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Biased small-molecule ligands for selective inhibition of HIV-1 cell entry via CCR5

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Keywords
CCR5, drug development, efficacy switch, fusion, HIV-1, ligand bias, small-molecule inhibitors.

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Abstract
Since the discovery of HIV’s use of CCR5 as the primary coreceptor in fusion, the focus on developing small-molecule receptor antagonists for inhibition hereof has only resulted in one single drug, Maraviroc. We therefore investigated the possibility of using small-molecule CCR5 agonists as HIV-1 fusion inhibitors. A virus-free cell-based fusion reporter assay, based on mixing “effector cells” (expressing HIV Env and luciferase activator) with “target cells” (expressing CD4, CCR5 wild type or a selection of well-described mutations, and luciferase reporter), was used as fusion readout. Receptor expression was evaluated by ELISA and fluorescence microscopy. On CCR5 WT, Maraviroc and Aplaviroc inhibited fusion with high potencies (EC50 values of 91 and 501 nM, respectively), whereas removal of key residues for both antagonists (Glu283Ala) or Maraviroc alone (Tyr251Ala) prevented fusion inhibition, establishing this assay as suitable for screening of HIV entry inhibitors. Both ligands inhibited HIV fusion on signaling-deficient CCR5 mutations (Tyr244Ala and Trp248Ala). Moreover, the steric hindrance CCR5 mutation (Gly286Phe) impaired fusion, presumably by a direct hindrance of gp120 interaction. Finally, the efficacy switch mutation (Leu203Phe) – converting small-molecule antagonists/inverse agonists to full agonists biased toward G-protein activation – uncovered that also small-molecule agonists can function as direct HIV-1 cell entry inhibitors. Importantly, no agonist-induced receptor internalization was observed for this mutation. Our studies of the pharmacodynamic requirements for HIV-1 fusion inhibitors highlight the possibility of future development of biased ligands with selective targeting of the HIV–CCR5 interaction without interfering with the normal functionality of CCR5.

Abbreviations
AIDS, acquired immunodeficiency syndrome; CCR5, C-C chemokine receptor type 5; CD4, cluster of differentiation 4; CXCR4, C-X-C chemokine receptor type 4; ECL2, extracellular loop 2; gp120, glycoprotein 120; GPCR, G-protein-coupled receptor; HAART, highly active antiretroviral therapy; HIV-1, human immunodeficiency virus type 1; RLU, relative light units; TM, transmembrane domain; V3, third variable loop; WT, wild type.

Introduction
Human immunodeficiency virus type 1 (HIV-1) belongs to the Retroviridae family of single-stranded RNA viruses, replicating through the process of reverse transcription. The principal factors enabling entry of HIV-1 into a human host cell are CD4 and a chemokine coreceptor, the most important of which are CCR5 and CXCR4. These two distinct chemokine receptors were originally discovered in relation to their role as the primary coreceptors for HIV-1 cell entry (Alkhatib et al. 1996; Bleul et al. 1996; Deng et al. 1996; Feng et al. 1996; Oberlin et al. 1996),...
and have since been the targets of multitudes of anti-HIV drug candidates (Steen et al. 2009; Chen et al. 2012). The viral capsid of HIV-1, containing single-stranded RNA and enzymes needed in the viral life cycle, enters the host cell by fusion of the viral envelope with the cell membrane. The fusion process is initiated by binding of the viral envelope-associated glycoprotein 120 (gp120) to human CD4, which facilitates secondary binding to a chemokine coreceptor via exposure of the V3 loop (Biscone et al. 2006; Huang et al. 2007). During the early stages and throughout most of the infection, HIV-1 typically exerts a preference toward the use of CCR5 and is designated R5 tropic. The V3 loop supposedly binds to CCR5 in a two-step fashion, not unlike the binding of endogenous chemokines, the first step being recognition of the receptor through interaction with the N-terminus and extracellular loop 2 (ECL2) (Rucker et al. 1996; Huang et al. 2007; Thiele and Rosenkilde 2014; Kufareva et al. 2015). Second, interactions with residues in the TM domain are necessary to permit the conformational changes of the viral envelope protein required for entry into host cell (Garcia-Perez et al. 2011a; Tamamis and Floudas 2014).

The current first-line treatment of HIV is a combination therapy (highly active antiretroviral therapy, HAART) consisting of several antiretroviral drugs such as nucleoside and non-nucleoside reverse transcriptase inhibitors, protease inhibitors, and integrase inhibitors. These all target essential viral enzymes in HIV’s replication cycle; however, the high viral turnover and error-prone nature of reverse transcription causes rapid mutation (Wei et al. 1995; Preston et al. 1997), which eventually leads to development of resistance (Werb et al. 2010; Hughes and Andersson 2015). With recent changes in the international treatment guidelines (WHO, 2015) in light of the START study (Group et al. 2015) – suggesting start of treatment at the time of diagnosis – there is an increased risk of cumulative toxicity from the antiviral drugs, and potential problems with adherence may further lead to increased prevalence of resistance (Babiker et al. 2013). Targeting the human component, for example, the chemokine fusion coreceptors, the development of resistance is theoretically delayed, making such drugs desirable anti-HIV agents. Aside from their coreceptor function in HIV/AIDS, the chemokine receptors are involved in a number of physiological processes including homeostasis and cell migration during development and immune responses, as well as in the pathophysiology of autoimmune disease and cancer (Bacheslerie et al. 2014). Thus, targeting the human chemokine system is not without risks, and roughly 20 years after the discovery of HIV-1’s exploitation of chemokine receptors, the attempts to create effective HIV-1 entry inhibitors have only resulted in the approval of a single drug, the CCR5 antagonist Maraviroc (FDA, 2007). Other drug candidates, such as Aplaviroc and Vicriviroc, have failed to complete clinical trial due to concerns about toxicity and side effects resulting from off-site targets of these antagonists (Nichols et al. 2008). Future development of drugs that alone inhibit the interaction between HIV and CCR5 and/or CXCR4 without interfering with the natural chemokine-induced activity of the receptors (so-called biased ligands with functional selectivity) are necessary to avoid side effects caused by disruption of the chemokine receptor function. The recently published crystal structure of CCR5 (Tan et al. 2013) has helped improve the understanding of the interactions between the receptor and gp120; however, this structure was of a Maraviroc-bound, inactive conformation, and thus some limitations apply to the model. Nonetheless, the key interaction points of gp120–V3 have been found to be similar to those of Maraviroc and Aplaviro (Maeda et al. 2006; Kondu et al. 2008; Garcia-Perez et al. 2011a; Tan et al. 2013), suggesting that the antagonists function through interference with the secondary binding step. Furthermore, the inactive Maraviroc-bound CCR5 conformation might also play a role in the prevention of fusion (Garcia-Perez et al. 2011b; Tan et al. 2013).

In order to improve the knowledge needed for the design of small-molecule ligands with functional selectivity toward HIV-1 fusion inhibition, we investigated the conformational receptor requirements for HIV-1 interaction using CCR5 as a model system. By utilizing previously well-described CCR5 mutations (Steen et al. 2013, 2014a,b), we applied inactive and constitutively active receptor conformations in HIV-1 gp120-mediated fusion, including some with bias toward G-protein activation and absent β-arrestin recruitment. Furthermore, we studied not only small-molecule receptor antagonists, but also small-molecule agonists in the structural requirements of HIV-1 inhibition by use of so-called efficacy switch mutations in CCR5 (Steen et al. 2013, 2014b), where the antagonist is converted to an agonist, which makes it possible to test whether small-molecule agonists are comparable to antagonists in their ability to inhibit HIV-1 fusion. Mimicking infection of human cells with HIV-1, a virus-free cell–cell fusion assay was utilized (Hong et al. 1999; Herschhorn et al. 2011) to highlight the potential possibilities of designing improved CCR5-targeting drugs for treatment of HIV-1.

Materials and Methods

Cell cultures and transfection

CHO-K1 cells were grown in 5% CO2 at 37°C in RPMI 1640 supplemented with 10% fetal bovine serum (FBS),
2 mmol/L GlutaMAX™ (Gibco), 180 units/mL penicillin, and 45 µg/mL streptomycin. Transfection of CHO-K1 cells was performed by lipofection using Lipofectamine® 2000 (Invitrogen, Carlsbad, CA) as described previously (Benned-Jensen and Rosenkilde 2010).

**Plasmids**

The human wild-type (WT) CCR5 cDNA was cloned from a spleen-derived cDNA library into the expression vector pcDNA3.1(+) (Invitrogen, Carlsbad, CA). Mutations were constructed using QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. All mutations were verified by DNA sequence analysis carried out by GATC Biotech (Konstanz, Germany). CD4 was kindly supplied by Mark Marsh (Cell Biology Unit, Medical Research Council Laboratory for Molecular Cell Biology, University College London, London, England). The CD4 construct was transferred into the pcDNA3.1 Hygro(+) vector by PCR cloning. DNA sequencing of CD4 revealed a N64I mutation, which however had no influence on the fusion capacity of CD4 and it was therefore used. The GAL4-VP16 activator gene was generated by fusing the activation domain of the HSV1-encoded VP16 gene to the GAL4 DNA-binding domain of *Saccharomyces cerevisiae*. The GAL4-VP16 gene was synthesized and cloned into the pUC57 vector by GenScript (Piscataway, NJ). The gene was then transferred into the pcDNA3.1(+) vector. The reading frame was confirmed by DNA sequencing. Firefly luciferase reporter pGL4.31 was acquired from Promega (Fitchburg, WI). R5-tropic HIV-1 Env (pJR-FL) was kindly provided by Joseph Sodroski (Dana-Farber Cancer Institute, Boston, Massachusetts, USA).

**Ligands**

The small-molecule antagonist Maraviroc was acquired from Sigma-Aldrich. Aplaviroc was kindly provided by Gary Bridger (AnorMED, Langley, British Columbia, Canada). Both ligands were reconstituted at a stock concentration in DMSO, with a final assay DMSO concentration of less than 0.5%.

**Cell–cell fusion assay**

Subconfluent CHO-K1 cells were cotransfected with R5-tropic HIV-1 Env and the GAL4-VP16 activator (“effector cells”). Another CHO-K1 cell culture was cotransfected with CD4, CCR5, and the pGL4.31 reporter (“target cells”). One day after transfection, target cells were seeded into 96-well plates (2 × 10^4 cells per well) and preincubated with ligand in 100 µL of growth medium (w/o added pen/strep) for 60 min. To initiate cell–cell fusion, 2 × 10^4 effector cells containing an equimolar ligand concentration were overlaid for each well and coincubated overnight. After coincubation, cells were washed, then lysed, and assayed for luciferase activity with Steadylite Plus™ (PerkinElmer Waltham, MA) according to the manufacturer's instructions, using a 2104 EnVision® Multilabel Reader (PerkinElmer, Waltham, MA). Luminescence registered as relative light units (RLU). Determinations were made in triplicate.

**ELISA**

CHO-K1 cells were transfected with FLAG-tagged CCR5. One day after transfection, the cells were seeded into 96-well plates (4 × 10^4 cells per well) and incubated with ligand in growth medium overnight. Cells were washed in Tris-buffered saline (TBS) and then fixed with 3.7% formaldehyde for 15 min at room temperature. After fixation, cells were washed and incubated in a blocking solution (TBS with 2% BSA) for 30 min. Cells were then incubated for 2 h with anti-FLAG antibody (Sigma-Aldrich) at 2 µg/mL in TBS with 1 mmol/L CaCl_2 and 1% BSA. After washing with TBS/CaCl_2/BSA, the cells were incubated with goat anti-mouse HRP-conjugated antibody (Abcam, Cambridge, UK) at a 1:1000 dilution. Following additional washing, the immunoreactivity was revealed by the addition of TMB Plus substrate (Kem-En-Tec, Taastrup, Denmark), and the reaction was stopped with 0.2 mol/L H_2SO_4 after 5 min. Absorbance was measured at 450 nm. Determinations were made in triplicate.

**Calculations**

Standard errors of the mean (SEM) were calculated from row means and IC_{50} values were determined by nonlinear regression using the GraphPad Prism 6.0 software (GraphPad, San Diego, CA). P values were determined by analysis of variance (ANOVA) using Dunnett’s multiple comparison test with statistical significance defined as P < 0.05.

**Fluorescence microscopy**

CHO-K1 cells were transfected with FLAG-tagged CCR5. One day after transfection, the cells were seeded into 24-well plates (5 × 10^4 cells per well) on fibronectin-coated glass slides and incubated with ligand in 500 µL growth medium overnight. Forty-eight hours post transfection, the cells were fixed with 3.7% formaldehyde for 15 min. To reduce unspecific staining, the cells were blocked in 2% BSA for 30 min and then permeabilized with 0.2% saponin in PBS before incubation with the anti-FLAG-tag
antibody (M2) conjugated to Alexa Fluor 647 (10 μg/mL; Cell Signaling) for 1 h at RT. The cells were washed three times with PBS before the glass slides were mounted on microscope slides, sealed, and visualized at 64 × resolution with oil using an Upright Laser Scanning Confocal Microscope LSM700 (Zeiss).

Results

Effect of CCR5 alterations on baseline gp120-mediated fusion

A selection of CCR5 mutations was made based on their previously described phenotypes (Fig. 1A) with residue positions designated in accordance to the Baldwin–Schwartz/Ballesteros nomenclature (Baldwin 1993; Schwartz et al. 1994; Ballesteros and Weinstein 1995), including (1) inactive mutations (Y244A and W248A in TM-6, position VI:09/6.44 and VI:13/6.48) (Steen et al. 2013), (2) constitutively active mutations (L203F in TM-5, position V:13/5.47, G286F in TM-7, position VII:09/7.42, and the double mutation L203F;G286F) (Steen et al. 2013, 2014b), two of which with lack of β-arrestin recruitment (G286F and L203F;G286F), (3) a mutation with removed small-molecule antagonist key anchor point (E283A in TM-7, position VII:06/7.39) (Rosenkilde and Schwartz 2006; Thiele et al. 2011, 2012), and (4) a mutation with removal of a residue suggested as an anchor point for Maraviroc (Garcia-Perez et al. 2011a; Tan et al. 2013), but not for Aplaviroc (Y251A in TM-6, position VI:16/6.51) (Maeda et al. 2006; Kondru et al. 2008). To investigate the effect of the mutations on the fusion process, effector cells and target cells were mixed, and the level of fusion was measured as firefly luciferase activity (Fig. 2A). The receptor mutations were tested in parallel with WT CCR5 and revealed markedly different levels of fusion (Fig. 2B and Table 1). The luciferase activity for WT CCR5 was matched only by L203F, while a decreased signal was revealed for all of the remaining mutations with a ~50% reduction for Y251A and E283A, followed by Y244A and W248A at ~20–25% of WT level, and with the lowest level of fusion for G286F together with the combined mutation in L203F;G286F. The level of background activity was found to be negligible (174 ± 89 RLU).

Figure 1. Overview of mutations, ligands, and pharmacodynamic phenotypes included in current study. (A) Helical wheel structure of CCR5. Mutated residues (white on black) marked with positions and phenotypes. Most conserved residue of every TM is indicated (black on gray). (B) Chemical structure of the small-molecule antagonists Maraviroc and Aplaviroc.
To ensure that the low level of fusion was not caused by the absence of receptors, the surface expression of the mutations was evaluated by ELISA against an N-terminal FLAG-tag and compared to that of WT CCR5 (Fig. 2C). Two mutations matched the surface expression of WT CCR5—L203F and E283A—while the remaining mutations showed a lowered surface expression level. The relationship between fusion signal and level of receptor expression was further investigated by titrating different amounts of WT CCR5 DNA during transfection and measuring fusion (Fig. 2D). An increased signal was observed in response to increasing amounts of receptor DNA, indicating that cell–cell fusion was more prevalent for target cells expressing larger amounts of CCR5. However, this relationship was not linear, likely due to the nature of the cell–cell fusion assay, where only one gp120–CCR5 connection in theory is necessary for enabling fusion and thus luciferase production. Furthermore, we investigated how incubation time affected the readout by incubating WT CCR5-expressing target cells for 1 to 27 h (Fig. 2E). This revealed a highly time-dependent reaction achieving a strong signal after 6 h with lasting effect for the remainder of the tested incubation period (up to 27 h), which is comparable to previous findings for the assay (Hong et al. 1999). A relatively high background signal was observed immediately following coincubation, but decreased with time, reaching its minimum after approximately 10 h of incubation (Fig. 2E). Consequently, overnight incubation was chosen for maximum specific window of fusion in the following studies.

The surface expression of all CCR5 mutations was sufficient to allow fusion, which enabled testing of ligands on these mutations, however due to its markedly impaired fusion capabilities, G286F (and the combined mutation L203F;G286F) was excluded from the following studies of ligand interaction.

The fusion assay is sensitive to small alterations in the ligand binding pocket

By employing the selected CCR5 mutations, we tested the effectiveness of the small-molecule CCR5 antagonists, Maraviroc and Aplaviroc (Fig. 1B), in the fusion assay. We first focused on the key anchor point of most small molecules targeting CC-chemokine receptors, Glu283 in TM-7 (Rosenkilde and Schwartz 2006; Thiele et al. 2011, 2012). As expected, mutation to an alanine resulted in loss of function for both Maraviroc and Aplaviroc...
Table 1. Surface expression and fusion level of CCR5 WT and mutations as measured in ELISA and cell fusion, and inhibition of cell fusion by Maraviroc and Aplaviroc.

<table>
<thead>
<tr>
<th>Position</th>
<th>Residue</th>
<th>E0 ± SEM</th>
<th>n</th>
<th>Fusion</th>
<th>F0 ± SEM</th>
<th>n</th>
<th>Fold</th>
<th>Maraviroc</th>
<th>Log IC50 ± SEM</th>
<th>IC50</th>
<th>n</th>
<th>Fold</th>
<th>Fusion inhibition</th>
<th>nM</th>
<th>Aplaviroc</th>
<th>Log IC50 ± SEM</th>
<th>IC50</th>
<th>n</th>
<th>Fold</th>
<th>Fusion inhibition</th>
<th>nM</th>
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<tbody>
<tr>
<td>CCR5 WT</td>
<td></td>
<td>100 ± 0.03</td>
<td>6</td>
<td></td>
<td>12,357 ± 1284</td>
<td>20</td>
<td>1.00</td>
<td></td>
<td>−7.04 ± 0.06</td>
<td>91</td>
<td>10</td>
<td>1.00</td>
<td>−6.30 ± 0.09</td>
<td>501</td>
<td>10</td>
<td>1.00</td>
<td></td>
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<tr>
<td>TM-V L203F</td>
<td></td>
<td>121 ± 4.2</td>
<td>6</td>
<td></td>
<td>15,761 ± 3084</td>
<td>18</td>
<td>1.3</td>
<td></td>
<td>−7.31 ± 0.04</td>
<td>49</td>
<td>9</td>
<td>1.9</td>
<td>−6.31 ± 0.09</td>
<td>488</td>
<td>9</td>
<td>1.0</td>
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<tr>
<td>TM-VI Y244A</td>
<td></td>
<td>10 ± 3.8</td>
<td>6</td>
<td></td>
<td>3185 ± 660</td>
<td>18</td>
<td>0.3</td>
<td></td>
<td>−8.35 ± 0.03</td>
<td>4.5</td>
<td>9</td>
<td>21</td>
<td>−7.98 ± 0.05</td>
<td>11</td>
<td>9</td>
<td>48</td>
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<tr>
<td>W248A</td>
<td></td>
<td>12 ± 3.0</td>
<td>6</td>
<td></td>
<td>2386 ± 426</td>
<td>16</td>
<td>0.2</td>
<td></td>
<td>−8.18 ± 0.12</td>
<td>6.6</td>
<td>8</td>
<td>14</td>
<td>−7.77 ± 0.27</td>
<td>17</td>
<td>8</td>
<td>30</td>
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<tr>
<td>TM-VII Y251A</td>
<td></td>
<td>17 ± 4.0</td>
<td>6</td>
<td></td>
<td>6770 ± 1553</td>
<td>17</td>
<td>0.6</td>
<td></td>
<td>No inhibition</td>
<td>8</td>
<td></td>
<td></td>
<td>−7.29 ± 0.07</td>
<td>51</td>
<td>9</td>
<td>10</td>
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<tr>
<td>E283A</td>
<td>G286F</td>
<td>102 ± 9.2</td>
<td>6</td>
<td></td>
<td>7453 ± 3366</td>
<td>14</td>
<td>0.6</td>
<td></td>
<td>No inhibition</td>
<td>7</td>
<td></td>
<td></td>
<td>Not tested</td>
<td></td>
<td></td>
<td>Not tested</td>
<td></td>
<td></td>
<td></td>
<td>Not tested</td>
<td></td>
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<tr>
<td>LM-VII L203F;G286F</td>
<td></td>
<td>21 ± 3.0</td>
<td>6</td>
<td></td>
<td>779 ± 110</td>
<td>18</td>
<td>0.1</td>
<td></td>
<td>Not tested</td>
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<td>Not tested</td>
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<td>Not tested</td>
<td></td>
<td></td>
<td></td>
<td>Not tested</td>
<td></td>
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<tr>
<td>% of WT</td>
<td></td>
<td></td>
<td></td>
<td>Raw baseline cell fusion (F0) shown in relative light units (RLU). Fusion inhibition by Maraviroc and Aplaviroc shown as log IC50 determined by nonlinear regression, and fold change compared to WT CCR5. Number of individual experiments designated by n.</td>
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on fusion. When inducing an efficacy switch of a ligand/receptor pair, the effect of the ligand is reversed, that is, an antagonist/inverse agonist becomes an agonist and vice versa. Mutation of Leu203 in position V:13/5.47 to a phenylalanine causes an increase in constitutive activity of CCR5 through G proteins and thereby a shift toward an active conformation that in turn leads to the efficacy switch of Aplaviroc, which locks this active conformation (Steen et al. 2014b). In addition to a surface expression level close to that of WT CCR5, the functionality of L203F in terms of affinity, efficacy, and potency of the endogenous ligands, CCL3 and CCL5, is also similar to WT CCR5 (Steen et al. 2014b), making it an ideal target for the test. Furthermore, Aplaviroc works as a biased agonist on L203F CCR5 as it is capable of stimulating G-protein activity but not β-arrestin recruitment (Steen et al. 2014b), thus providing insight into the direct inhibitory effect of small-molecule agonists by preventing β-arrestin-associated receptor internalization.

By targeting the efficacy switch mutation with Maraviroc and Aplaviroc, we investigated how small-molecule agonists (biased toward G-protein activation) affect the fusion process in the absence of agonist-induced receptor internalization. Both Maraviroc and Aplaviroc displayed potent inhibition of fusion on L203F (Fig. 6A and B), closely matching the effect on WT CCR5. No decrease in surface expression in response to increasing concentration of ligand was observed (Fig. 6C and D), which was confirmed by fluorescence microscopy (Fig. 6E–H). This suggests that a small-molecule agonist can work as an effective, direct inhibitor of R5-tropic HIV-1 fusion, without this effect being attributed to internalization of the chemokine receptor.

**Discussion**

By studying receptor activity states and pharmacodynamic requirements for fusion inhibitors, we here highlight the possibility of development of functionally selective (i.e., biased) small-molecule ligands targeting the HIV–CCR5 interaction without interfering with the normal receptor functionality. We used a cell–cell fusion assay as a tool for investigation of the gp120 interaction, and through an efficacy switch mutation in CCR5 we show that small-molecule agonists function as effective inhibitors of HIV-1 fusion without decreasing receptor surface expression, indicating no internalization and thus using another mechanism (i.e., a more direct inhibition of the fusion process) than described for the scarce existing small-molecule agonists (Saita et al. 2006; Ferain et al. 2011).
Sensitivity of the fusion assay and general implications of the experiments

The observed potencies of Maraviroc and Aplaviroc on WT CCR5 in the cell–cell fusion assay were comparatively worse than initial studies have shown (Maeda et al. 2004; Dorr et al. 2005). Since confluent cell seeding is required to ensure membrane contact and only one gp120/CCR5 connection is required for cell fusion, inhibition of the signal is potentially more difficult and requires higher inhibitor concentrations, and thus has an inherent risk of a right-shifted dose–inhibition curve, which explains this discrepancy. This can also explain why the Y251A and E283A mutations were able to support substantial fusion despite their impairment in gp120 binding (Maeda et al. 2006). Nonetheless, we were able to confirm what has been suggested for Maraviroc and Aplaviroc binding on CCR5 as both inhibitors lose their effect upon removal of the common Glu283 and specific Tyr251 anchor point (Fig. 3). We observed an increase in potency of the inhibitors for all TM-6 mutations (Table 1). Since we previously showed that the affinity of Aplaviroc is WT-like on...
W248A and Y251A (Steen et al. 2013), this increase in potency can be explained by disrupted gp120 binding to the receptor, and indeed impaired fusion was confirmed for these three mutations (Fig. 2B and Table 1). Aside from the increase in potency, a paradoxical increase in cell fusion was observed for W248A at high ligand
concentrations. This increase in cell fusion was accompanied by a slight but significant increase in receptor surface expression (Fig. 5E) resulting from mobilization of intracellular receptor stores (Fig. 5F+G). A previous study found that high Maraviroc concentrations increased WT CCR5 expression after long incubation (>24 h) by enhancing oligomerization of CCR5 on a synthesis level (Nakano et al. 2014). Since the incubation time in our experiment is shorter, thus insufficient to see a significant effect of altered synthesis, and the increase in surface expression of the mutations was seen at lower ligand concentrations as well, the explanation here is likely different. The intracellular stores likely consist of “discarded” misfolded or otherwise defective receptors resulting from the mutagenic manipulation. Mobilization of these stores to the cell surface can be explained by the small molecules entering the cells, binding to and somewhat stabilizing the intracellular receptors allowing transport to the surface, thus acting as molecular chaperones. Indeed, the long incubation time makes cellular uptake or membrane penetration of the small molecules a possibility. It is possible that this population of defective receptors has a decreased affinity for the inhibitors while still supporting gp120 binding, or allows for use of ligand bound receptor, thus contributing to the observed biphasic inhibition pattern. Similarly, a biphasic infectivity curve has been described for another CCR5 antagonist, Vicriviroc, using peripheral blood mononuclear cells and resistant HIV-1 strains (Anastassopoulou et al. 2009). A model based on the existence of CCR5 subpopulations with varying affinity for Vicriviroc was proposed as an explanation to this phenomenon, which in essence is supported by a recent study showing HIV’s exploitation of low-chemokine-affinity G-protein-uncoupled CCR5 populations (Colin et al. 2013). This explanation fits with our observations for the W248A mutation.

Of notice, the mutation suffering the biggest fusion impairment, G286F comprising a steric hindrance, is located in TM-7, pointing directly toward TM-6, indicating that the interface between TM-6 and TM-7 toward the main binding pocket (Rosenkilde et al. 2010) is essential for gp120 interaction with CCR5 as suggested in previous studies (Garcia-Perez et al. 2011a; Tan et al. 2013; Tamamis and Floudas 2014). It has previously been shown that gp120 binding and HIV-1 infection are independent of G-protein activation (Amara et al. 2003; Colin et al. 2013), and our data support this notion. We tested several CCR5 mutations with different activity states (Steen et al. 2013, 2014b) and found no clear connection between active/inactive conformations and gp120-mediated fusion (Fig. 2B and Table 1), indicating that HIV-1 entry inhibitors are not required to stabilize a certain conformation, thus improving the probability of successful drug design.
Improving drugs targeting CCR5 in HIV-1 fusion

Since the discovery of CCR5 and CXCR4’s role in HIV-1 infection, the focus has been on the development of small-molecule entry inhibitors in the form of antagonists (Steen et al. 2003; Dean et al. 1996; Liu et al. 1996; Alkhatib et al. 1996; Bleul et al. 1996; Oberlin et al. 1996; Simmons et al. 1997). These large peptides assert their effect through orthosteric blockade of gp120, as well as the agonistic property of β-arrestin recruitment leading to a reduction in coreceptors at the cell surface. More efficient variants hereof have been designed (Simmons et al. 1997; Elsner et al. 2000); however, due to the low bioavailability, short half-life, and high production cost, these peptides were not successful agents in the treatment of HIV. Thus, a small molecule is preferable, however only a limited number of such agonists with anti-HIV activity have been described (Saita et al. 2006; Ferain et al. 2011), and their effect has been solely attributed to internalization of CCR5. A valid concern for such agonists is the possibility of proinflammatory side effects, resulting from activation of the classical signaling pathways, for example, Gzα.

Lately, the concept of biased ligands has entered the field of drug development, and G-protein-coupled receptors (GPCRs) are obvious targets. For the treatment of HIV, the theoretically most effective small-molecule agonist would be biased toward β-arrestin recruitment and receptor internalization, thus reducing receptor surface expression without G-protein activation and thereby diminishing the proinflammatory adverse effects associated with the Gzα pathway. Reduction in cell surface expression is a valid method for inhibition of HIV infection as proven by the fact that individuals carrying the CCR5A32 mutation are resistant to HIV by reduced CCR5 expression (Dean et al. 1996; Liu et al. 1996; Samson et al. 1996; Ditzel et al. 1998). Targeting the human component instead of viral proteins should in theory decrease the risk of viral resistance development. Nonetheless, resistance toward Maraviroc has been described previously (Macarthur and Novak 2008; Flynn et al. 2013). A biased drug as described above could conceivably further reduce this risk as fewer drug-bound receptors are exposed. Since it does not seem to be of importance to the fusion process whether the blocking of gp120 is induced by an antagonist or an agonist, we can furthermore speculate on the possibility that a “silent” drug (i.e., with no intrinsic effect on receptor signaling, internalization, and chemokine binding) could have a selective effect in HIV entry inhibition. This function sparing probe-dependent allostery has in part been described and discussed for the CCR5 antagonist TAK652 showing a more potent effect on HIV-1 entry inhibition compared to inhibition of CCL3L1-induced CCR5 internalization (Muniz-Medina et al. 2009). Here, we show effective fusion inhibition by small-molecule agonists with no decrease in CCR5 surface expression, in accordance with their lack of β-arrestin recruitment in L203F CCR5 (Steen et al. 2014b). This fits into the ideas that have previously been considered for small-molecule antagonists, and adds the possibility of designing and introducing biased or function sparing agonists as direct inhibitors of HIV entry in treatment. Design of such a drug will pose a novel strategy in combating the virus, and has the potential benefits of reducing the risk of drug resistance as well as proinflammatory adverse effects otherwise associated with chemokine receptor agonists. In light of the changes in the HIV/AIDS treatment guidelines, suggesting an early start of treatment, problems with resistance against the traditional HAART regimen could potentially escalate, providing the need for such new and improved drugs. Even though toxicity will also increase when using multiple drugs for an extended period of time, monotherapy is currently not ideal in terms of antiviral activity. Thus, an improved, functionally selective CCR5 ligand could prove a rational supplement to the current treatment options.

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Author contributions

C.B., H.R.L., and M.M.R. designed the experiments. C.B. performed the cell–cell fusion and ELISA. K.S. performed the fluorescence microscopy. C.B. and M.M.R. analyzed the results and prepared the manuscript.

Disclosure

The authors declare that they have no conflicts of interest with the contents of this article.

References


HIV Cell Entry Inhibition Independent of Activity

C. Berg et al.


