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Inhibition of CCR5-mediated infection by diverse R5 and R5X4 HIV and SIV isolates using novel small molecule inhibitors of CCR5: Effects of viral diversity, target cell and receptor density

Samantha Willey[a,b], Paul J. Peters[a], W. Matthew Sullivan[a], Patrick Dorr[c], Manos Perros[c], Paul R. Clapham[a,*]

[a] *Center for AIDS Research, Program in Molecular Medicine, Department of Molecular Genetics and Microbiology, University of Massachusetts Medical School, Biotech II, 373 Plantation St, Worcester, MA 01605, USA*
[b] *Wohl Virion Centre, Department of Immunology and Molecular Pathology, Windeyer Institute of Medical Sciences, 46 Cleveland Street, London W1T 4JF, UK*
[c] *Pfizer GRD, Sandwich Laboratories, Kent CT13 9NJ, UK*

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Abstract

Highly active anti-retroviral therapy (HAART) has been very effective in reducing viral loads in human immunodeficiency virus (HIV)-1 patients. However, current therapies carry detrimental side effects, require complex drug regimes and are threatened by the emergence of drug-resistant variants. There is an urgent need for new anti-HIV drugs that target different stages of the replication cycle. Several synthetic small organic molecules that inhibit HIV infection by binding to the CCR5 coreceptor without causing cell activation have already been reported. Here, we have exploited a series of CCR5 antagonists to investigate their effects on diverse HIV and the simian counterpart (SIV) isolates for infection of a variety of cell types via different concentrations of cell surface CCR5. These inhibitors show no cross-reactivity against alternative HIV coreceptors including CCR3, CCR8, GPR1, APJ, CXCR4 and CXCR6. They are able to inhibit a diverse range of R5 and R5X4 HIV-1 isolates as well as HIV-2 and SIV strains. Inhibition was observed in cell lines as well as primary PBMCs and macrophages. The extent of inhibition was dependent on cell type and on cell surface CCR5 concentration. Our results underscore the potential of CCR5 inhibitors for clinical development.

Keywords: HIV coreceptors; HAART; CCR5; Coreceptor inhibitor

1. Introduction

Human immunodeficiency virus (HIV), and the simian counterpart (SIV) enter cells by inducing fusion of viral and cellular membranes and releasing the viral core into the cell cytoplasm. Initially, the surface subunit of the trimeric envelope glycoprotein, gp120, interacts with the primary receptor CD4 (Dalgleish et al., 1984; Klatzmann et al., 1984). This induces conformational changes that result in the formation, or exposure, of a coreceptor binding site, allowing binding to the coreceptor and subsequent membrane fusion. All HIV and SIV coreceptors are members of the seven-transmembrane G-protein coupled receptor family, and although many members of this family, as well as related 'orphan' receptors, can support infection by various HIV and SIV strains in vitro, the chemokine receptors CCR5 and CXCR4 remain the two major coreceptors implicated in vivo (Alkhatib et al., 1996; Berger et al., 1999; Clapham and McKnight, 2002; Deng et al., 1996; Dimitrov et al., 1998; Doranz et al., 1997; Dragic et al., 1996; Willey et al., 2003).

Of the two major HIV coreceptors, CCR5 plays an essential role in HIV transmission and pathogenesis. The majority of transmitted viruses exclusively use CCR5 as a coreceptor, and these R5 isolates remain present throughout the duration of infection. Variants able to exploit CXCR4 usually

* Corresponding author. Tel.: +1 508 856 6281; fax: +1 508 856 4283.
E-mail address: paul.clapham@umassmed.edu (P.R. Clapham).

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only emerge during the later stages of disease in up to 50% infected individuals (de Roda Husman et al., 1999; Tersmette et al., 1988; van Rij et al., 2002). In addition, the natural CCR5 ligands RANTES, MIP-1α and MIP-1β, and modified analogues such as AOP-RANTES, are effective at inhibiting CCR5-mediated infection in vitro (Cocchi et al., 1995; Deng et al., 1996; Dragic et al., 1996; Oravecz et al., 1996; Simmons et al., 1997). Moreover, individuals homozygous for a 32-base pair deletion in CCR5 (rendering it non-functional) are largely resistant to infection whilst remaining in good health (Dean et al., 1996; Huang et al., 1996; Liu et al., 1996; Samson et al., 1996). All these factors make CCR5 an ideal candidate for novel therapeutic strategies.

Current anti-retroviral therapy (HAART) exploits varying combinations of nucleoside reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors, protease inhibitors (all oral), and a (less widely available) injectable fusion inhibitor (Cohen et al., 2002; Yeni et al., 2002). These regimens are highly effective at reducing viral loads, allowing some recovery of the immune system and increasing the life span of infected individuals (Gulick et al., 1997; Hammer et al., 1997; Kaufmann et al., 2000; Palella et al., 1998). However, the complex drug administration regime, detrimental side effects and increasing evolution of resistant viral variants reflect the urgent need for new drugs targeting alternative stages in the viral replication cycle (Brenner et al., 2002; De Clercq, 2002). Small molecule inhibitors of HIV entry would provide a significant advance on current HAART treatments. Several such molecules have been described including TAK-779 (an anilide derivative) and the piperidine SCH-C, which both target the coreceptor CCR5, while the bicyclam molecule AMD3100 targets CXCR4 (Baba et al., 1999; De Clercq et al., 1994; Donzella et al., 1998; Strizki et al., 2001). Such organic molecules are advantageous over natural chemokine ligands as HIV inhibitors, since they reduce the risk of potential inflammatory responses, are cheaper to produce than small proteins, and can be administered orally as opposed to intravenously. These coreceptor inhibitors (TAK-779, SCH-C, AMD3100) have proven to be effective HIV inhibitors in vitro, and have been superseded by superior compounds that hold great promise, with several currently in clinical trials (Barber, 2004; Hendrix et al., 2000; Maeda et al., 2004; Seto et al., 2005; Tagat et al., 2004).

In this study, we have exploited a series of six related small molecule compounds to investigate their effects on CCR5-mediated infection by diverse HIV-1 and HIV-2 isolates. These compounds were made as part of the campaign towards the discovery of maraviroc (MVC, UK-427,857), which retains high potency as a CCR5 inhibitor for broad cross-clade reactivity against HIV-1, has oral bioavailability, and is currently undergoing phase 2b/3 clinical trials (Abel et al., 2003; Dorr et al., 2003; Napier et al., 2003; Pozniac et al., 2003). The two most potent and the least potent compound(s) were extensively analysed for their ability to inhibit a range of R5 and R5X4 isolates of HIV-1, HIV-2 and SIV. Their effect on infection of different cell types, including cells expressing varying cell surface concentrations of CD4 and/or CCR5, as well as primary PBMCs and macrophages, the major cellular targets of HIV-1 in vivo, is described.

2. Materials and methods

2.1. Viruses

SF162 is a non-syncytium-inducing (NSI) molecular clone of HIV-1, which primarily uses CCR5 as a coreceptor (Cheng-Mayer et al., 1989). The HIV-1 strain C3 is a molecular cloned variant of JR-CSF able to infect T-cell lines via very low levels of CCR5 (Boyd et al., 1993; Dejucq et al., 1999). The HIV-1 isolates E80 (R5), 2044 (X4), and 2076 and 2028 (R5X4), are primary isolates described previously (Simmons et al., 1996). The HIV-1 isolate RU570 was from Dr. A. Bobkov and Dr. Jonathan Weber, and obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (Bobkov et al., 1994). The HIV-1 strain 89.6 is an R5X4 molecular clone of HIV-1 (Collman et al., 1992), as is the isolate HAN-2 (Sauermann et al., 1990). All isolates of HIV-1 used in this study are clade B, with the exception of RU570, which is clade G. The HIV-2 isolates used in this study are primary isolates from individuals in Portugal of West African descent. ALI was derived from a patient with AIDS-related complex, and TER from a patient with AIDS (Clapham et al., 1999; McKnight et al., 1998). SIVman4 is a T-cell line adapted variant of SIVsmB670 (Murphey-Corb et al., 1986). SIVmac239 is a molecular clone derived from a rhesus macaque isolate in 1985 (Daniel et al., 1985). All virus stocks were propagated in PBMCs purified from whole blood by density gradient centrifugation, stimulated for 2 days in phytohemagglutinin (PHA, 1 μg/ml) and 2 days in human recombinant IL-2 (10 units/ml, Roche, Inc.).

2.2. Cell lines

Human glioma-derived cell lines (U87 and NP2) stably expressing CD4 and different chemokine receptors or orphan receptors were as follows: U87/CD4 expressing CCR5 (Bjorndal et al., 1997; Deng et al., 1997; Willett et al., 1997), and NP2/CD4 expressing CCR3, CCR5, CCR8 and GPR1 (Soda et al., 1999). These cells were cultured in DMEM supplemented with 10% FCS, gentamycin (10 μg/ml, Gibco Invitrogen Corporation) and puromycin at 1 μg/ml. NP2/CD4/Apj cells were made by transfecting parental NP2/CD4 cells with a pBabe (puro)-Apj construct and selecting for stable transfectants in puromycin-containing selection medium. The T-cell line MOLT4 clone 8 was obtained from the NIH AIDS Research and Reference Reagent Program and was maintained in RPMI 1640ŠŠŠ (Gibco Invitrogen Corporation) medium containing gentamycin (10 μg/ml, Gibco Invitrogen Corporation) and 10%

FCS. GHOST/CXCR6 cells were cultured in DMEM containing 10% FCS and gentamycin (Cecilia et al., 1998; Daniel et al., 1988; Kikukawa et al., 1986). HeLa cells expressing low (1×10^4 molecules/cell) or high (4×10^5 molecules/cell) levels of CD4, and three levels (low, medium and high, corresponding to 2×10^3, 10^4 and 10^5 molecules/cell, respectively) of CCR5 were maintained in DMEM supplemented with 4% FCS (Platt et al., 1998). Immunostaining and FACS analysis confirmed that these different clones expressed low and high CD4, and low, medium and high levels of CCR5 on their cell surfaces (Platt et al., 1998).

2.3. Preparation of PBMCs and macrophages

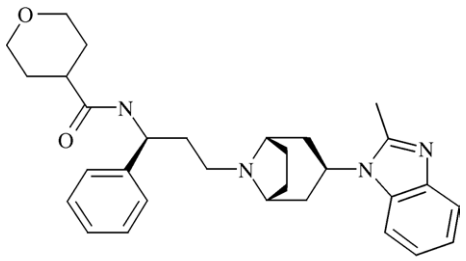
Lymphocytes were purified from whole blood derived from volunteer donors (University of Massachusetts IRB approved protocol) or buffy coats (Hospital Transfusion Services, Brentwood, UK) by density gradient centrifugation on Ficoll-paque, as previously described (Simmons et al., 1997). PBMCs were stimulated with PHA and IL-2, as described above, before infecting. Macrophages were prepared from white blood cells by adherence, as previously described (Simmons et al., 1997) or by elutriation (Kalter et al., 1991). After culture for 5–7 days, macrophages were treated with versene (Gibco Invitrogen Corporation) and removed with a cell scraper before seeding for infection.

2.4. Inhibitors and antibodies

The chemokine RANTES was purchased from PeproTech, Inc., Rocky Hill, NJ. The chemokines I309 and eotaxin, and the human herpesvirus 8 (HHV8)-encoded chemokine vMIP-I were purchased from R&D Systems, Inc. The small molecule inhibitor, TAK-779, was obtained through the AIDS Research and Reference Reagent Program, NIAID, NIH (Baba et al., 1999). The Apj ligand, Apelin-36, was from Peptide Institute, Inc., Osaka, Japan. The CXCR4 antagonist AMD3100 was provided by AnorMED, Inc., Langley, Canada. CCR5 small molecule inhibitors (UK-396,794, UK-383,990, UK-387,323, UK-387,323, UK-400,343, UK-403,341) made as part of the synthesis campaign towards the discovery and development of maraviroc (MVC, UK-427,857) were provided by Pfizer GRD, Sandwich, Kent, UK. The structure of UK-396,794 is shown in Fig. 1. The CCR5 monoclonal antibody (mab) 2D7 was from the NIH AIDS Research and Reference Reagent Program, and the CCR5 mab MAB182 was from R&D Systems, Inc.

2.5. Inhibition assays

Adherent cells were seeded the day before infection in 48-well plates at 3×10^4 cells/ml (NP2/CD4, GHOST coreceptor-expressing cell lines), 2×10^4 cells/ml (U87/CD4 coreceptor expressing cell lines) or 2.5×10^5 cells/ml (macrophages) in 500 μl of the appropriate medium. On the day of infection, medium was removed and cells were



UK-396, 794

Fig. 1. The structure of UK-396,794.

incubated for 1 h with 75 μl of inhibitor (i.e. chemokines, small molecule inhibitors) at 2× final concentration before exposing for 3 h to approximately 100 focus-forming units (FFU) of virus in 75 μl, a titer determined by infection of NP2/CD4/CCR5 or U87/CD4/CCR5 coreceptor-expressing indicator cells. Cells were washed once in medium before inhibitor was replaced at 1× concentration and cells left at 37 °C for 72 h. PBMCs were seeded in 100 μl in v-bottom 96-well plates at 1×10^6 cells/ml before centrifuging for 5 min at 1000 rpm and resuspending in 50 μl of growth medium containing twice the final concentration of inhibitor. Following incubation for 1 h at 37 °C, 50 μl of virus at titers at 1×10^4 FFU/ml or higher was added, mixed and cells were incubated at 37 °C for 3 h. After incubation, cells were washed three times by centrifuging for 5 min at 1000 rpm in medium and resuspended in 150 μl growth medium containing the appropriate final inhibitor concentration. Cells were left at 37 °C and cell-free supernatant was harvested on days 3, 6, 9, 12, 15 and 18 of infection to monitor virus production. Inhibitor was replenished at each harvest.

2.6. Measurement of virus infectivity

HIV infection of PBMCs was determined by measuring RT activity in cell supernatants by RT-ELISA (CavidiTech, Uppsala, Sweden). Infected adherent cells were detected by immunostaining for intracellular p24 as previously described (Clapham et al., 1992; McKnight et al., 1994). In brief, cells were rinsed in PBS before fixing in a cold (−40 °C) 1:1 methanol:acetone mix for 5–10 min, rinsing once in PBS and once in PBS/1% FCS. For HIV-1, cells were stained using a 1:1 mix of anti-HIV-1 gag monoclonal antibodies 38:96 K and EF7 (MRC AIDS Reagent Program, Potters Bar, England) diluted 1:40, whilst HIV-2 infected cells were stained using a mix of 6 HIV-2^+ serum samples (WHO panel C; MRC ARP) diluted 1:4000. Infected cells were detected by a 1:400 dilution of a goat anti-mouse or goat anti-human β-galactosidase conjugate for HIV-1 and HIV-2, respectively (Southern Biotechnology Associates, Inc.) and revealed with an X-Gal substrate (PBS with 3 mM potassium ferricyanide, 3 mM potassium ferrocyanide, 1 mM magnesium chloride and 0.5 mg/ml X-gal).

2.7. Down-modulation of CCR5

1×10^5 purified and activated PBMC were pelleted and resuspended in 50 μl medium, UK-396,794 or RANTES at 0 and 100 nM. Samples were incubated at 37 °C for 0 or 90 min and cooled to 4 °C on ice. Inhibitors were removed by washing with cold PBS/1% FCS/0.1% azide before cells were stained on ice with an anti-CCR5 mab (MAB182) or isotype control (IgG_{2B}, Dako) at 5 μg/ml. Cells were washed twice with cold PBS/1% FCS/0.1% azide before resuspending in goat anti-mouse FITC-conjugated antibody (Dako) diluted 1 in 40. Cells were washed once with cold PBS/1% FCS/0.1% azide and twice with cold PBS/0.1% azide before resuspending in 500 μl 2% formol saline and detecting bound antibody by flow cytometry (FACScan, BD Biosciences) using the CellQuest software package. The relative cell surface expression of CCR5 was calculated as: 100 × (mean channel fluorescence [chemokine + α-CCR5 Mab] − mean channel fluorescence [isotype control])/(mean channel fluorescence [medium alone + α-CCR5 Mab] − mean channel fluorescence [isotype control]).

3. Results

3.1. Small molecule antagonists of CCR5 inhibit infection by HIV

The capacity of the six CCR5-specific small molecule inhibitors to block infection of the $CD4^+CCR5^+$ NP2 glioma cell line was evaluated. Each of the six compounds blocked infection by both SF162 and E80, two R5-tropic HIV-1 strains. Compounds UK-396,794 and UK-400,343 were the most potent inhibitors, blocking infection almost completely at 50 nM (Fig. 2). Compounds UK-383,990 and UK-403,341 were the least effective, inhibiting SF162 infection by 75–94%, and E80 by 50 and 60% at 500 nM. Compounds UK-387,323 and UK-387,323 were intermediate in their capacity as HIV-1 inhibitors. Since the order of efficiency of inhibition by the six compounds was consistent against both virus isolates (UK-396,794 and UK-400,343 > UK-387,323 and UK-387,323 > UK-383,990, UK-403,341), further experiments on these inhibitors were carried out only with the two most effective (UK-396,794, UK-400,343) and one of the least effective (UK-403,341) inhibitors in this series.

3.2. Specificity of inhibition by CCR5 inhibitors

The cross-reactivity of UK-400,343 against other HIV coreceptors was assessed. Indicator cell lines stably expressing CD4 and a range of coreceptors were pre-treated with UK-400,343 or an appropriate coreceptor-specific natural chemokine ligand/small molecule inhibitor (Fig. 3). Treated and untreated cells were then infected with an HIV or SIV isolate, depending upon coreceptor usage. The HIV-2 strain TER was used for infection via CCR5, CCR8 and GPR1; HIV-1 strain 2044 for CXCR4; HIV-1 HAN-2 for CCR3; HIV-1 89.6 for APJ and SIVman4 for CXCR6.

In CCR5-expressing cells, compound UK-400,343 was a more potent inhibitor than the CCR5 ligand RANTES, reducing infection by 80% at 50 nM, in comparison to 25% reduction with RANTES (Fig. 3). Thus, UK-400,343 had no significant effect on infection via the coreceptors CCR3, CCR8, CXCR4, CXCR6, GPR1, or APJ in comparison to their specific inhibitors.

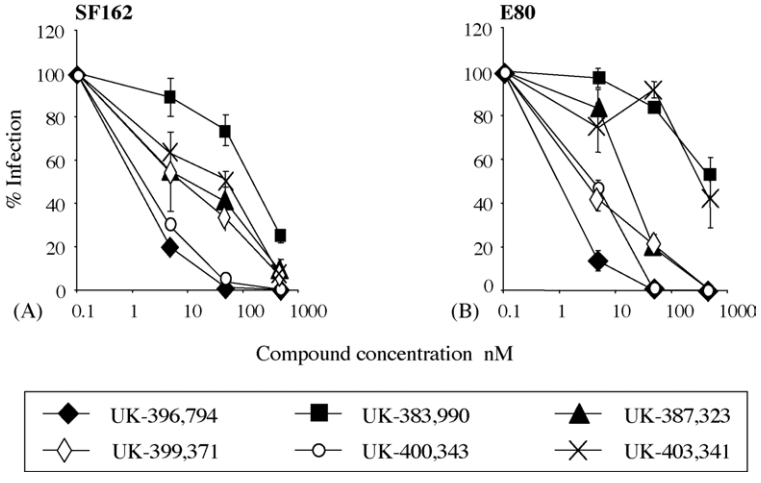


Fig. 2. Small molecule antagonists of CCR5 inhibit infection by HIV-1. U87/CD4/CCR5 cells were pre-treated with 0, 10, 100 and 1000 nM of the CCR5 inhibitors for 1 h before infecting with the R5 HIV-1 isolates SF162 (A) and E80 (B). Infected cells were detected by immunostaining for viral antigens. 100% infection was calculated for each virus independently and represents the mean number of infected foci in the absence of inhibitors. All samples were done in duplicate, and error bars represent the standard deviation of the mean. Data is representative of at least three independent assays.

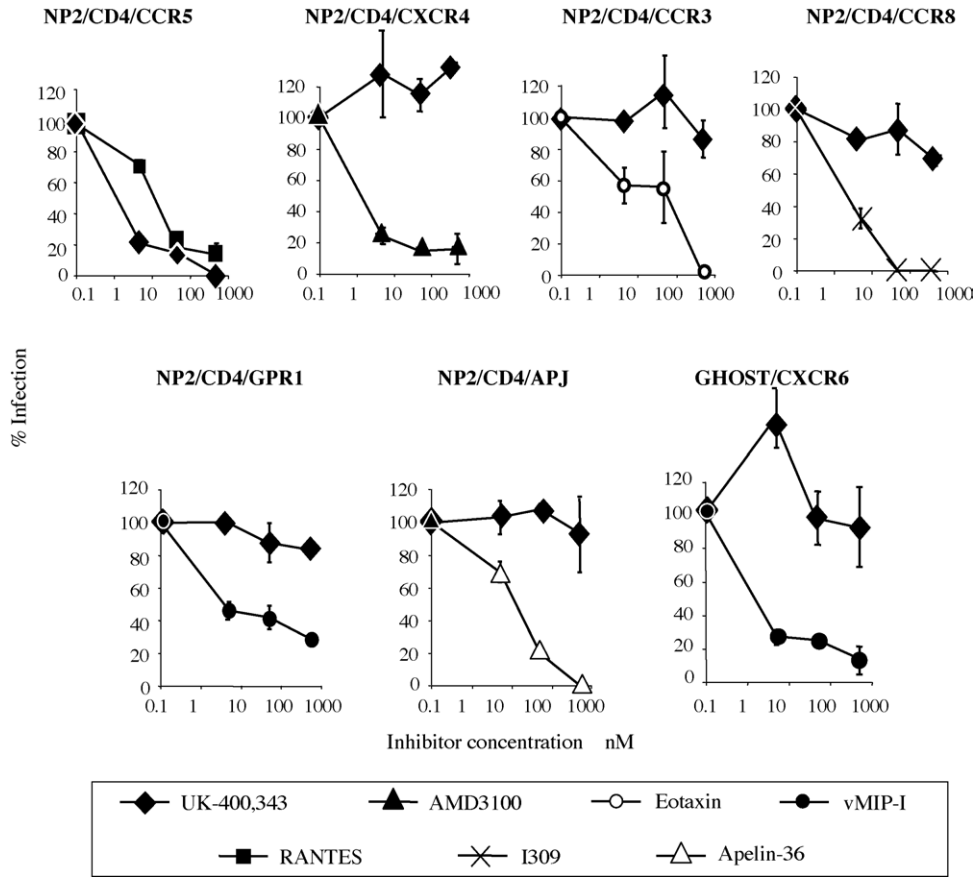


Fig. 3. UK-400,343 specifically inhibits CCR5-mediated infection by HIV. Indicator cell lines stably expressing CD4 and a range of coreceptors were pre-incubated with UK-400,343 or the natural ligand/synthetic inhibitor for each coreceptor tested before infection with HIV or SIV. The value of 100% infection was calculated for each coreceptor cell line independently, and represents an average of the number of foci in the absence of inhibitors. This value was in the range of 75–250 focus-forming units (FFU) for each assay. The percent infectivity in the presence of inhibitors was calculated from the relevant 100% value. All samples were done in duplicate, and the error bars represent the standard error of the mean. Graphs are representative of at least two independent assays.

3.3. CCR5-specific small molecule inhibitors of HIV do not inhibit infection via receptor down-modulation

Chemokines and chemokine analogues, such as RANTES and AOP-RANTES, inhibit CCR5-mediated HIV infection by inducing receptor down-modulation (Mack et al., 1998). Thus, the effect of the two most potent CCR5-specific small molecules on cell surface expression of CCR5 was determined. CCR5 expression levels were identical in samples incubated in the presence of either RANTES, the small molecule inhibitors or no inhibitor, indicating that none of these inhibitors prevented the binding of the CCR5-specific monoclonal antibody 2D7 (data not shown). PBMCs were incubated with UK-396,794, UK-400,343 (the two most potent CCR5 inhibitors) or RANTES. Following 90 min incubation with increasing concentrations of RANTES, CCR5 expression was successively reduced to 30% of levels in the absence of the chemokine (Fig. 4), similarly to previously published reports (Mack et al., 1998). At the same concentration, the two most potent CCR5 inhibitors had no effect on the surface expression of CCR5.

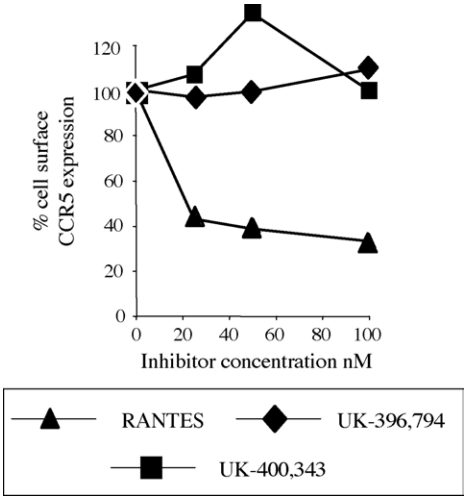


Fig. 4. Inhibition of HIV infection does not occur by ligand-induced receptor internalization. CCR5 internalization was determined by incubating PHA, IL-2 activated PBMCs with increasing concentrations of inhibitor for 90 min. Cell surface expression levels of CCR5 were analysed by quantitative flow cytometry, and relative % expression was calculated by the formula as stated in Section 2.

3.4. R5X4-tropic isolates of HIV-1 are inhibited more efficiently than R5-tropic isolates

Previous studies have indicated that virus isolates able to use CXCR4 in addition to CCR5 for entry are more susceptible to inhibition by CCR5 inhibitors as tested in cell systems that do not express CXCR4 (Kledal et al., 1997). The capacity of these small molecule inhibitors to block infection by a panel of R5 and R5X4 isolates of HIV-1 was therefore examined. All four R5 strains (SF162, RU570, E80, C3) were completely inhibited by compound UK-400,343 at 50 nM, and by compound UK-396,794 at 500 nM (Fig. 5A). Again, compound UK-403,341 was the least efficient, reducing RU570, E80 and C3 infection by no more than 60%. Infection of CCR5-expressing cells by the four R5X4 isolates (2076, 2028, 89.6, HAN-2) was more sensitive to inhibition by all three compounds. UK-396,794 and UK-403,341 were 10-fold more potent against the R5X4 strains, which were all inhibited at 5 nM (Fig. 5B). In contrast to inhibition of R5 viruses, infection by three of the R5X4 isolates (2076, 2028, 89.6) was completely inhibited by compound UK-403,341 at 500 nM.

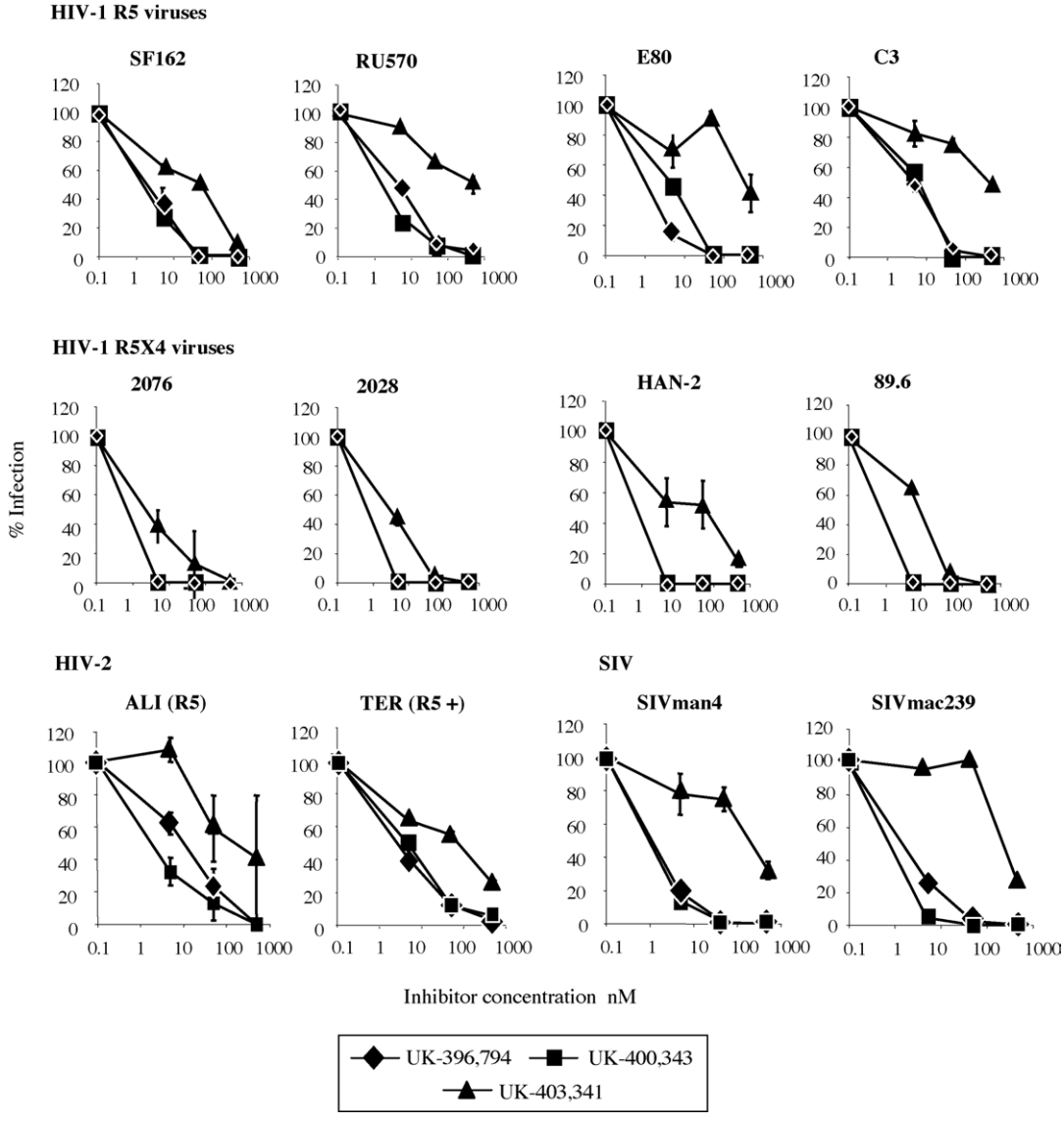


Fig. 5. Inhibition of HIV-1 R5 and R5X4, HIV-2 and SIV virus isolates. U87/CD4/CCR5 cells were pre-incubated with 0, 10, 100 or 1000 nM of UK-396,794, UK-400,343 or UK-403,341 for 1 h, before infecting with a range of (A) R5 (SF162, RU570, E80, C3), (B) R5X4 (2076, 2028, HAN-2, 89.6) HIV-1 isolates, (C) HIV-2 (ALI, TER) and (D) SIV (man4, mac239). 100% infection represents the average number of foci for each virus independently in the absence of inhibitor, and was used to calculate the % infection in the presence of inhibitor. 100% infection was in the range of 50–250 FFU. Error bars represent the standard error of the mean of at least three independent assays. Note that R5X4 HIV-1 isolates are inhibited more efficiently than R5 isolates.

3.5. Small molecule inhibitors are able to block infection of HIV-2 and SIV isolates including SIVmac239, which preferentially uses CCR5 E2

Fig. 5 also shows that the small molecule inhibitors were effective against two HIV-2 isolates and two SIV strains. Overall, the HIV-2 strains were slightly less sensitive than the HIV-1 strains to these inhibitors, but the order of efficiency of the three compounds remained the same (UK-400,343 > UK-396,794 > UK-403,341) (Fig. 5C). SIVman4, a T-cell line adapted SIV strain that uses CXCR4 in addition to CCR5 albeit inefficiently, was also inhibited (Fig. 5D). The T-tropic SIVmac239 was blocked more efficiently than the other HIV-2 and SIV strains (Fig. 5D), indicating that the preferential use of CCR5 E2 (rather than the N-terminus) reported for this virus (Edinger et al., 1997) did not adversely affect sensitivity to CCR5 inhibitors.

3.6. UK-396,794 is a more potent inhibitor of HIV-1 infection than RANTES or TAK-779

HIV infection can be inhibited by coreceptor-specific molecules, such as the CCR5-binding chemokine RANTES, and small molecules targeted to this chemokine receptor, such as TAK-779. The relative efficiency of UK-396,794 as a CCR5 inhibitor in comparison to RANTES and TAK-779 was therefore determined. UK-396,794 was consistently the better inhibitor, giving 100% inhibition at 5 nM (all R5X4 isolates) and 500 nM or less (R5 isolates) (Fig. 6). Like UK-396,794, RANTES was more effective against R5X4-tropic isolates of HIV-1, with most strains being completely blocked at 50 nM. As previously observed, RANTES was very inefficient at inhibiting R5 strains of HIV on the CCR5-expressing cell line used. Like UK-396,794 and RANTES, TAK-779 was more effective against R5X4 isolates than R5. Both R5X4 isolates tested were completely inhibited at 50 nM, one R5 strain (SF162) was completely inhibited at 500 nM and one reduced to 30% at the same concentration (RU570).

3.7. The antiviral potency of small molecule inhibitors is dependent upon cell type and cell surface concentration of CCR5

The cell lines U87/CD4/CCR5 and NP2/CD4/CCR5 are both astroglioma-derived, yet they conferred different sensitivities to CCR5 inhibitors. Thus, infection of the NP2 cell line with both R5 and R5X4 isolates of HIV-1 was more resistant to inhibition by all three compounds, with up to a 10-fold higher concentration of inhibitor being needed to completely block NP2 infection in comparison to U87 infection (data not shown). The relative expression level of CD4 on the two cell types (measured by flow cytometry) was not significantly different. However, CCR5 expression levels were far greater in the NP2/CD4/CCR5 cells (data not shown).

In order to determine if the differences in sensitivity to these CCR5 inhibitors were due to the higher cell surface

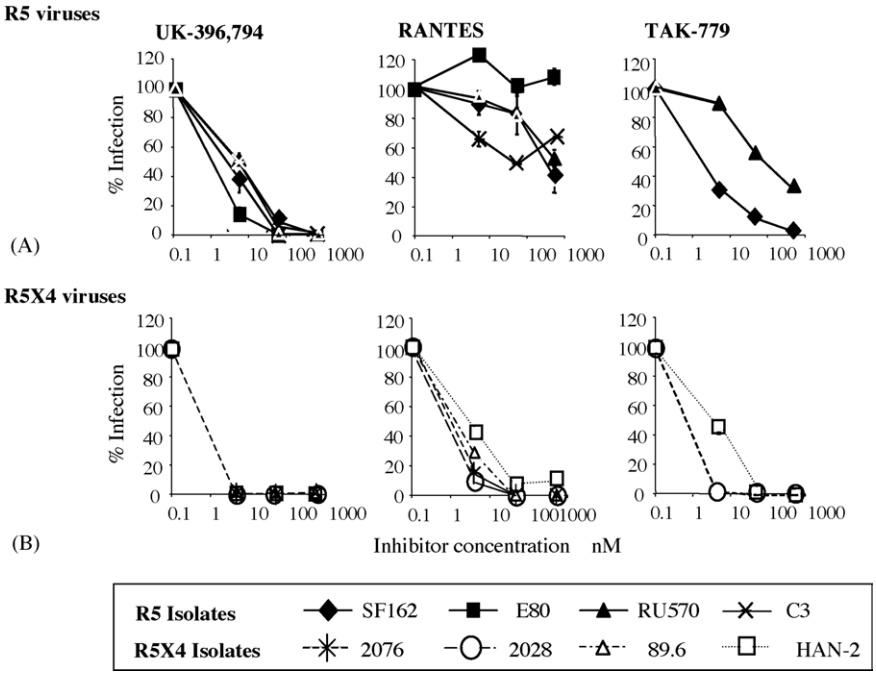


Fig. 6. Novel CCR5 inhibitors are more efficient than RANTES and TAK-779. U87/CD4/CCR5 cells were pre-incubated with 0, 10, 100 or 1000 nM of UK-396,794, RANTES or TAK-779 before infecting with HIV-1 R5 (A) or R5X4 (B) isolates. TAK779 inhibition was tested for just two isolates from each category (R5 strains SF162 and RU570, and R5X4 strains 2028 and HAN-2). 100% infection represents the average number of foci for each virus independently in the absence of inhibitor, and was used to calculate the percent infectivity in the presence of inhibitor. 100% infection was in the range of 50–250 FFU depending on the assay. Error bars represent the standard error of the mean of at least two independent assays.

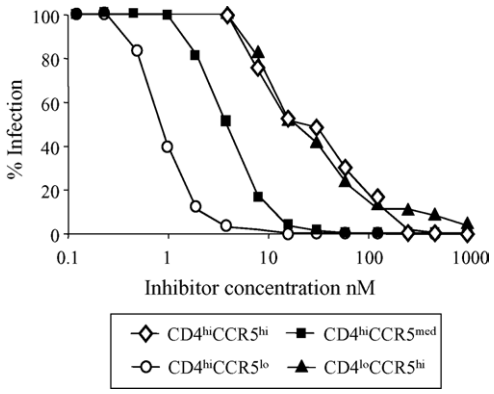


Fig. 7. The antiviral potency of small molecule inhibitors is dependent upon cell surface concentration of CCR5. HeLa cells expressing different levels of CD4 and CCR5 ($CD4^{hi}CCR5^{hi}$, $CD4^{hi}CCR5^{med}$, $CD4^{hi}CCR5^{lo}$ and $CD4^{lo}CCR5^{hi}$) were pre-incubated for 1 h with 0, 62.5, 125, 250, 500, 1000 or 2000 nM of UK-400,343 before infecting with R5-using SF162.

levels of CCR5, or due to other cell-specific factors, we tested the capacity of UK-400,343 to inhibit SF162 infection of HeLa cells expressing varying cell surface concentrations of both CD4 and CCR5 (Platt et al., 1998). The amount of cell surface CCR5 had a major influence on efficiency of inhibition for the CCR5 inhibitors (Fig. 7). While infection of $CCR5^{lo}$ HeLa cells was efficiently inhibited, much weaker inhibition was observed if high levels of CCR5 were present. This weak inhibition of $CCR5^{hi}$ cells was unaffected by a decrease in CD4 concentration. These observations likely explain the different sensitivities of inhibition observed on NP2/CD4/CCR5 and U87/CD4/CCR5 cells.

3.8. Novel CCR5 inhibitors block infection of primary PBMCs and macrophages by both R5 and R5X4 isolates of HIV-1

To gain a more physiologically relevant evaluation of this series of CCR5 inhibitors, their capacity to block a range of R5 and R5X4 HIV-1 isolates in primary untransformed human PBMCs and macrophages was tested. Previous reports have demonstrated that PBMC infection by R5 strains of HIV-1 is inhibited more efficiently by chemokines or chemokine derivatives than cell line infection (Simmons et al., 1997). Indeed, PBMC infection by two R5 strains (SF162 and RU570) was more efficiently inhibited by RANTES, with approximately 90% inhibition being observed at 50 nM, in comparison to only 20% inhibition on the cell line U87/CD4/CCR5 at the same concentration (Figs. 8A and 5A, respectively). Unlike inhibition observed with chemokines, these small molecules were slightly less effective in PBMCs than in cell lines, although they were still potent. Infection by SF162 was almost completely reduced by compounds UK-396,794 and UK-400,343 at 500 nM, whereas it was relatively resistant to inhibition by compound UK-403,341 (Fig. 8A). In contrast to the results observed in cell lines, yet in accordance with data reported for other CCR5 inhibitors, infection of PBMCs by the R5 clade G isolate RU570 was relatively resistant to inhibition by all three small molecule inhibitors (Figs. 8A and 5A) (Strizki et al., 2001). All three compounds (UK-396,794, UK-400,343, UK-403,341) could only reduce infection by this virus between 40 and 50% at a concentration of 500 nM.

As PBMCs express both CCR5 and CXCR4, inhibition of the R5X4 isolates 2076 and 2028 was inefficient and reduced by no more than 60% (2076) or 25% (2028). However, in an excess of the CXCR4 inhibitor AMD3100, these isolates were potently inhibited. Thus, PBMC infection by these R5X4 HIV-1 isolates was substantially more sensitive to inhibition than R5 isolates (Fig. 8A), as was true in cell lines (Fig. 5B). Both 2076 and 2028 were completely inhibited by RANTES, UK-396,794 and UK-400,343 at 50 nM or less in cell lines and PBMCs (Figs. 5B and 8A). UK-403,341, however, was extremely inefficient against these R5X4 strains on PBMCs, reducing infection by only 30% (2028) or not at all (2076). The order of efficiency of the compounds has thus remained consistent, irrespective of virus tropism and cell type.

3.9. Inhibition of macrophage infection by novel CCR5 inhibitors

Early studies demonstrated that macrophage infection via CCR5 varied in sensitivity to inhibition by β-chemokines (RANTES, MIP-1α, MIP-1β). However, the modified chemokine AOP-RANTES was a consistent inhibitor of HIV-1 infection of macrophages via CCR5 (Moriuchi et al., 1996; Simmons et al., 1997). The ability of small molecule antagonists of CCR5 to inhibit HIV-1 replication in primary macrophages was therefore investigated. These compounds completely inhibited infection by the R5 isolate SF162, with 100% inhibition being observed at 500 nM of inhibitor (Fig. 8B). In accordance with previous observations, the chemokine RANTES was not as effective as the small molecule inhibitors, achieving only 65% inhibition at 500 nM. The second R5 isolate, RU570 was also completely inhibited by both UK-396,794 and UK-400,343 at 5 nM (Fig. 8B). UK-403,341 was less effective, only giving 80% inhibition at 500 nM. RANTES gave similar inhibition profiles to that observed with SF162, reducing infection by 90 and 65%, respectively. Thus, RU570 infection of macrophages is sensitive to inhibition by CCR5 inhibitors, contrasting with the relative resistance of this isolate for PBMC infection.

4. Discussion

In this study, we have characterised antiviral properties of six representatives of a novel series of small molecule inhibitors of CCR5. These compounds were derived from

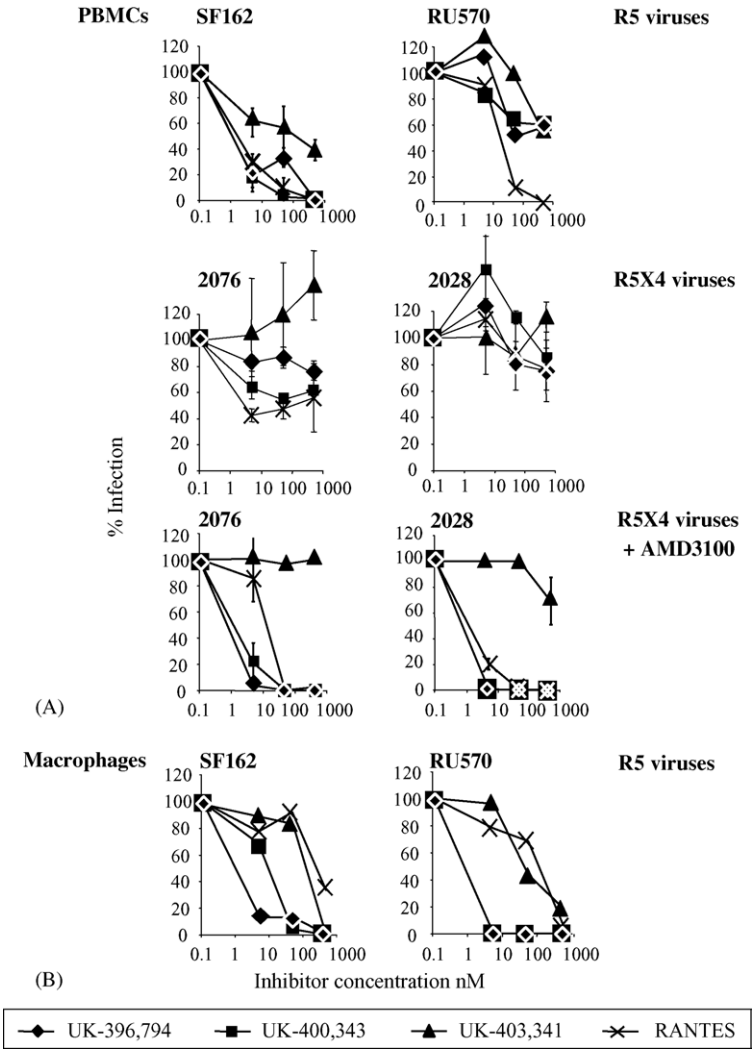


Fig. 8. Inhibition of PBMC and macrophage infection by CCR5 inhibitors. (A) PBMCs were pre-incubated for 1 h with 0, 10, 100 or 1000 nM of UK-396,794, UK-400,343, UK-403,341 or RANTES alone, or in combination with AMD3100 at 1000 nM before infecting with two R5 (SF162 and RU570) and two R5X4 (2076 and 2028) HIV-1 isolates. 100% infection for each virus represents RT activity in cell supernatant in the absence of inhibitors on day 9 of infection, was in the range of 500–2500 ng/ml, and was used to calculate all other values. All samples were done in triplicate, and error bars represent the standard error of the mean. Data is representative of at least two independent experiments. (B) Macrophages were pre-incubated with UK-396,794, UK-400,343 or UK-403,341 or RANTES at 0, 10, 100 or 1000 nM before infecting with R5 (SF162 and RU570) HIV-1 isolates. Cells were stained for viral antigens on day 15 of infection. 100% infection represents the number of infected cells in the absence of inhibitor (80–200 FFU/ml). All samples were done in duplicate, and error bars represent the standard error of the mean of two independent experiments.

the UK-427,857 discovery programme, which is being progressed through advanced stage clinical trials for HIV infection by Pfizer GRD. We have demonstrated potent inhibition of CCR5-dependent infection by diverse HIV and SIV strains and for varied cell types.

The efficient inhibition by the UK CCR5 antagonists applied to a range of HIV-1, HIV-2 and SIV strains with diverse coreceptor requirements, including the isolate SIVmac239, which preferentially utilises the second extracellular domain of CCR5 (CCR5 E2) for infection (Edinger et al., 1997). Thus, HIV variants that naturally escape CCR5 inhibitors were not readily apparent even among very diverse viruses. An intriguing exception to the efficiency of these inhibitors against PBMC infection was observed with the clade G HIV-1 isolate RU570. Infection of PBMCs by this isolate was sensitive to inhibition by RANTES, yet relatively insensitive to inhibition with all three inhibitors of this series, despite being well inhibited on cell lines and primary macrophages. RU570 has been reported to be resistant to SCH-C in PBMC culture, yet susceptible to UK-427,857 (Dorr et al., 2003; Strizki et al., 2001). The basis of these differences in susceptibility remains unclear, although this may be due to subtle differences in the binding site occupancy as reported for various CCR5 antagonists. For example, Westby et al. (2005) reported structurally related HIV coreceptor antagonists bound to similar regions of CCR5 but

had differential activities against maraviroc (UK-427,857)-resistant primary isolates. The cause of the relative resistance of RU570 to inhibition on PBMCs compared to macrophages and cell lines tested here remains unexplained. Moreover, we found no evidence to suspect the use of an alternative coreceptor on PBMCs. Thus, RU570 infection of PBMCs was blocked by RANTES, while significant replication of RU570 in PBMCs that lack CCR5 was not detected (data not shown).

Clearly, the potency of these CCR5 inhibitors is heavily influenced by the target cell used for infection. For instance, the cell line U87/CD4/CCR5 was consistently more sensitive to inhibition by the azabicycloalkane molecules than the similar astroglioma-derived NP2/CD4/CCR5 cell line. Flow cytometric analysis of these cell lines showed similar expression levels of CD4, but significantly higher levels of CCR5 on the NP2 cell line. CCR5 concentration was previously shown to influence the efficiency of CCR5 inhibitors (Platt et al., 2005; Reeves et al., 2002). In this study, the effect of CCR5 concentration on inhibition was remarkable when tested on HeLa cells expressing different amounts of CD4 and CCR5 (Platt et al., 1998). The results show an inverse correlation between CCR5 concentration and efficiency of inhibition and likely explain the different sensitivities to inhibition observed on U87/CD4/CCR5 and NP2/CD4/CCR5 cells.

It has been demonstrated that PBMC infection by R5 isolates of HIV-1 is more sensitive to inhibition by β-chemokines, such as RANTES and MIP-1α/β, than cell line infection (Simmons et al., 1997). Although PBMC infection was slightly less sensitive to the inhibitors than cell lines, they were still very potent against both R5 and R5X4 isolates of HIV-1. This data agrees with that reported by Strizki et al., who showed similar IC_{50} figures for inhibition of single-cycle replication of HIV pseudotypes in U87/CD4/CCR5 cells, and replication of a range of diverse HIV isolates in PBMCs (Strizki et al., 2001). The capacity of β-chemokines to suppress CCR5-mediated infection of primary macrophages (unlike PBMCs) has been reported to be variable (Dragic et al., 1996; Ketas et al., 2003; Moriuchi et al., 1996; Simmons et al., 1997). Few studies have examined the sensitivity of macrophage infection to small molecule CCR5 inhibitors. Thus, Ketas et al. (2003) showed that SCH-C is a more effective inhibitor of PBMC than macrophage infection. Here, all compounds in this series of inhibitor were more effective against RU570 and SF162 infection of macrophages than of PBMCs.

Although the effects of the inhibitors TAK-779 and SCH-C on HIV-1 R5 isolates have been extensively reported (Baba et al., 1999; Dragic et al., 2000; Ketas et al., 2003; Strizki et al., 2001; Trkola et al., 2002), their effect on R5X4 isolates is less well understood. CCR5 agonists, e.g. RANTES, inhibit R5X4 infection of $CCR5^+$ cell lines more effectively than R5 viruses (Kledal et al., 1997). All three compounds investigated here (UK-396,794, UK-400,343, UK-403,341) were also consistently more effective against a range of R5X4 HIV-1 isolates than a range of R5 isolates. Thus, when the HIV coreceptor binding site evolves to exploit CXCR4 in addition to CCR5 for entry, the affinity for and the strength of envelope interaction with CCR5, may be compromised. A precedent was reported by Reeves et al. (2004), who showed that mutations introduced into the coreceptor binding regions of gp120 conferred decreased CCR5 affinity but an increase in sensitivity to coreceptor inhibitors (Reeves et al., 2004). It is possible that continued CCR5-use is required to support the evolution of R5X4 variants as they adapt to and efficiently exploit CXCR4. If this is the case, then CCR5 inhibitors could act to efficiently repress the emergence of such variants.

A concern for the use of coreceptor-specific HIV inhibitors as therapy is the potential evolution of viral escape mutants. Such mutants could escape inhibition by adapting to exploit CXCR4, as seen with the chemokine analogues AOP-RANTES and N^{α}-nonanoyl-RANTES[2-68] (NNY-RANTES), a process which could potentially induce a faster progression to AIDS (Bjorndal et al., 1997; Mosier et al., 1999). In vitro experiments have indicated that HIV resistance to CCR5 antagonists is relatively difficult to achieve, requires extensive passaging in the presence of antagonist, and generates resistant isolates that still gain entry via CCR5 despite the presence of a CCR5-specific inhibitor. This situation was described for AD101 (a derivative of the potent CCR5 antagonist SCH-C) and UK-427,857 (Trkola et al., 2002; Westby et al., 2004). Nevertheless, a subsequent study demonstrated that escape from the CCR5 inhibitor AD101 was mainly conferred by just four changes within the third hypervariable loop within gp120, which did not correspond to a decrease in receptor affinity (Kuhmann et al., 2004). Thus, the concern remains that variants that escape CCR5 inhibitors will eventually emerge in vivo. We previously described a variant of the HIV-1 JR-CSF strain that was able to infect cells via low CCR5 levels (Dejucq et al., 1999) and recently reported the presence of highly macrophage-tropic envelopes in the brain of patients with neuropathology (Peters et al., 2004). These envelopes were able to exploit low levels of CD4 for infection but were also able to infect cells expressing trace levels of CCR5. Such variants may survive, replicate and eventually evolve to escape, if CCR5 inhibitors are only partially effective. This possibility will be severely limited by the use of CCR5 inhibitors in combination with drugs, that target other events in the HIV replication cycle, but remains a risk at sites in vivo where drugs may penetrate inefficiently, e.g. the brain.

In conclusion, we have characterised several small molecule CCR5 antagonists that are extremely effective at inhibiting infection by a very diverse set of HIV and SIV isolates. Inhibition of infection was demonstrated for a range of cell lines and primary cell cultures, including T-cells and macrophages that represent the main cell types targeted and infected by HIV in vivo. Several coreceptor antagonists are currently in clinical trials and hold much promise as HIV therapeutic agents.

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