of 125I-mMIP-1α by MIP-1αP, RANTES, or their AOP variants from CHO cells expressing CCR1 (A), CCR5 (B), and D6 (C). Experiments were performed on 2 x 10⁶ cells incubated at room temperature with 125I-mMIP-1α and competitor chemokine in the presence of 0.5% azide for 90 min, before washing three times in ice-cold PBS. Cells were lysed and remaining 125I-mMIP-1α in the lysate was counted. Each point was done in triplicate and the mean and standard deviation calculated. Repeat experiments gave similar displacement profiles. Diamonds, AOP-MIP-1αP; squares, MIP-1αP; filled triangles, AOP-RANTES; crosses, RANTES.

**Fig. 2.** MIP-1αP and AOP-MIP-1αP show similar signaling potency through CCR1 and CCR5. HEK293 cells transfected with CCR1 (panels A and C) or CCR5 (panels B and D) were loaded with Fura-2, and fluorescence emission recorded (λex, 340 nm; λem, 500 nm) for up to 3 min during which chemokines were added, indicated by the arrows labeled 1 and 2. A, plot a, 5 nM MIP-1αP (arrow 1), 50 nM MIP-1αP (arrow 2); plot b, 5 nM AOP-MIP-1αP (arrow 1), 50 nM MIP-1αP (arrow 2). B: plot a, 2.5 nM MIP-1αP (arrow 1), 25 nM MIP-1αP (arrow 2); plot b, 2.5 nM AOP-MIP-1αP (arrow 1), 25 nM MIP-1αP (arrow 2). C: plot a, 20 nM RANTES (arrow 1); plot b, 20 nM AOP-RANTES (arrow 1). D: plot a, 5 nM RANTES (arrow 1); plot b, 5 nM AOP-RANTES (arrow 1).

**Characterization of AOP-MIP-1αP**

Signaling Activity of AOP Chemokine Variants—Our previous work (39) has shown that for variants of MIP-1α, greater affinity for CCR1 or CCR5 in heterologous displacement studies is associated with more potent chemokine signaling through these receptors, as assessed by the induction of calcium ion fluxes. Thus, it was anticipated that the AOP chemokines would elicit more robust fluxes into cells than the unmodified chemokines. To examine this, HEK293 cells expressing CCR1 or CCR5 were assessed for their ability to mobilize Ca²⁺ in response to ligand stimulation. Initially, radioligand displacement experiments were done and importantly showed that, as with the CHO cell transfectants above, the AOP variants had enhanced affinity for CCR1 and CCR5 expressed in these cells (not shown). Curiously, however, when Ca²⁺ flux assays were performed, AOP-MIP-1αP signaling through CCR1 and CCR5 induced fluxes that were of similar magnitude to those seen with MIP-1αP (Fig. 2, A and B, fluxes after arrow 1). Additionally, despite its enhanced ability to displace mMIP-1α, AOP-RANTES showed weaker signaling through CCR1 than an equimolar concentration of RANTES (Fig. 2C). However, AOP-
RANTES induced a more robust Ca\(^{2+}\) flux through CCR5 than an equivalent concentration of RANTES (Fig. 2D). These observations were seen across a range of ligand concentrations (not shown). Also, the ability of AOP-MIP-1αP to block a second signal induced with the unmodified protein was examined. For CCR5, a second flux induced with a 10-fold molar excess of MIP-1αP was considerably smaller if AOP-MIP-1αP had been used as the first stimulant (Fig. 2A and B, fluxes after arrow 2). Likewise, with the CCR1 transfectants (Fig. 2A), 5 nM AOP-MIP-1αP completely prevents a detectable second flux induced with 50 nM MIP-1αP, while 5 nM MIP-1αP does not. Thus, while the addition of AOP to MIP-1αP does not cause enhanced coupling to Ca\(^{2+}\) flux induction, increased receptor occupancy appears to result in a more effective block of subsequent MIP-1αP-induced signals.

**Chemotactic Activity of Chemokines and Their AOP Variants**—Signals induced by the AOP variants through CCR1 and CCR5 will regulate their chemotactic activity. To examine chemotaxis, MIP-1αP, RANTES, and their AOP variants were used in Transwell chemotaxis assays to attract CEM-CCR5 cells, or THP1 cells (that express CCR1 but not CCR5) (Fig. 3). While they gave similar profiles, MIP-1αP consistently appeared to be marginally more active than AOP-MIP-1αP at attracting CEM-CCR5 cells (Fig. 3A). Thus, 0.1 nM MIP-1αP in the bottom of the chemotaxis plate attracted significant numbers of cells through the filter, but the same concentration of AOP-MIP-1αP was ineffective. However, equal numbers of CEM-CCR5 cells were attracted toward 0.3 nM of each chemokine. With THP1 cells, MIP-1αP and AOP-MIP-1αP produced near identical dose-response profiles (Fig. 3B). AOP-RANTES, on the other hand, was a more potent chemotactic agent for CEM-CCR5 cells than RANTES (Fig. 3C): considerably more cells migrated when 1 or 10 nM AOP-RANTES was added to the bottom well of the chemotaxis plate compared with the same concentration of RANTES. In contrast, AOP-RANTES was less effective at attracting THP1 cells through CCR1 (Fig. 3D) with virtually no cells being attracted toward 10 nM AOP-RANTES, while the same concentration of RANTES was an effective stimulus.

Thus, with MIP-1αP, RANTES, and their AOP derivatives, the Ca\(^{2+}\) flux data closely mirrors the chemotaxis data, suggesting that although AOP variants of MIP-1αP and RANTES have enhanced interactions with CCR1 and -5, this does not consistently result in an equivalent enhancement of bioactivity.

D6-mediated signaling or chemotaxis was not tested. This receptor has shown no signaling or chemotactic activity when expressed in heterologous cells under assay conditions in which other chemokine receptors show robust signals.\(^2\)

**AOP-MIP-1αP Is the Most Potent Chemokine-based Inhibitor of HIV Entry through CCR5**—We have shown previously (39) that, in vitro, MIP-1αP is a potent inhibitor of HIV entry through CCR5, and is consistently as effective as AOP-RANTES in this context. Thus, it was anticipated that AOP-MIP-1αP may show even greater potency at inhibiting viral entry due to its enhanced ability (compared with MIP-1αP) to bind to CCR5. To test this, the ability of AOP-MIP-1αP to prevent pseudotyped reporter virus entry into CEMx174-CCR5 cells was compared with MIP-1αP and AOP-RANTES. Remarkably, 0.1 nM AOP-MIP-1αP was able to almost completely inhibit entry of JR-FL and ADA pseudotyped virus (Fig. 4). AOP-RANTES and MIP-1αP were less effective than AOP-MIP-1αP, and only reduced viral entry by 60–70% at 0.1 nM. Indeed, a 10-fold higher concentration of these ligands was required before complete inhibition of entry of JR-FL pseudotyped virus was seen, while some viral entry (5–10%) was still seen with the ADA-pseudotyped virus at this concentration. Infection by viruses pseudotyped with envelope from a CXCR4-tropic virus, JC2 (which uses CXC4R on CEMx174 cells to gain entry) or an amphotropic murine leukemia virus, was unaffected by all the chemokines tested (data not shown). These assays show AOP-MIP-1αP to be the most effective chemokine-based inhibitor of CCR5-mediated HIV entry currently known.

**Internalization and Recycling of CCR5**—CCR5 internalization and recycling may contribute to the HIV entry inhibitory properties of chemokines (38). Thus, we wished to compare the ability of MIP-1αP and RANTES, and their AOP derivatives, to internalize and recycle CCR5 in CEM-CCR5 cells using FACS analysis to detect cell surface CCR5. First, cells were incubated with 5 nM of each chemokine and CCR5 expression determined over time. As seen in Fig. 5A, AOP-MIP-1αP more rapidly internalizes CCR5 from the surface of transfected CEM cells, than MIP-1αP, RANTES, or AOP-RANTES. With AOP-MIP-1αP, nearly 60% of detectable cell surface CCR5 had been removed within 5 min, with a further 20% internalized by 30 min. When MIP-1αP or AOP-RANTES were used, 50% of the surface CCR5 was removed after 30 min, while RANTES hardly internalized any of the CCR5 (Fig. 5A). However, by 45 min, equivalent levels of CCR5 remained on the surface of the CEM cells treated with MIP-1αP or AOP-MIP-1αP. Thus, the AOP-coupled chemokines more rapidly cause the internalization of CCR5 into these cells than the unmodified chemokines, with AOP-MIP-1αP being more effective than AOP-RANTES. With 100 nM MIP-1αP, AOP-MIP-1αP, or AOP-RANTES, CCR5 internalization was broadly similar with more than 80% of cell surface CCR5 internalized by 15 min (Fig. 5B). However, RANTES removed only about 50% over 45 min.

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\(^2\) R. J. B. Nibbs and G. J. Graham, unpublished data.
Characterization of AOP-MIP-1αP

Next, we wished to examine whether CCR5 was able to recycle to the cell surface after internalization by MIP-1αP or AOP-MIP-1αP. CEM-CCR5 cells were treated for 45 min with 100 nM chemokine, washed thoroughly, reincubated in medium, and the re-emergence of CCR5 assessed by FACS analysis (Fig. 5C). After 2 h, cells treated with MIP-1αP have 65% of the control levels of CCR5 back on the surface while those treated with AOP-MIP-1αP have just above 30%. Therefore, AOP-MIP-1αP brings about a more persistent down-regulation of cell surface CCR5 than an equimolar concentration of MIP-1αP. There are also reproducible differences in the rate of re-emergence between the two proteins, particularly over the first 30 min after washing away the chemokine. Very little AOP-MIP-1αP associated CCR5 returns to the surface over this time period, while cells treated with MIP-1αP double their detectable cell surface CCR5 protein levels. Similarly, AOP-RANTES more effectively prevented the re-emergence of internalized CCR5 compared with RANTES in these assays (not shown).

DISCUSSION

Here we have described the biological properties of a form of MIP-1αP modified at the NH₂ terminus by the addition of an aminooxypentane group. AOP-MIP-1αP has enhanced affinity for CCR1 and CCR5, but surprisingly, does not show dramatically altered Ca²⁺ flux signaling or chemotactic potency compared with MIP-1αP. Importantly, AOP-MIP-1αP is markedly more potent than MIP-1αP and AOP-RANTES in inhibiting entry through CCR5 of viruses pseudotyped with the envelope protein of R5 HIV strains, and therefore, appears to be the most potent chemokine-based inhibitor of CCR5-mediated HIV entry currently known.

Modifying the amino-terminal domains of chemokines can dramatically alter receptor affinity, and change the ability of the ligated receptor to couple to the signaling machinery of the cell (5–16). How AOP addition alters the CCR1 and CCR5 binding properties of MIP-1αP is uncertain, but this hydrophobic chain may become embedded more readily in the hydrophobic core of the receptor to increase receptor affinity. However, AOP-MIP-1αP should be viewed as a partial, rather than a full, agonist of these receptors with respect to coupling to Ca²⁺ fluxes and chemotaxis, because increased receptor occupancy at a given concentration by AOP-MIP-1αP results in little change in activity in these assays. Some, or all, of these AOP-MIP-1αP-ligated receptors must not be contributing as fully to chemotaxis and Ca²⁺ flux induction as when an equivalent number of receptors are bound to MIP-1αP. Nonetheless, this increased receptor occupancy results in AOP-MIP-1αP and MIP-1αP being distinct in their ability to prevent subsequent Ca²⁺ fluxes induced by MIP-1αP. Thus, the use of AOP-MIP-1αP as the first stimulus is a more effective inhibitor of subsequent stimulation because the amount of remaining receptor available is reduced.

AOP-RANTES, like AOP-MIP-1αP, showed an enhanced ability to displace mMIP-1α from CCR1 compared with RANTES, yet this increased receptor occupancy is not effectively coupled to Ca²⁺ fluxes and chemotaxis. This suggests that AOP-RANTES could also be classified as a partial agonist of CCR1. This is supported by the observation that, while treatment of CCR1-expressing cells with RANTES or AOP-RANTES gives starkly different Ca²⁺ fluxes, it can still bring about comparable reductions in a second Ca²⁺ flux induced with RANTES (not shown). On CCR5, AOP addition to RANTES increases mMIP-1αP displacement, but also markedly increases its chemotactic potency and its ability to couple to Ca²⁺ fluxes. We are currently uncertain as to why the AOP moiety only increases the activity of RANTES in these assays, and only through CCR5. However, this emphasizes the complexity of chemokine/receptor interactions, and demonstrates the difficulty in predicting the outcome of amino-terminal modification. Our results with AOP-RANTES are, to some extent, in contrast to published observations that, despite showing similar changes in Ca²⁺ flux induction through CCR1 (44) or CCR5 (38) by AOP addition to RANTES, revealed little change in binding affinity (10, 44). We are uncertain why these discrepancies exist, but they could be explained by the different protocols, radiolabeled ligands, and/or cell lines used for analyzing receptor affinity.

It is of note, that in contrast to CCR1 and CCR5, D6 has a reduced affinity for AOP variants of MIP-1αP and RANTES compared with their unmodified counterparts. It is tempting to speculate that this is indicative of an atypical receptor conformation of D6, perhaps linked to the current “non-signaling” status of this receptor. It will be interesting to see if mutants of D6 that exhibit weak signaling ability³ show altered interaction with AOP variants.

The increased receptor occupancy seen on addition of AOP to MIP-1αP, while not changing chemotaxis and Ca²⁺ flux induction, increases the rate of CCR5 internalization and slows

³ R. J. B. Nibbs, unpublished data.
receptor re-emergence. Similarly, AOP-RANTES induces more rapid and effective CCR5 internalization, and slower recycling, than RANTES. Chemokine receptor internalization is believed to be controlled by signals emanating from the ligated receptor that alter the receptor phosphorylation status and allow its recognition by the endocytotic machinery (45, 46). Our results suggest that CCR5 internalization uses signal transduction pathways that, unlike those coupled to Ca\(^{2+}\) fluxes and chemotaxis, are more effectively stimulated by CCR5 bound to AOP-MIP-1\(\alpha\)P than the unmodified counterpart. It has been shown that AOP-RANTES triggers more rapid CCR5 phosphorylation and association with GRK2 and \(\beta\)-arrestin1 (proteins involved in receptor desensitization and endocytosis) (47), and similar pathways may be involved in mediating the rapid AOP-MIP-1\(\alpha\)P-induced CCR5 internalization. An alternative possibility is that AOP-linked chemokines cause CCR5 to cluster more effectively thereby encouraging internalization, and indeed AOP-MIP-1\(\alpha\)P does form higher order aggregates more readily than MIP-1\(\alpha\)P.\(^4\) Once inside the cell, the slowed CCR5 recycling seen with AOP variants may be a consequence of greater receptor affinity, or perhaps increased aggregation tendency, preventing receptor-ligand dissociation and subsequent CCR5 recycling. As proposed by Mack and colleagues (38), such receptor complexes may be more readily targeted for degradation.

The primary aim at the outset was to generate a molecule with enhanced potency at inhibiting HIV entry through CCR5. This has proved successful: AOP-MIP-1\(\alpha\)P is markedly more active (approximately 10-fold) than MIP-1\(\alpha\)P and AOP-RANTES at preventing the entry of R5-pseudotyped reporter viruses into CEMx174-CCR5 cells. A simple explanation for this is that enhanced affinity for CCR5 causes more receptor to become internalized or blocked, leaving less available to be used by the virus. Indeed, this view is widely held as the mechanism by which chemokines prevent HIV entry. However, closer examination of the data suggests that this may not be an adequate explanation, since the concentration of AOP-MIP-1\(\alpha\)P (and MIP-1\(\alpha\)P and AOP-RANTES) required to block >95% of viral entry into CEMx174-CCR5 cells is so low. Indeed, in most reports (10, 11, 48–51), concentrations of chemokine that have been used to prevent HIV entry are insufficient to result in many CCR5 receptors becoming occupied or internalized. For example, in our hands, the use of 0.1 nM AOP-MIP-1\(\alpha\)P (that blocks >95% of viral internalization) is not able to cause any detectable internalization of CCR5 from the surface of CEM-CCR5 cells (not shown), although chemotaxis is stimulated at this concentration showing some CCR5 molecules are occupied (Fig. 3A). However, from the heterologous displacement studies in Fig. 1B, and homologous displacement studies with \(^{125}\)I-AOP-MIP-1\(\alpha\)P (not shown), it appears that CCR5 occupancy at 0.1 nM AOP-MIP-1\(\alpha\)P is extremely low. Indeed, incubating CCR5-expressing cells with 0.1 nM \(^{125}\)I-AOP-MIP-1\(\alpha\)P results in barely detectable binding of the radioligand (not shown). Similarly, the concentrations of MIP-1\(\alpha\)P and AOP-RANTES that completely block viral entry cause little receptor occupancy or internalization (not shown). Thus, steric interference of HIV binding, CCR5 internalization, or slowed receptor

\(^4\) K. Ottersbach and G. J. Graham, unpublished data.
recycling (all of which have been proposed to play a role in inhibiting HIV entry), are unlikely to be of much importance at the low chemokine concentrations that effectively inhibit viral entry.

These observations lead us to propose that an alternative mechanism is at play, and we are currently investigating whether signals emanating from CCR5 at low receptor occupancy are involved in chemokine-mediated inhibition of HIV entry through this receptor. While signaling appears not to be required for HIV entry through CCR5 (52–54), it may be important for the inhibition of viral entry seen at low chemokine concentrations. While we have shown here that MIP-1αP and AOP-MIP-1αP show similar potency in the Ca²⁺ flux and chemotaxis assays with CCR5-expressing cells, nonetheless other signaling pathways coupled to CCR5 may be more potently stimulated by AOP-modified, rather than wild-type, MIP-1αP and contribute to the enhanced inhibition of viral internalization seen with this protein. Indeed, as we have discussed above, the faster rate of CCR5 internalization upon binding AOP-MIP-1αP, rather than MIP-1αP, may reflect enhanced signaling activity by AOP chemokines. Also, it is worth considering that in the viral internalization assays chemokine stimulation is over a longer time period than in the Ca²⁺ flux and chemotaxis assays. Such chronic, low-level CCR5 stimulation may elicit quite distinct intracellular signals. Experiments are underway to attempt to answer some of these questions.

In summary, the addition of aminooxypentane to MIP-1αP enhances interaction with CCR1 and CCR5, increases CCR5 internalization, and creates a more potent inhibitor of HIV entry through CCR5 without affecting its chemotactic potency or its ability to induce Ca²⁺ fluxes. These studies suggest that an alternative mechanism, other than steric interference or receptor internalization, may be involved in chemokine inhibition of viral entry. Finally, the properties of AOP-MIP-1αP may make it useful experimentally to down-regulate MIP-1αP receptors, and potentially therapeutically applicable in the treatment of HIV infection and inflammatory disease.

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