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Effects of CC chemokine receptor 5 (CCR5) inhibitors on the dynamics of CCR5 and CC-chemokine-CCR5 interactions

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Abstract

Background: This study aimed to examine how CC chemokine receptor 5 (CCR5) inhibitors (aplaviroc [APL], TAK779 and maraviroc [MVC]) interact with CCR5 and affect its dynamics and physiological CC-chemokine-CCR5 interactions.

Methods: A yellow fluorescent protein (YFP)-tagged CCR5-expressing U373-MAGI cell line was generated and a stable CCR5-expressing clonal population, YFPCCR5UM16, was prepared. YFPCCR5-UM16 cells were exposed to RANTES, macrophage inflammatory protein (MIP)-1 α or MIP-1 β (all 100 ng/ml) with or without CCR5 inhibitors and YFPCCR5 internalization was visualized with real-time by laser scanning confocal microscopy. The mobility of YFPCCR5 was also examined in the presence of CCR5 inhibitors with fluorescence recovery after photobleaching (FRAP) imaging.

Results: Following the addition of each CC chemokine, intracellular fluorescence intensity increased whereas membranous fluorescence decreased, signifying YFPCCR5 internalization. All three CCR5 inhibitors failed to induce YFPCCR5 internalization. All three CCR5 inhibitors blocked the CC-chemokine-induced internalization at a high concentration of 1 μ M; however, the ratio of APL concentration that blocked RANTES-induced internalization by 50% over APL concentration that blocked HIV type-1 (HIV-1) replication by 50% was 16.4, indicating that APL permits CC-chemokine-induced internalization to a much greater extent compared with TAK779 and MVC, having ratios of 1.1 and 0.9, respectively. The examination of YFPCCR5 mobility with FRAP imaging revealed that YFPCCR5 continuously underwent rapid redistribution, which none of the three inhibitors blocked.

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Disclosure statement

All authors declare no competing interests.

Conclusions: The finding that APL moderately blocked the RANTES-triggered ^{YFP}CCR5 internalization despite the highly potent anti-HIV-1 activity of APL strongly suggests that development of CCR5 inhibitors, which do not overly inhibit physiological CC-chemokine–CCR5 interactions, is practically feasible.

Introduction

Alkhatib *et al.* [1] reported in 1996 that CC chemokine receptor 5 (CCR5), a G-protein-coupled seven transmembrane segment receptor [2], is one of the two essential coreceptors for HIV type-1 (HIV-1) entry to human CD4⁺ T-cells, thereby serving as an attractive target for possible intervention of infection by HIV-1 that uses CCR5 as a coreceptor (R5-HIV-1) [1,3]. To date, scores of newly designed and synthesized CCR5 inhibitors have been reported to be potent against R5-HIV-1 [4], and one such inhibitor, maraviroc (MVC) [5,6], has recently been approved by the US Food and Drug Administration (FDA) for treatment of HIV-1-infected individuals who do not respond to any existing antiretroviral regimens. However, recent reports suggest that the absence of CCR5 could lead to adverse consequences, such as the greater risk for lethal infection by West Nile virus and abnormalities of liver function in CCR5-2 homozygous individuals [7–10]. Considering that the interactions between CC chemokines and CCR5 are important factors in the human immune defence and the aberrations of such interactions have been related to various disorders [11,12], the sustained long-term suppression of CC-chemokine–CCR5 interactions in those who carry wild-type CCR5 and who might not have a possible compensatory mechanism(s) for the absence of CCR5 might produce adverse effects. In the present study, we established a new assay system to investigate the dynamics of cellular CCR5 and to quantify the levels of CC-chemokine-induced internalization to determine the effects of CCR5 inhibitors on CC-chemokine–CCR5 interactions in living cells by confocal microscopy. We also examined the mobility of yellow fluorescent protein (YFP)-tagged CCR5 (^{YFP}CCR5) in the presence of CCR5 inhibitors with fluorescence recovery after photobleaching (FRAP) imaging.

Methods

Reagents

Aplaviroc (APL) is an experimental CCR5 inhibitor containing a spirodiketopiperazine core as previously described by Maeda *et al.* [13,14]. The method for the synthesis of APL has been reported elsewhere [15]. TAK779 and MVC were synthesized according to the data published by Baba *et al.* [16] and Dorr *et al.* [5]. RANTES (also known as CCL5), macrophage inflammatory protein (MIP)-1 α . (CCL3) and MIP-1 β (CCL4) were purchased from PeproTech, Inc. (Rocky Hill, NJ, USA).

Cells

The U373-MAGI (UM) cell line, obtained from the AIDS Research and Reference Reagent Program, NIAID, National Institutes of Health (Bethesda, MD, USA), was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (HyClone Laboratories, Logan, UT, USA), 200 μ g/ml G418 and 100 μ g/ml hygromycin B.

Construction of YFPCCR5-expressing UM cells

The human wild-type CCR5 (^{WT}CCR5)-encoding gene was obtained from pZeoSV (Invitrogen, Carlsbad, CA, USA) carrying the CCR5 gene (pZeoSV-CCR5) [17]. The CCR5-encoding gene was inserted into the pEYFP-N1 vector (Clontech, BD Biosciences, Palo Alto, CA, USA) using *Nhe*-I and *Xho*-I digestion, generating a plasmid that expresses CCR5 with YFP at the C terminus of CCR5 (pEYFP-N1-CCR5). UM cells were then transfected with pEYFP-N1-CCR5 using Lipofectamine 2000 (Invitrogen). The transfectants were magnetically sorted with a CCR5-specific monoclonal antibody (mAb 2D7; BD Pharmingen, San Diego, CA, USA) bound to Dynabeads M-450 (DynaL AS, Oslo, Norway), and the most rapidly growing clone (^{YFP}CCR5-UM16) was selected and used throughout the present study.

Flow cytometry analyses

^{YFP}CCR5-UM16 cells (10^6) were incubated in the presence or absence of 100 ng/ml RANTES at 37°C for 1 h, cooled on ice and washed 3× in cold phosphatebuffered saline. The cells were subsequently fixed with phosphate-buffered saline containing 1% formaldehyde and 1% fetal calf serum. The fixed cells were labelled with phycoerythrin (PE)-conjugated CCR5 mAb (2D7) at 4°C, thoroughly washed and analysed for the amount of CCR5 using an Epics XL (Beckman-Coulter, Fullerton, CA, USA). Unlabelled ^{YFP}CCR5-UM16 cells were also subjected to the same analysis.

Determination of CCR5 dynamics using confocal microscopy

^{YFP}CCR5-UM16 cells were cultured overnight on poly-D-lysine-coated glass cover slips in 35 mm tissue culture dishes at 3×10^3 per 100 µl with DMEM. In examining the effects of each CCR5 inhibitor on the interactions of CC-chemokine and CCR5, UM16^{CCR5-YFP} cells were incubated with a CCR5 inhibitor for 1 h and subsequently exposed to each chemokine (100 ng/ml). A Zeiss LSM510 confocal microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA) with a 40× C-apochromat (numerical aperture 1.2) objective lens was used for fluorescence time-lapse live cell imaging experiments. Cells were imaged every 2 min over a 40 min period using 488 nm laser excitation, 1.2 µm confocal slice thickness and 0.22 µm X–Y pixel sampling. Digital image data were organized into figures and prepared for presentation using Adobe Photoshop version 6.0 (Adobe Systems, Mountain View, CA, USA).

Study of CCR5 mobility on the cell membrane using FRAP

FRAP imaging of ^{YFP}CCR5-UM16 cells was conducted as previously described [18–20]. Briefly, a pre-bleach image was collected, a specific rectangular subcellular region of interest of consistent size was photobleached and subsequent images were collected every 20 s until 8 min post-bleach. To measure fluorescence recovery, images were background subtracted, compensated for bleaching caused by imaging (rate measured from control non-bleached cells) and double-normalized, first for pre-bleach intensity (set to 100%) and then initial post-bleach intensity (set to zero). The resultant FRAP recovery curves were plotted using the Microsoft Excel spreadsheet programme. To examine the influence of each CCR5 inhibitor (1 µM) and methylβ-cyclodextrin (10 mM) on the recovery of fluorescent signal,

YFPCCR5-UM16 cells were pretreated with each agent at 37°C for 1 h, followed by FRAP imaging.

Results

Generation and characterization of YFPCCR5-UM16 cells We generated a plasmid carrying the CCR5-encoding gene connected to YFP at the C terminus of CCR5 (YFPCCR5), transfected adherent human astroglia-derived UM cells [21,22] with the plasmid, sorted out stable CCR5-expressing UM cells using magnetic sorting and obtained clonal populations that expressed consistent levels of CCR5 on the cell membrane. With the limiting dilution technique, we obtained a rapid-growing CCR5-expressing clone, YFPCCR5-UM16 (Figure 1A). The constitutive expression of the YFPCCR5 molecules was confirmed by labelling with PE-conjugated antiCCR5 mAb (2D7), which recognizes the second extracellular loop of CCR5 [23]. The YFPCCR5 positivity was 92.1% and the 2D7-PE and YFPCCR5 double-positive population was 87.3% (Figure 1B). We also confirmed that YFPCCR5-UM expressed neither CCR1 nor CCR3 by flow cytometry (HN *et al.*, data not shown).

It was also noted that YFPCCR5 molecules were mostly present on and near the cellular membrane as examined by confocal microscopy (Figure 1A), corroborating the results of flow cytometry.

RANTES-induced CCR5 internalization in YFPCCR5-UM16 cells

We asked whether YFPCCR5 functioned as physiologically similar to WTCCR5 with regard to CC-chemokine-induced CCR5 internalization. To determine this, YFPCCR5-UM16 cells were exposed to RANTES, the most potent CC chemokine, at a concentration of 100 ng/ml, incubated for 40 min under confocal microscopy, and the images of YFPCCR5-UM16 cells were digitally recorded every 2 min. As shown in Figure 1C (panel i), YFPCCR5 was primarily located on and near the cellular membrane. When the cells were not exposed to RANTES, the YFP fluorescence pattern did not significantly change in culture over 40 min. The images of the cells at 20 and 40 min are shown in Figure 1C (panels ii and iii), respectively. However, when the cells were exposed to 100 ng/ml RANTES (Figure 1C, panels iv–vi), by 20 min, YFPCCR5 had redistributed, with a slight increase in the speckled fluorescent intensity in the cytoplasm and, by 40 min, the shift of fluorescence from the cellular membrane and rim into the cytoplasm was more significant and denser speckles were observed in the cytoplasm (Figure 1C, panel vi).

We subsequently examined whether the shift and increase in the size and intensity of the speckles represented the actual internalization of YFPCCR5 (Figure 2A, 2B and 2C). When we measured the integrated fluorescence intensity of YFPCCR5-UM16 (derived from YFPCCR5), it did not significantly change regardless of the addition of 100 ng/ml RANTES (Figure 2A), suggesting that despite the shift and the increase in the size and intensity of the speckles, the total YFP fluorescence intensity remained virtually the same because the measured integrated intensity represents the sum of cytoplasmic and membranous fluorescence. However, when YFPCCR5-UM16 cells were stained with PE-conjugated mAb 2D7, the PE-derived fluorescence intensity substantially decreased because of the

internalization of membranous CCR5, resulting in loss of PE-derived fluorescence over 1h following the addition of RANTES by 16% (Figure 2B). Taken together, these results confirmed that the increase in YFP fluorescence intensity observed in the cytoplasm represented the internalization of YFPCCR5. In addition, we examined chemokine-induced Ca²⁺ responses in YFPCCR5-UM16 cells using fluorescence spectrometer to confirm the functionality of YFPCCR5. Of particular note, the magnitude of the Ca²⁺ response in YFPCCR5-UM16 cells was less than that in WTCCR5-UM cells; however, significant intracellular Ca²⁺ flux was observed following the exposure of both cell populations to RANTES in a dose-dependent manner (Figure 2D). Taken together, these results indicate that YFPCCR5-UM16 cells exhibit appropriate physiological functions to monitor CCR5-chemokine interactions.

We subsequently determined the effects of the CCR5 inhibitors on the chemokine-induced CCR5 internalization. When YFPCCR5-UM16 cells were pre-exposed to 0.01 or 0.1 μ M APL for 1 h in a regular incubator, and subsequently exposed to 100 ng/ml RANTES, we observed a weak but similar fluorescence shift and an increasing speckle pattern, suggesting that 0.01 and 0.1 μ M APL did not fully block the RANTES-elicited mobilization of YFPCCR5 (Figure 1C, panels vii–xii).

Quantification of the magnitudes of YFPCCR5 internalization

In order to determine the magnitude of the CCchemokine-induced CCR5 internalization, we randomly chose YFPCCR5-UM16 cells under confocal microscopy and digitally encircled the intracytoplasmic area on the images of cells (Figure 3A), and quantified the magnitudes of CCR5 internalization by computationally integrating the intracytoplasmic fluorescence intensity in each cell. In monitoring the changes in the integrated intracytoplasmic fluorescence intensity, we quantified the intensity every 5 min and the values were determined as percentage relative to integrated fluorescence intensity (%RFI) using the intensity at time 0 as a reference (100%). This YFPCCR5 internalization quantification assay revealed that the addition of CC-chemokines resulted in a significant increase of %RFI over 40 min, although no significant %RFI increase was observed in the cells without the addition of CC chemokines (Figure 3). The increase of %RFI values occurred consistently for the first 30–40 min after the exposure of the cells to CC chemokines and the values generally reached a peak after approximately 40 min; thereafter, the values plateaued. The values began to decrease by 60 min under observation. As shown in Figure 3B, with 100 ng/ml of RANTES, the %RFI value increased by 67% at 40 min. The increase of %RFI values at 40 min following the addition of MIP-1 α and MIP-1 β was by 45% and 40%, respectively (Figure 3C and 3D). As expected, three CCR5 inhibitors, the addition of APL, TAK779 or MVC (1 μ M each) produced no significant integrated fluorescence increase, indicating that neither exerted agonistic activity (Figure 3E, 3F and 3G).

Effects of CCR5 inhibitors on CC-chemokine-induced YFPCCR5 internalization

We next examined whether CCR5 inhibitors affected the CC-chemokine-induced internalization of YFPCCR5 using the above described YFPCCR5 internalization quantification assay. The YFPCCR5-UM16 cells were pretreated with 0.001, 0.01, 0.1 or 1 μ M of APL, TAK779 or MVC for 1 h, exposed to RANTES (100 ng/ml) in the presence of

each inhibitor, and the integrated intracytoplasmic fluorescence intensity was determined. Pretreatment with APL or TAK779 demonstrated the blockade of the RANTES-elicited internalization in a concentration-dependent manner, whereas exposure to 0.01 μM MVC almost completely blocked the internalization (Figure 3H). APL substantially permitted the increase of %RFI at 0.001, 0.01 and 0.1 μM by 52%, 36% and 14%, respectively, and TAK779 similarly did so by 58%, 41% and 18%, respectively. However, both APL and TAK779 completely blocked the internalization at 1 μM . We also examined the effects of the CCR5 inhibitors on the MIP-1 α - and MIP-1 β -elicited internalization (both at 100 ng/ml) employing the same quantification assay. TAK779 at 0.001, 0.01 and 0.1 μM failed to block the internalization and the %RFI increased by 43%, 35% and 13% for MIP-1 α (Figure 3I), and 37%, 30% and 12% for MIP-1 β (Figure 3J), respectively. Although APL and MVC almost completely blocked the MIP-1 α and MIP-1 β -elicited internalization at concentrations >0.01 μM , both inhibitors permitted the MIP-1 α - and MIP-1 β -elicited internalization at 0.001 μM with the increment of %RFI, 13% and 24% for APL and 23% and 24% for MVC, respectively (Figure 3I and 3J). These results strongly suggest that APL preferentially permits the interaction between CCR5 and RANTES, but not between CCR5 and MIP-1 α and MIP-1 β .

CCR5 inhibitors do not inhibit the redistribution of YFP^{CCR5} on cell membrane

Physiological CCR5 has been shown to continuously mobilize and redistribute on the cellular membrane. Thus, we examined how the binding of CCR5 inhibitors to CCR5 affected the mobility and redistribution of CCR5 using YFP^{CCR5} -UM16 cells and FRAP assay. As shown in Figure 4A, the area indicated was exposed to a short pulse of high 488 nm laser intensity that effectively irreversibly photobleached the YFP signal in the target. At 0 min post-bleach, the subcellular bleach area was devoid of fluorescence (Figure 4A, panel ii). While monitoring the cells over time, the photobleached area gradually regained fluorescence and, by 8 min post-bleach, the area reacquired substantial fluorescence signal, indicating that YFP^{CCR5} underwent diffusion and redistribution in the photobleached area (Figure 4A, panel iii). Quantification of fluorescence levels in the targeted area showed that by 8 min postbleach the fluorescence signal recovered to $84\% \pm 4.4$ of the fluorescence level relative to the level before the photobleaching (Figure 4B). In the same assay, we also exposed the YFP^{CCR5} -UM16 cells to 10 mM methyl- β -cyclodextrin, which is known to decrease the fluidity of cellular membrane through its cholesterol-depleting property [20], and in the presence of the agent, FRAP was conducted and the fluorescence signal recovery was quantified. As expected, there was only marginal level of fluorescence in the cells exposed to methyl- β -cyclodextrin. By contrast, the fluorescence signals following the photobleaching on APL-, TAK779- or MVC-pretreated cells were all comparable ($85\% \pm 2.5$, $82\% \pm 3.2$ and $87\% \pm 5.6$, respectively) to the untreated control cells. These data strongly suggest that the CCR5 inhibitors examined in this study do not significantly affect the mobility and redistribution of CCR5.

Discussion

In developing CCR5 inhibitors as therapeutics for HIV-1 infection and AIDS, CCR5 blockade by CCR5 inhibitors was presumed to be fairly tolerated mainly based on the

findings that multiple and overlapping interactions among CC chemokines and their receptors have been identified [24,25] and that humans, who genetically lack CCR5 because of the deletion of 32 base pairs (CCR5-32), are apparently healthy and live normal life spans [26–29]. However, recent reports suggest that the absence of CCR5 could lead to adverse consequences, such as the greater risk for lethal infection by West Nile virus and abnormalities of liver function in CCR5-32 homozygous individuals [7–10]. Thus, the sustained long-term suppression of CC-chemokine–CCR5 interactions, in particular in patients who carry ^{WT}CCR5 and might not have a possible compensatory mechanism(s) for the absence of CCR5, may result in adverse effects. Cautions should be used in blocking CC-chemokine–CCR5 interactions, especially in view of the notion that the function of CCR5 in healthy ^{WT}CCR5-carrying individuals is not fully understood and that there are currently no data regarding the safety of sustained suppression of the interactions.

The present data demonstrated that CC-chemokine-induced CCR5 internalization occurs relatively slowly but steadily over 40 min in contrast to previously published data that CC-chemokine-induced internalization of CCR5 occurred within 20 min as examined using CCR5-specific mAb in human CCR5-expressing non-human Chinese hamster ovarian cells [30,31]. The difference could be explained by evidence that the dynamics of human CCR5 on the membrane of human cells and hamster cells could differ as YFPCCR5-UM16, a human-derived cell line, was used in the present study, whereas hamster-derived cells were used in the studies by Mueller *et al.* [30] and Signoret *et al.* [31]. It was also possible that the attachment of YFP to CCR5 could slow down the internalization of CCR5 in YFPCCR5-UM16 cells because of the bulkier size of YFPCCR5 and possible functional alterations (Figure 2A, 2B and 2C) [32–34].

In the present work, we examined the effects of three CCR5 inhibitors on CC-chemokine–CCR5 interactions using the current assay system with YFPCCR5-UM16 cells. To quantify the effects of CCR5 inhibitors against CC-chemokine-induced internalization in regard to their anti-HIV-1 activity, we determined the ratios of the 50% effective dose for chemokine-induced internalization over the 50% inhibitory concentration values for HIV-1 inhibition for the three inhibitors, APL, TAK779 and MVC (Table 1). The ratios for APL, TAK779 and MVC with RANTES were 16.4, 1.1 and 0.9, respectively, indicating that APL is more permissive to allow RANTES to elicit CCR5 internalization, whereas the ratios for MIP-1 α - and MIP-1 β -induced internalization with all three chemokines ranged from 0.8 to 2.5. These data suggest that APL exerts its anti-HIV-1 activity preserving the RANTES–CCR5 interactions. In this regard, we previously reported that APL at a concentration of 0.01 μ M had only moderate inhibition (approximately 20%) of the binding of ¹²⁵I-RANTES to CCR5 [13]. By contrast, APL completely blocked the binding of MIP-1 α to CCR5 and efficiently blocked that of MIP-1 β . The complete inhibition of the binding of MIP-1 α is not surprising because in the initial search of lead compounds in the development of CCR5 inhibitors, we sought compounds that blocked the binding of ¹²⁵I-labelled MIP-1 α to CCR5-expressing CHO cells and MIP-1 α -elicited cellular Ca²⁺ mobilization as described previously [35]. MIP-1 β is a close analogue of MIP-1 α and is presumed to have a binding feature close to that of MIP-1 α . It is noteworthy that all three CCR5 inhibitors we examined in this study have been reported to get lodged in the same hydrophobic pocket of CCR5 located within CCR5 in the proximity of the interface between the extracellular domain and the

transmembrane domain [14,36,37], although the shape and size of the hydrophobic cavity substantially differ because of the structural and biochemical difference of CCR5 inhibitors [14,36]. Indeed, such preservation of CC-chemokine function(s) in a small molecule CCR5 inhibitor, TAK652, has also been reported by MunizMedina *et al.* [38]. In their study, TAK652 showed that the selectivity ratio for CCL3L1-induced internalization versus HIV-1 entry inhibition was 11.9 in human osteosarcoma cells and 12.7 in peripheral blood mononuclear cells, respectively. Thus, the different features of inhibition by the three inhibitors are to be expected. Further detailed structural molecular analyses of the CCR5 inhibitors-CCR5 interactions will elucidate how these differences are produced. This information should help design more potent and effective CCR5 inhibitors.

It is of note that although APL, at a concentration of 1 μ M, permitted the binding of 125 I-RANTES to CCR5 by >50% [13,37], the same concentration of APL completely blocked the RANTES-induced CCR5 internalization in the present study (Figure 3H). The difference between the suppression of 125 I-RANTES binding by APL, RANTES-induced CCR5 internalization and RANTES-induced chemotaxis should be explained by evidence that even if APL fairly permitted the binding of CC chemokine to CCR5, APL that is lodged within CCR5 more efficiently suppressed the signal transduction following the binding of RANTES. Nevertheless, it is argued that, as described above, APL still permitted RANTES-elicited cellular functions at concentrations that effectively blocked the infectivity of HIV-1.

It should be noted that because APL selectively preserves in part both the binding and the cell signalling capacities of RANTES at concentrations that are compatible with full R5-HIV-1 blockade, the combination of both APL and chemokine effects could result in a net partial agonist effect of RANTES leading to CCR5 down-regulation, whereas the interactions of the envelop glycoprotein-CD4 complex with CCR5 are blocked. This suggests that a CCR5 inhibitor, such as APL, is an ideal anti-HIV agent that prevents R5-HIV-1 cellular entry by a dual antiviral mechanism integrating its allosteric inhibition of envelope glycoprotein-CD4 complex interactions with CCR5 and CCR5 down-regulation that would preserve the physiological functionality of the receptor.

The FDA approved the first CCR5 entry inhibitor, MVC, for treatment of HIV-1-infected adults who do not respond to other existing antiretroviral regimens. The results of precedent Phase 3 clinical trials showed that MVC did not cause significant adverse effects [39]. Moreover, the recent analysis of the MERIT study with the enhanced sensitivity tropism test that effectively excludes individuals with CXCR4-tropic HIV-1 from the data obtained has revealed that patients receiving MVC achieved significant suppression of the virus and larger and faster CD4⁺ T-cell increases were observed in the MVC arm compared with the efavirenz arm [40]. It should be emphasized, however, that it remains to be determined whether longer-term suppression of CC-chemokine-CCR5 interactions affects the physiological functions of CCR5, including immune response.

We have previously identified the amino acid residues in CCR5 that are critical for CC chemokine binding to CCR5 by performing the residue-by-residue examination of binding affinity (K_d) alterations with a single amino acid substitution [14]. More recently, we reported that amino acid residues in the β -hairpin structural motif of ECL2 are crucial for

HIV-1-elicited fusion and binding of APL and its analogues to CCR5 [36]. The direct ECL2-engaging property of APL likely produces an ECL2 conformation, which HIV-1 gp120 cannot bind to, but also prohibits HIV-1 from utilizing the ‘inhibitor-bound’ CCR5 for cellular entry - a mechanism of HIV-1 resistance to CCR5 inhibitors [41,42]. It should be noted that it remains to be determined whether the preservation of physiological CC-chemokine–CCR5 interactions brings about benefit in the treatment with CCR5 inhibitors. However, the present data clearly demonstrate that it is feasible to design CCR5 inhibitors that are more potent against HIV-1, preserve CCR5 inhibitor–CCR5 interactions, and prevent or delay the emergence of resistant HIV-1 variants. The data also show that YFP⁺CCR5-UM16 cells should be of utility in examining the dynamics of CCR5 and its interactions with CC chemokines.

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Figure 1.
CCR5 expression and RANTES-induced YFP CCR5 internalization in YFPCCR5-UM16 cells

Figure 2.
YFP-CCR5 internalization and calcium mobilization induced by RANTES in YFP-CCR5-UM16 cells

Figure 3.
Quantification of CC-chemokine-induced YFP^{CCR5} internalization and the effects of CCR5 inhibitors on this internalization

Figure 4.
Influences of CCR5 inhibitors against the redistribution of YFP^{CCR5}

Quantification of the effects of CCR5 inhibitors against CC-chemokine-induced internalization in regard to their anti-HIV-1 activity

Table 1.

Effect	Measure	CCR5 inhibitor		
		APL	TAK779	MVC
Anti-HIV-1 effect				
HIV-1 _{JR-FL}	IC ₅₀ , nM ^a	0.81 ±0.11	14.6 ±2.89	1.25 ±0.29
Effect on CCR5 internalization				
RANTES	ED ₅₀ , nM ^b	13.3 ±1.94	16.3 ±4.92	1.13 ±0.65
	ED ₅₀ /IC ₅₀ ratio	16.4	1.1	0.9
MIP-1 α	ED ₅₀ , nM ^b	<1	36.2 ±10.8	<1
	ED ₅₀ /IC ₅₀ ratio	<1.2	2.5	<0.8
MIP-1 β	ED ₅₀ , nM ^b	1.56 ±0.67	35.7 ±11.5	1.96 ±0.84
	ED ₅₀ /IC ₅₀ ratio	1.9	2.4	1.6

^aThe 50% inhibitory concentrations (IC₅₀) were determined in UM cells expressing wild-type CC chemokine receptor 5 (WT-CCR5-UM) using MAGI assay. All assays were conducted in duplicate or triplicate, and the data shown represent means ± SD derived from the results of two independent experiments.

^bThe 50% effective dose (ED₅₀) values were determined based on the peak percentage relative integrated fluorescence intensity (%RFI) values within 40 min shown in Figure 4, setting the peak %RFI without inhibitors as a control. The ED₅₀ data shown represent means ± SD derived from the results of at least three independent experiments. Each ED₅₀/IC₅₀ ratio was determined by dividing the ED₅₀ value by the corresponding IC₅₀ value. APL, aplaviroc; HIV-1, HIV type-1; MIP, macrophage inflammatory protein; MVC, maraviroc.