A novel assay to identify entry inhibitors that block binding of HIV-1 gp120 to CCR5

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Abstract

HIV-1 infection is initiated by the interaction of the envelope glycoprotein gp120 with the cellular receptor CD4 that triggers conformational changes in gp120 necessary for subsequent interaction with a coreceptor CCR5 (or CXCR4). The CD4-induced (CD4i) conformation of gp120 can be mimicked by a full-length single chain (FLSC) protein consisting of gp120 linked with the D1D2 domains of CD4 by a 20-amino-acid linker. We have used this protein to establish a flow cytometry-based assay and an ELISA-based assay to identify inhibitors that block the binding of gp120 to CCR5. Both assays are specific for detecting the known CCR5 antagonist TAK-779, but the ELISA-based assay was more sensitive, simple, inexpensive, and rapid; thus, it can be adapted to high throughput screening (HTS). The ELISA-based method was validated with a diverse set of known antagonists, for example, TAK-779, AOP-RANTES, PSC-RANTES, and several mAbs.

Keywords: HIV; Envelope glycoprotein; Coreceptor; CCR5; Fusion; Entry inhibitor; Flow cytometry; Cell-based assay; ELISA

Introduction

The discovery of T-20 (Fuzeon; enfuvirtide) (Wild et al., 1994) and its recent approval by the United States Food and Drug Administration (FDA) (Cervia and Smith, 2003; Kilby et al., 1998) as a new class of anti-HIV-1 agents, termed entry inhibitors, resulted in renewed hope that the initial steps of HIV-1 infection, that is, fusion and entry, can be effectively blocked. Furthermore, this drug is effective in HIV-1-infected people who are resistant to other existing anti-HIV-1 drugs. Therefore, identification of compounds that inhibit the early steps of HIV-1 infection has drawn considerable attention lately. HIV-1 infection is mediated by attachment of the virus surface envelope glycoprotein gp120 to the CD4 receptor and subsequently to chemokine receptors, CXCR4 or CCR5 (Brelot and Alizon, 2001; Moore et al., 1997; Sodroski, 1999; Stein and Engleman, 1991). CCR5 belongs to the family of G-protein coupled receptors (GPCR) and was discovered as the major chemokine receptor for the macrophage tropic strains (M-tropic) of HIV-1 based on the observation that the β-chemokines, MIP-1α, MIP-1β, and RANTES inhibit infection of CD4+ cells by nonsyncytium-inducing (NSI) strains of HIV-1 (Cocchi et al., 1995). CCR5 is also the primary coreceptor for HIV-1 infection through sexual transmission (D’Ubaldo et al., 1999; O’Brien et al., 1998). The findings that some individuals homozygous for a defective CCR5 allele with an internal 32 base pair deletion (CCR5-D32) were resistant to infection with R5 viruses and appeared to be healthy (Liu et al., 1996; Marmor et al., 2001; Samson et al., 1996) reinforced the notion that CCR5 could be a possible target for early therapeutic intervention against HIV-1 infection.
Moreover, it has been demonstrated that virus–cell fusion can be effectively inhibited by addition of CCR5-specific ligands as well as CCR5-neutralizing antibodies to cells (Cocchi et al., 1995; Simmons et al., 1997). A plethora of publications, especially from pharmaceutical companies, have emerged recently reporting the discovery of new classes of small molecule CCR5 antagonists (Baba et al., 1999; Hale et al., 2001a; Kazmierski et al., 2003; Lynch et al., 2002; Maeda et al., 2001; Strizki et al., 2001). Some of the reported data has recently been used to map possible pharmacophore sites of CCR5 antagonists (Debnath, 2003; Finke et al., 2001). The results from early clinical trials also indicated that CCR5 coreceptor can be used as a viable target for antiretroviral therapy (Pozniak et al., 2003; Reynes et al., 2002).

Several methods to identify CCR5 antagonists have been reported (Chiba et al., 2001; Holland et al., 2004; Jenkinson et al., 2003a, 2003b; Nussbaum et al., 1994; Spenlehauer et al., 2001). The conventional CCR5 antagonist binding assay uses inhibition of chemokine binding and requires a radio-labeled chemokine, such as [I^{125}]RANTES or [I^{125}]MIP-1α or [I^{125}]MIP-1β as a competition agent (Baba et al., 1999; Hale et al., 2001b; Strizki et al., 2001). Recently, Jenkinson et al. (2003b), who developed a baculovirus based assay, pointed out that the labeled chemokine-based approach has several limitations: (1) missing inhibitors that do not bind directly to the chemokine binding site in CCR5 but may bind to other sites of the coreceptor responsible for gp120-CCR5 binding. It has been reported that at least one of the sites for gp120 binding on CCR5 is distinct from the chemokine binding site (Dragic et al., 1998); (2) failure to identify inhibitors that target the CCR5 binding site on gp120; (3) inhibitors of the gp120-CCR5 interaction due to allosteric interference may be missed; and (4) the radio-labeled ligands, in large scale use, may pose health risk to the laboratory personnel, especially to pregnant woman. Therefore, it is prudent to develop a simple assay system that can overcome all the above problems.

The interaction between gp120 and CD4 induces conformational changes in the envelope glycoprotein gp120 resulting in exposure of epitopes necessary for the subsequent binding of gp120 to the chemokine receptor CCR5 (Wu et al., 1996). These epitopes are recognized by two human monoclonal antibodies (mAbs), 17b and 48d (Thali et al., 1993). Characterization of the 17b binding site was provided from an X-ray crystal structure of the gp120 core complexed with a two-domain fragment of human CD4 and the Fab fragment of 17b (Kwong et al., 1998, 2000; Rizzuto et al., 1998; Wyatt et al., 1998). Binding of these mAbs blocked the interaction of gp120–CD4 complexes with CCR5 (Cocchi et al., 1995), suggesting that gp120 attachment to CD4 creates or exposes a high-affinity binding site for CCR5 that is involved in membrane fusion and virus entry.

The potential utility of the gp120–CD4 complex in subunit vaccine design has been reported by several groups (Devico et al., 1996; Fouts et al., 2000; He et al., 2003). Recently, a full-length single chain (FLSC) analogue of the gp120–CD4 complex, consisting of HIV-1 BaL gp120 joined to the D1D2 domains of CD4 by a 20-amino-acid linker, has been described (Fouts et al., 2000). This protein...
replicated structural, functional, and antigenic features of the gp120–CD4 complex, including increased exposure of epitopes of the coreceptor-binding site on gp120, and abolition of epitopes corresponding to the CD4 binding site on gp120 that are occluded by complex formation. Furthermore, FLSC bound specifically to the CCR5 coreceptor and blocked R5 virus infection (Fouts et al., 2000), suggesting that these single-chain chimeric molecules accurately mimicked the gp120–CD4 complex intermediate that arises during HIV-1 binding to CD4+ cells (Farber and Berger, 2002) (Fig. 1A). This further suggested the potential utility of this single-chain protein as a mimic for the gp120–CD4 complex (Dragic et al., 1998; Fouts et al., 2000). We hypothesize that an inhibitor that blocks FLSC from binding to the CCR5 chemokine receptor will function as an efficient CCR5 antagonist (Fig. 1B).

We have utilized FLSC to develop (a) a flow cytometry-based assay and (b) an ELISA-based assay. We report here the systematic development of these assays and its usefulness in identifying inhibitors that prevent the binding of gp120 to CCR5.

Results

Development of flow cytometry-based assay to identify CCR5 antagonists

We have confirmed the functional integrity of FLSC by a series of experiments. We first verified whether FLSC mimics the complex (made by mixing equal molar ratios of sCD4-183 and the BaL gp120 and designated as “complex” throughout the article) by measuring its binding to the mAb 17b, which recognizes the CD4-induced binding site on gp120. BaL gp120 alone was used as a control. mAb 17b bound to both FLSC and complex with almost identical affinity whereas BaL gp120 did not bind (Fig. 2A). We then verified whether mAb 17b could inhibit the binding of FLSC to CCR5 using flow cytometry. Because FLSC was used as the mimic for the gp120–CD4 complex, we chose CII2Th/synCCR5 cells for this purpose. These cells do not have CD4 receptors on their surface but express high level of CCR5 (Mirzabekov et al., 1999). Initially, D7324, a polyclonal sheep antibody, which recognizes the C terminal 15 amino acids of gp120, was used as antibody to detect FLSC. Although the results were reasonable, a vast improvement in sensitivity was achieved (data not shown) when we used instead a mouse antibody M-T441 that recognizes the D2 domain of CD4. Therefore, we have used phycoerythrin (PE)-conjugated anti-CD4 mAb M-T441 as detecting antibody and PE-conjugated mouse IgG2b isotype as control. Fig. 2(B) shows that mAb 17b inhibited the binding of FLSC and the complex to the CCR5 expressed cells in an almost identical dose-dependent fashion. These experiments confirmed that CD4-induced binding sites are similar in the FLSC chimeric protein and the complex, and proved that this protein could be used to design assays to identify inhibitors that prevent the interaction between gp120 and CCR5.

The same assay method used for 17b inhibition was used for identifying CCR5 antagonists. TAK-779 was used as a CCR5 antagonist (Baba et al., 1999; Este, 2001) and AMD-3100 as a CXCR4 antagonist (Bridger et al., 1999; Donzella et al., 1998) to validate the assay. Fig. 3A shows the results of flow cytometry analysis indicating that TAK-779 inhibited the interaction between both FLSC (c–e) and complex (h–j).
Fig. 3. Flow cytometry analyses of TAK-779 and AMD-3100 inhibition of binding of FLSC and complex to C12Th/synCCR5 cells. (A, a) Negative control (cells + FLSC + PE-conjugated mouse IgG2b isotype); (b) positive control (cells + FLSC + PE-conjugated M-T441); (c–e) in the presence of TAK-779 at 1000, 250, and 62.5 nM doses, respectively; (f and g) negative and positive controls, respectively, as in a and b, except that complex was used instead of FLSC; (h–j) TAK-779 inhibition of the binding of the complex to CCR5 at the same dose range as in c–e; (k–o) AMD-3100 inhibition using identical conditions as in a–e (the positive controls in b and l are from two different passage cells). (B) Dose–response plot of percent inhibition of TAK-779 calculated from MFI.
with CCR5-expressing cells in an almost identical fashion. On the contrary, AMD-3100 did not inhibit the binding between FLSC and CCR5 (m–o). The MFI was used to calculate the percent inhibition. TAK-779 inhibited the interaction (Fig. 3B) with an EC\textsubscript{50} of approximately 175 nM for both.

![Diagram](image.png)

Fig. 4. Selection of detecting antibody for the cell-based ELISA. Cf2Th/synCCR5 cells were fixed with 5% formaldehyde on a 96-well plate. The FLSC, the gp120, and the complex were added to the cells at varied concentrations (results using 1 μg/ml were shown here). The binding of FLSC was detected by sequentially adding (a) D7324, biotin-labeled goat-anti-sheep IgG, SA-HRP, and TMB, or (b) M-T441, biotin-labeled antimouse IgG, SA-HRP, and TMB. Absorbance at 450 nm was recorded in an ELISA reader. Experiments were done in triplicate.

Development of an ELISA-based assay for CCR5 antagonists

Although the flow cytometry-based assay is specific in identifying the CCR5 antagonist, TAK-779, the method requires large quantities of both FLSC and detecting antibodies. Therefore, it is not economical for high throughput screening (HTS). Based on our previous experience in developing an ELISA-based assay for identifying CXCR4 inhibitors (Zhao et al., 2003), we wanted to develop an ELISA-based assay in a micro-well plate format so that only low quantities of FLSC and antibodies would be required for the assay. Such method could be routinely run in the laboratory and would be amenable for high throughput screening. Furthermore, an ELISA-based assay does not require any sophisticated instrument as is the FACS caliber.

The flow cytometry-based assay described above confirmed that the interaction between FLSC and CCR5 can be detected using the anti-CD4 antibody M-T441 with higher sensitivity than with the antibody D7324. We wanted to confirm this finding in the ELISA-based assay before selecting M-T441 as the detecting antibody to determine the binding between FLSC and Cf2Th/synCCR5 cells. Fig. 4 shows that the mAb M-T441 was much more sensitive in detecting FLSC than was D7324. Therefore, we used M-T441 as the detecting antibody in all the subsequent experiments.

We have shown earlier that FLSC mimics the BaL gp120–CD4 complex in exposing the CD4i epitopes recognized by the antibody 17b. Fig. 4 shows that the

<table>
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\textsuperscript{a} All the compounds have <15% inhibition up to 1000 nM.
\textsuperscript{b} The antibodies and IgGs have <20% inhibition up to 15 μg/ml.
FLSC and the complex bound to CCR5 almost in similar fashion, when M-T441 was used for determination of FLSC binding, whereas BaL gp120 did not bind to CCR5. This result again confirmed that FLSC truly mimicked the complex.

The purpose of the development of this ELISA-based assay is to use it for identifying HIV-1 entry inhibitors, targeted to CCR5. To determine the optimum concentrations of both FLSC and M-T441 needed for obtaining the best possible results with minimum effect on the sensitivity, we measured the binding of FLSC to the Ci2Th/synCCR5 cells using graded concentrations of FLSC and M-T441 (15.6–2000 ng/ml). The optimum concentrations determined for FLSC and M-T441 were 125 and 250 ng/ml, respectively, and were selected for the ELISA-based assay.

To investigate the optimum fixation time of the Ci2Th/synCCR5 cells on 96-well plates, cells were fixed with 5% formaldehyde in 0.01 M phosphate-buffered saline (PBS) for 5, 20, and 30 min at room temperature. The longer fixation time did not improve the detection of the known CCR5 inhibitor (TAK-779) tested for this purpose. Therefore, in all subsequent assays, a fixation time of 5 min was used.

**Potential utility of the ELISA-based assay for identifying CCR5 antagonists**

In order to assess the validity and usefulness of this simple cell-based ELISA to identify CCR5 antagonists, we have selected several categories of possible CCR5 antagonists and tested their inhibitory activity in this assay. We selected TAK-779 (Baba et al., 1999) as the only small molecule CCR5 antagonist available from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. We also selected RANTES and its two analogs AOP-RANTES (Proudfoot et al., 1999; Simmons et al., 1997; Torre et al., 2000) and PSC-RANTES (Pastore et al., 2003), which were shown to effectively block gp120 binding to CCR5. Several monoclonal antibodies were also selected to test their potential inhibitory activity, for example, mAbs 2D7 and 3A9 were selected as anti-CCR5 antibodies (Wu et al., 1996, 1997a, 1997b). We have also selected three anti-CD4i mAbs, 17b (Kwong et al., 1998; Sullivan et al., 1998; Wyatt et al., 1998), 48d (Moore et al., 1993; Thali et al., 1993), and F425 A1g8 (Cavacini et al., 2003), and two anti-V3 loop mAbs, F425 B4a1 and F425 B4e8 (Cavacini et al., 2003). Several non-CCR5 targeted mAbs, mouse, and human IgG and small molecule entry inhibitors were chosen as negative controls. We chose b12, an anti-CD4 binding site mAb (Burton et al., 1991, 1994), and 12G5, an anti-CXCR4 mAb (Endres et al., 1996), AMD-3100 (Bridge, 1999; Donzella et al., 1998) and T22 (Murakami et al., 1999; Nakashima et al., 1992), two known CXCR4 antagonist as negative controls. A variety of other entry inhibitors reported were also selected, for example, chloropetin (Matsuzaki et al., 1994) was selected as an envelope glycoprotein gp120 inhibitor, meso-tetra (4-carboxyphenyl)-porphyrin (MTCPP) (Debnath et al., 1994; Neurath et al., 1995), a gp120 inhibitor targeted to the V3 loop, C34 (Chan et al., 1997; Lu and Kim, 1997) and T20 (Cervia and Smith, 2003; Wild et al., 1994), two known inhibitors targeted to the HIV-1 envelope glycoprotein gp41. Because some of the mAbs were mouse and human origin, respectively, we have also tested mouse IgG and human IgG as controls. Table 1 shows the corresponding results obtained in the cell-based ELISA assay. Except for the reagents used as negative controls, all other antagonists showed dose-dependent inhibition, and their corresponding EC50 values are shown in Table 1. The dose–response curve for TAK-779 is shown as an example (Fig. 5).

**Discussion**

The discovery of CCR5 and CXCR4 chemokine receptors and subsequent understanding of their importance in HIV-1 tropism and virus entry opened up an important field for AIDS research (Berger et al., 1999; Moore et al., 1997). CCR5 has emerged as the principal coreceptor because CCR5-using viruses are found exclusively during the early and the clinical latent phase of HIV-1 infection as well as during the late-stage of the disease in more than half of the infected people (Cilliers et al., 2003). Earlier studies showed that subtype C isolates almost exclusively use CCR5 as the coreceptor. HIV-1 subtype C now accounts for more than half of the new infections worldwide, especially in developing countries, such as South Africa, India, and China (Cilliers et al., 2003). These facts and the observation that some individuals who were homozygous for a defective CCR5 allele with an internal 32 base pair deletion (CCR5-Δ32) in the CCR5 gene were protected from HIV-1...
infection by R5 viruses and appeared to be healthy (Liu 
et al., 1996; Marmor et al., 2001; Samson et al., 1996) justify the use of CCR5 as target for developing a new class of anti-HIV-1 agents.

The clinical success of the peptide-based inhibitor T-20, which targets the HIV-1 envelope glycoprotein gp41 and its recent approval by the US FDA as the only entry inhibitors, so far, generated renewed hope for identifying inhibitors that target early steps of the HIV-1 life cycle, such as CCR5 antagonists. Identification of small molecule drugs against this target has been recently reviewed (Kazmierski et al., 2003). The first discovery of a CCR5 antagonist, TAK-779, was reported by Takeda Chemical Industries in Japan (Baba et al., 1999). Recently, several reports emerged on the systematic discovery of highly potent CCR5 antagonists as anti-HIV-1 agents (Castonguay et al., 2003; McCombie et al., 2003; Palani et al., 2001, 2002; Strizki et al., 2001; Tagat et al., 2001; Trkola et al., 2002; Tsamis et al., 2003; Willoughby et al., 2003). Results on phase I/II studies (Reynes et al., 2002) of one of the CCR5 antagonists, SCH-C, from Schering-Plough Research Institute were presented at the 9th Conference on Retroviruses and Opportunistic Infections (February 24–28, 2002, Seattle). In another report, presented at the 43rd Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC) (September 13–17, 2003, Chicago), an investigational CCR5 antagonist from Pfizer, UK-427857, showed encouraging results in phase I clinical trials (Pozniak et al., 2003).

High throughput screening (HTS) assays using radiolabeled 125I-RANTES or 125I-MIP-1α, natural ligands of CCR5 receptors, have been traditionally used to identify CCR5 antagonists. Although these methods were successful in identifying highly active CCR5 antagonists, they have limited utility in high throughput settings due to the reasons described in the introduction.

Although many studies focused on the interaction of CCR5 with HIV-1, the specific interaction sites in CCR5 and gp120 are yet not fully defined. Therefore, it was prudent to develop an assay that reflects the process of gp120 interaction with CCR5 during HIV-1 infection without targeting any specific sites on CCR5 or gp120. Toward this goal, we have utilized the C2Th/synCCR5 cell line and FLSC to set up a simple model to study the interaction between gp120 and CCR5. Because C2Th/synCCR5 cells do not express CD4, gp120 does not bind to these cells. This was demonstrated by both FAC5 analysis and cell-based ELISA. Furthermore, FLSC mimics the complex formed by gp120 and sCD4-183 as demonstrated by its binding to the antibody 17b and the inhibition of the binding of FLSC to CCR5. In our initial attempt to develop the assays, we used a flow cytometry-based readout technique. This method was able to successfully identify TAK-779 as an antagonist at a low nanomolar dose level, whereas AMD-3100, a CXCR4 antagonist, had no inhibitory activity at a 100 μM level (data not shown). This method, although simple, is not convenient for large scale use, especially in high throughput settings. Therefore, we developed an ELISA-based method using FLSC and the cell line C2Th/synCCR5. Because the cells were fixed in the cell-based assay, a mild fixing reagent (5% formaldehyde) and a short fixing time were used to preserve the conformational integrity of CCR5. After systematic optimization of the experimental conditions, the ELISA-based method was used to measure the inhibitory activity of several antagonists (Table 1). They can exert their effect on gp120 binding to CCR5 directly or through allosteric modulation (Rees et al., 2002) or bind to the CD4i epitopes or to the V3 loop of gp120 and thereby prevent the binding of gp120–CD4 complexes (FLSC or complex) to CCR5. We have used TAK-779 as the only available small molecule CCR5 antagonist that directly binds to CCR5. The chemokine analogs AOP-RANTES and PSC-RANTES, two very potent CCR5 antagonists, were shown to exert their inhibitory activity through induction of CCR5 receptor internalization and prevention of recycling of CCR5 to the cell surface (Mack et al., 1998). The binding of FLSC to CCR5-expressing cells could be effectively blocked at low nanomolar doses by the CCR5 antagonist TAK-779 (EC_{50} = 5.12 nM) and by RANTES analogs in a dose-dependent manner. RANTES by itself, though active, showed less activity compared with TAK-779 and the RANTES analogs. The apparent lower sensitivity of the flow cytometry-based method in detecting TAK-779 (EC_{50} = 175 nM) compared to the above result in the cell-based ELISA could probably be attributed to the fact that in the flow cytometry-based assay, FLSC concentrations needed for detection were much higher (2 μg/ml) compared with FLSC concentration in the cell-based ELISA (0.125 μg/ml). Therefore, a higher concentration of TAK-779 was required to compete with FLSC.

Two anti-CCR5 mAbs, 2D7 and 3A9, respectively, inhibited the binding. The inhibitory potency of 2D7 was much better than that of 3A9, in agreement with published reports (Wu et al., 1997a, 1997b). Because the method measures the inhibition of FLSC binding to CCR5, we also selected three mAbs that recognize CD4i epitopes and two mAbs targeted to the V3 loop of gp120. All these mAbs showed potent inhibition. We tested meso-tetra(4-carboxyphenyl) porphyrin (MTCPP), shown to bind to the V3 loop of gp120 (Debnath et al., 1994; Neurath et al., 1995). MTCPP also had inhibitory activity (Table 1). Because the CCR5 binding site on gp120 also involves the V3 loop (Cormier and Dragic, 2002), it is expected that this method will be able to detect such inhibitors. We have used two small molecule inhibitors AMD-3100 and T-22, which are known to target CXCR4, as controls. Two mAbs, such as b12, an anti-CD4 binding site antibody, and 12G5, an anti-CXCR4 antibody, and both normal mouse IgG2a and human IgG, were also used as controls. None of these reagents showed any appreciable activity up to the dose levels tested (Table 1). The data presented in this study validate the cell-based ELISA assay and confirm that the assay is simple and capable of detecting inhibitors targeted
to a variety of sites on CCR5 or gp120 and the CD4i epitopes, which prevent the binding of FLSC to the CCR5.

Although CD4 and gp120 could have been used to perform the same assay, their cost for routine use is prohibitive. Dragic et al. (2000) used a similar method as described above but they have used a complex of gp120 and CD4-IgG2 to determine the inhibitory effect of TAK-779. The method was sensitive but CD4-IgG2 is a proprietary complex from Progenics (Terrytown, NY) and off limit to most researchers. Therefore, this newly developed cell-based assay is simple, rapid, economical, and sensitive, and the method has the potential to be adapted in high throughput settings. This assay does not require the use of any infectious agents; therefore, no special biosafety measures are needed in the laboratory for the experiments.

Materials and methods

Reagents

The plasmids expressing recombinant FLSC (pEF6-FLSC) and Bal gp120 (pBal gp120) were provided by Dr. Abraham Pinter at the Public Health Research Institute with the permission from Dr. Anthony L. DeVico (Fouts et al., 2000). Stable cell lines expressing recombinant proteins were established by transfecting plasmids into 293T cells using FuGene 6 (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer’s protocol. Soluble FLSC and Bal gp120 were purified from cell culture medium by lectin chromatography with Galanthus nivalis snowdrop agglutinin (Sigma-Aldrich, St. Louis, MO) as described (Gilljam, 1993).

Peptides T22, T20, and C34 were synthesized by a standard solid-phase FMOC method in the MicroChemistry Laboratory of the New York Blood Center. Chloroepetin was a generous gift from Dr. Satoshi Oumara of Kitasato University, Japan. MTCPP was purchased from Porphyrin Products (Logan, UT). AOP-RANTES and PSC-RANTES were generously provided by Drs. Robin Offord and Oliver Hartley, Université de Genève, Switzerland.

The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: Cf2Th/synCCR5 cell line from Drs. Tajib Mirzabekov and Joseph Sodroski; sCD4-183, the first two domains of the soluble CD4, from Garlick RL et al.; mAbs against gp120 CD4 binding site (IgG1 b12 from Drs. Dennis Burton and Paul Parren), against CD4i sites (17b and 48d from Dr. James E. Robinson, and F425 A1g8 from Drs. Marshall Posner and Lisa Cavacini), against V3 loop (F425 B4a1 and F425 B4e8 from Dr. Marshall Posner and Dr. Lisa Cavacini), and against CCR5 (2D7 and 3A9 from Millenium Pharmaceuticals, Inc. and PharMingen); TAK-779 from Takeda Chemical Industries; and AMD-3100 from AnorMED. M-T441, biotinyl-M-T441, and PE-conjugated M-T441 which recognize the domain two region of CD4 and the isotype control (IgG2b) and PE-conjugated mouse IgG2b was purchased from Ancell (Bayport, MN). D7324, a polyclonal sheep antibody raised against a peptide derived from the C-terminal 15 amino acids of gp120, was purchased from Cliniqa (Fallbrook, CA). Anti-sheep IgG and anti-mouse IgG (both biotin-labeled) were from Zymed (S. San Francisco, CA) and Chemicon (Temecula, CA), respectively. RANTES was purchased from BioSource International (Camarillo, CA). Recombinant soluble CD4 (sCD4) was obtained from Immunodiagnostics Inc. (Woburn, MA). The complex was formed by mixing the equal molar concentrations of sCD4-183 and the gp120 (Bal). mAb 12G5 against CXCR4, mouse IgG2a, and human IgG were purchased from R&D systems (Minneapolis, MN) and Zymed, respectively.

Flow cytometry analysis

Cf2Th/synCCR5 cells (1 × 10^6) were incubated for 15 min at room temperature in Hanks’ balanced salt solution (HBSS) (Sigma) with FLSC at the final concentration of 2 µg/ml in the presence or absence of the compounds tested. After thorough washing, the binding of FLSC to CCR5 was detected by PE-conjugated M-T441. PE-conjugated IgG2b isotype was used as control. Both these PE-conjugated Abs at 5 µg/ml were added and incubated at room temperature for 15 min. After extensive washing, cells were resuspended and analyzed in a Becton Dickinson FACSCalibur flow cytometer (Mountain View, CA) using CellQuest software. Dead cells were excluded by propidium iodide staining. Live cells were gated on their forward and side light scatter characteristics and the MFI was measured.

When D7324 was used as the detecting antibody, normal sheep IgG was used as control. A concentration of 10 µg/ml each was used. The cells were analyzed using FITC-conjugated anti-sheep IgG (1:200) as the second read-out antibody following the method described above.

Cell-based ELISA

Cf2Th/synCCR5 cells in DMEM medium supplemented with 10% FBS, 1% penicillin/streptomycin, 500 µg/ml G418, 500 µg/ml zeocyn, and 3 µg/ml puromycin were seeded into 96-well plates and cultured into a monolayer of 90% confluency at 37 °C overnight. Cells were fixed with 5% formaldehyde in 0.01 M phosphate-buffered saline (PBS) for 5 min at room temperature. The plates were washed three times with PBS containing 0.05% Tween 20 (PBST) and blocked with 5% nonfat dry milk in 0.01 M PBS (pH 7.2) at 4 °C overnight. The FLSC, the gp120, or the complex was added at graded concentrations (0.0625–4 µg/ml) and incubated at 37 °C for 1 h. Detecting Abs and their corresponding control Abs were added, respectively, as previously described in the Flow cytometry analysis section. After incubation at 37 °C for 1 h, the unbound antibodies were removed by washing plates three times with PBST. Then 100 µl of biotin-labeled second Abs (anti-sheep IgG
and anti-mouse IgG, respectively) were added, followed by incubation at 37 °C for 1 h. After extensive washes, 100 μl of streptavidin-labeled horseradish peroxidase (SA-HRP) (0.125 μg/ml) was added and incubated at 37 °C for 1 h. Subsequently, the substrate 3,3′,5,5′-tetramethyldiethylene diamine (TMB) was added. After 5 min at room temperature, reactions were terminated by addition of 1 N H2SO4. Absorbance at 450 nm (A450) was recorded in an ELISA reader (Dynatech Laboratories, Inc., Chantilly, VA). Each sample was tested in triplicate.

For measuring the inhibitory activity of anti-HIV-1 agents on the binding of FLSC to the CCR5-expressing cells, the test compounds were added to the fixed CF2Th/synCCR5 cells before addition of the FLSC. Binding of FLSC to the CCR5-expressing cells was measured as described above (in case of mouse antibodies, e.g., 2D7, 3A9, etc., biotinyl-M-T441 was used as detecting antibody), and the inhibitory activity of the compounds on FLSC binding was calculated using the following formula: [1 − (E − N)/(P − N)] × 100%. N represents the absorbance at 450 nm in the negative control (the control Ab), while P corresponds to that in positive control (detecting Ab). E corresponds to the absorbance in experimental group where the test compounds were added before addition of FLSC. EC50 (the concentration for 50% inhibition) values were calculated using the GraphPad Prism software (GraphPad Software Inc., San Diego, CA).

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References


