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ScienceDirect
Antiviral Research 74 (2007) 125–137
Antiviral Research
www.elsevier.com/locate/antiviral

Novel CCR5 monoclonal antibodies with potent and broad-spectrum anti-HIV activities

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Received 18 July 2006; accepted 8 November 2006

Abstract

To identify monoclonal antibodies (mAbs) with high potency and novel recognition sites, more than 25,000 of mouse hybridomas were screened and 4 novel anti-human CCR5 mAbs ROAb12, ROAb13, ROAb14, and ROAb18 showing potent activity in cell–cell fusion (CCF) assay were identified. These mAbs demonstrated potent antiviral activities in both single-cycle HIV infection (IC_{50} range: 0.16–4.3 μg/ml) and PBMC viral replication (IC_{50} range: 0.02–0.04 μg/ml) assays. These potent antiviral effects were donor-independent. All 4 mAbs were also highly potent in the PhenoSense assay against 29 HIV isolates covering clade A through G. In all antiviral assays, these mAbs showed potency superior to the previously reported mAb 2D7 in side-by-side comparison studies. All 4 mAbs were also fully active against viruses resistant to HIV fusion inhibitor enfuvirtide and CCR5 antagonist maraviroc. Although ROAb12, ROAb14, and ROAb18 inhibited RANTES, MIP1α and MIP1β binding and cell activation, the other novel mAb ROAb13 was inactive in inhibiting cell activation by these three ligands. Furthermore, highly synergistic antiviral effects were found between mAb ROAb13 and 2D7 or ROAb12. In addition, none of these mAbs showed agonist activity or caused internalization of the CCR5 receptor.

Keywords: HIV; CCR5; Monoclonal antibody; Entry inhibitor; Synergism

1. Introduction

Human immunodeficiency virus (HIV) enters the host cell via the interaction of the viral envelope (Env) protein gp160 and host cell membrane proteins. Synthesized as a single polypeptide precursor, Env is subsequently cleaved by a cellular protease to generate two non-covalently associated subunits, gp120 and gp41. Gp120 binds to the cell surface, whereas the membrane-spanning gp41 subunit mediates membrane fusion. The primary receptor for HIV-1 is CD4, however, a second receptor termed coreceptor is required for efficient viral entry into host cells (James et al., 1996; Lapham et al., 1996; Trkola et al., 1996). Binding of gp120 to CD4 results in multiple conformational changes in gp120, mediating the interaction with the co-receptor. Binding of gp120 to coreceptor triggers structural changes in gp41, leading to virus-host cell fusion. HIV gp41 can be partitioned into extracellular, transmembrane, and cytoplasmic domains. The extracellular portion of gp41 contains an amino-terminal hydrophobic fusion peptide and two heptad repeats (HR). Upon coreceptor triggering, the fusion peptide of gp41 is exposed and inserted into the cell membrane to form a bridge between the virus and the cell. The C-terminal HR (HR2) domains then pack into the hydrophobic grooves between the 3 N-terminal HR (HR1) domains on the triple-stranded coiled-coil, thus forming a six-helix bundle (Chan et al., 1997; Weissenhorn et al., 1997). This helical condensation moves the viral membrane in close proximity to the cell membrane and results in the formation of a fusion pore and entry of the viral core to the host cytoplasm.

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doi:10.1016/j.antiviral.2006.11.003

Highly active anti-retroviral therapy (HAART) using generally three or more anti-HIV agents from different classes in combination have become the standard treatment regimen for HIV-infected patients. This treatment strategy has greatly improved the effectiveness of infection control and the survival of AIDS patients (Barbaro et al., 2005). However, the emergence of drug resistance has resulted in the failure of treatments in large numbers of infected patients, it thus necessitates the development of new classes of anti-HIV drugs. One of the most promising steps in the viral life cycle for therapeutic intervention is the viral entry process (Barbaro et al., 2005; Borkow and Lapidot, 2005; Lazzarin, 2005; Pierson et al., 2004). Host and viral molecules involved in various stages of HIV entry have been explored as novel targets for the discovery of anti-HIV agents. Enfuvirtide (ENF) is the first FDA-approved HIV entry inhibitor in the market. ENF is a HR2-derived peptide inhibitor that targets at the trimeric coiled-coil formed by HR1s, whereby preventing the formation of six-helix bundle (Lazzarin, 2005).

Recently, the HIV coreceptor CCR5 has been intensively studied as a novel drug target (Lalezari et al., 2005; Maeda et al., 2004; Watson et al., 2005; Wood and Armour, 2005). The CC-chemokine receptor CCR5 is the major coreceptor for HIV and plays a pivotal role in HIV transmission and pathogenesis. Viral tropism is defined by the choice of coreceptor used during the entry process. The majority of primary HIV-1 strains use CCR5 as coreceptor (termed R5 virus), whereas some viruses are able to use another chemokine receptor, CXCR4, as coreceptor (termed X4 virus) or use both CCR5 and CXCR4 as coreceptors (termed R5X4 virus) (Coakley et al., 2005; Eckert and Kim, 2001). Almost all primary infections are caused by R5 viruses. Although R5/X4 and/or X4 HIV viruses appear at later stages of viral infection in many patients, often accompanying dramatic decline of $CD4^+$ cells and rapid progression of AIDS; the R5 viruses still persist in these patients. A significant contribution to the understanding of the important role of CCR5 in HIV infection is the observation that genetically CCR5-deficient (Δ32) individuals are essentially protected against infection by HIV-1 in high risk populations (Liu et al., 1996; Samson et al., 1996); and heterozygous Δ32 individuals are not protected against HIV-1 infection but are often long-term non-progressors (Eugen-Olsen et al., 1997). Because of its role as the predominant coreceptor of the clinical HIV populations, and the normal physiology within the human genetic knockout population, CCR5 has become a very attractive target for anti-HIV therapy. A number of small-molecule CCR5 antagonists have been identified that demonstrated potent antiviral effects both in cell culture and in clinical trials (Lalezari et al., 2005; Maeda et al., 2004; Strizki et al., 2005; Watson et al., 2005; Wood and Armour, 2005). Several anti-human CCR5 monoclonal antibodies (mAbs) have also been discovered for therapeutic use and are in preclinical or clinical trials (Trkola et al., 2001).

CCR5 belongs to the family A of G protein-coupled receptors (GPCR) with characteristic seven-transmembrane (7-TM) domains (Alkhatib et al., 1996; Deng et al., 1996; Dragic et al., 1996). CCR5 has an N-terminal exodomain and three extracellular loops. Therefore, it can offer multiple epitopes for recognition by mAbs. The binding properties, including recognition sites, affinity, and on- or off-rate, may determine the antiviral potency and resistant profiles of the mAbs. To identify mAbs with high potency and novel recognition sites, we screened more than 25,000 mouse hybridomas. Here we describe 4 novel CCR5 mAbs with potent antiviral activities.

2. Materials and methods

2.1. Reagents

All cell culture media and supplements and fetal bovine sera were purchased from Invitrogen (Carlsbad, CA). Human CCR5 monoclonal antibody 2D7 and PE- and FITC-conjugated goat anti-mouse antibodies were purchased from BD Pharmingen (San Diego, CA). CCR5 antagonist maraviroc (MVC), non-nucleoside reverse transcriptase inhibitor (NNRTI) efavirenz, and the HIV fusion inhibitor enfuvirtide (ENF) were synthesized in house.

2.2. Cell lines

For the generation of CCR5-expressing stable cell lines, human CCR5 cDNA was cloned by using polymerase chain reaction (PCR) from OriGene's TrueClone (Cat. No. TC110858, OriGene Technologies, Rockville, MD). The following primers were used in the PCR reaction: 5′-primer, 5′-ATATATTAATCT-AGAACCATGGATTATCAAGTGTCAAGTC-3′; 3′-primer, 5′-ATATATTCTAGAGCGGATCCTCACAAGCCCACAGATAT-TTC-3′. The 1.1 kb PCR product was digested with *Xba*I and *Bam*HI and cloned into mammalian expression vector pcDNA3.1 (−) (Invitrogen, Carlsbad, CA). The resulting clone pcDNA3.1-hCCR5 was sequence verified to be identical to the published human CCR5 coding sequence (Accession no. NM_000579). CCR5-expressing stable cell lines based on CHO-G16α and the pre-B lymphoma cell line L1.2 cells (both obtained from ATCC, Manassas, VA) were generated by transfection with pcDNA3.1-hCCR5 FuGene 6 (Roche Applied Science, Indianapolis, IN) transfection reagent was used for transfecting CHO-G16α cells and the CCR5-positive stable cell population (CHO-CCR5) was obtained by several rounds of FACS sorting using CCR5 mAb 2D7-PE. CHO-CCR5 cells were maintained in F12 media supplemented with 10% FBS, 50 μg/ml penicillin/streptomycin, and 0.4 mg/ml hygromycin and 0.4 mg/ml of G418. L1.2 cells were transfected with linearized pcDNA3.1-hCCR5 by electroporation. The Positive clone (L1.2-CCR5) was obtained by FACS sorting using 2D7-PE, and it was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 0.8 mg/ml G418, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μg/ml penicillin/streptomycin, and 50 μM β-mercaptoethanol. One day before the use for immunization, L1.2-hCCR5 cells were treated with 1 mM of sodium butyrate to boost the expression of CCR5.

2.2.1. Other cell lines

Packaging cell line 293FT (Invitrogen, Calsbad, CA) was maintained in Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% FBS, 0.5 mg/ml G418, 1× Pen-Strep,

and 2 mM L-glutamine. Indicator cell line JC53-BL (TZM-bl, NIH AIDS Research and Reference Reagent Program) was maintained in DMEM supplemented with 10% FBS, 1× Pen-Strep, and 2 mM L-glutamine). Cells constitutively expressing various chemokine receptors HEK293-hCCR1, CHO-G16α-hCCR2b, L1.2-hCCR3, CHO-G16α-hCCR4, and CHO-G16α-hCCR6 were obtained from in-house. Their expression has been verified by FACS analysis using appropriate antibodies. These cells were used in the binding assays. U87.CD4.CCR5 cell line that expresses human CD4 and CCR5 was obtained from the NIH AIDS Reagents Program (No. 4035).

2.3. Generation of mouse anti-human CCR5 mAbs

Female Balb/c mice were given a primary intraperitoneal immunization with 10^7 CCR5-expressing cells (CHO-CCR5 or L1.2-CCR5) with complete Freund's adjuvant. The second immunization was done 4–6 weeks later similarly except incomplete Freund's adjuvant was used with the cells. The mice were then boosted at 4–6 week intervals with 10^7 CHO-CCR5 or L1.2-CCR5 cells in phosphate-buffered saline (PBS) with no adjuvant. The last immunization was carried out intraperitoneally with 10^7 CCR5-expressing cells or intravenously with 2×10^6 CCR5-expressing cells on the third day before fusion. The spleen cells of the immunized mice were fused with myeloma cells according to Galfre and Milstein (1981). Ten days after fusion, the supernatants were tested for specific antibody production by cell-based ELISA. Hybridomas that produced the most potent supernatants in the CCR5-dependent cell–cell fusion (CCF) assay were then cloned by limiting dilution or FACS sorting. The mAbs were individually purified to >95% homogeneity by protein A chromatography. All mAbs were stored at 4 °C in PBS.

2.4. FACS analysis

CHO-CCR5 cells were washed twice in phosphate-buffered saline (PBS) containing 0.5% FBS (FACS buffer), then resuspended in FACS buffer at 4×10^6 cells/ml. For each reaction, 25 μl of cells (1×10^5) were incubated with 1 μg/ml of primary antibodies and isotype controls on ice for 30–45 min. Cells were washed three times in FACS buffer and incubated with labeled anti-mouse secondary antibodies for 30 min on ice. After three washes, the stained cells were analyzed with a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

2.5. Radioligand binding assays

^{125}I-RANTES (regulated on activation normal T cell expressed and secreted), ^{125}I-MIP-1α (macrophage inflammatory protein-1α), ^{125}I-MIP-1β, ^{125}I-MIP-3α, ^{125}I-MCP-1 (monocyte chemotactic protein), ^{125}I-eotaxin, ^{125}I-TARC (thymus and activation-regulated chemokine), and ^{125}I-SDF-1 (stromal cell-derived factor-1) were purchased from PerkinElmer Life Sciences Inc. (Shelton, CT). Binding assays were performed on whole cells expressing various chemokine receptors with ^{125}I-labeled corresponding ligands: RANTES, MIP-1α, and MIP-1β for CCR5; SDF-1 for CXCR4; MIP1α for CCR1; MCP-1 for CCR2b, eotaxin for CCR3; TARC for CCR4; MIP-3α for CCR6. Cells were plated in 96-well culture plates at 1.5×10^5 cells/well in ice cold binding buffer (phenol red-free F12 medium supplemented with freshly made 0.1% BSA and 0.1% NaN_3). Serially diluted CCR5 mAbs were added to the cells, followed by addition of 100 pM of the corresponding ^{125}I-labeled ligands. After 2 h of incubation at room temperature with gentle shaking, cells were harvested onto GF/C UniFilter plates (PerkinElmer Life Sciences Inc.) using cell harvester. UniFilter plates were pretreated with 0.3% PEI/0.2% BSA for 30 min prior to harvest. Filter plates were washed five times with 25 mM pH 7.1 HEPES buffer containing 500 mM NaCl, 1 mM $CaCl_2$ and 5 mM $MgCl_2$. Plates were dried in 70 °C oven for 20 min and 40 μl scintillation fluid was added and radioactivity was measured using TopCount NXT (PerkinElmer). In all experiments, each data point was assayed in duplicate. Data are presented as the percentage of counts obtained in absence of cold competing ligand. Curve fitting and subsequent data analysis were carried out using GraphPad PRIZM software (Intuitive Software for Science, San Diego, CA) and IC_{50} values were calculated using non-linear regression analysis.

2.6. CCR5-mediated intracellular Ca^{2+} flux

Assessment of antibody function on CCR5 receptors was accomplished using a fluorometric imaging plate reader assay (FLIPR). Briefly, 24 h prior to the assay, CHO-CCR5 cells were plated in clear bottom, black-walled 96-well plates (Becton-Dickinson, Franklin Lakes, NJ, USA) at a density of 5×10^4 cells per well. On the day of screening, the cells were incubated in a buffer consisting of Hank's Balanced Salt Solution (HBSS) containing 20 mM HEPES, pH 7.4, 1.5 mM $CaCl_2$, 2.5 mM probenecid, followed by the addition of 4 μM of the calcium sensitive fluorescent dye Fluo4-AM (Invitrogen, Carlsbad, CA). After 1 h incubation at 37 °C, the cells were washed three times with the above buffer, then immediately placed in FLIPR (Molecular Devices, Sunnyvale, CA) to monitor changes in fluorescent signal at λ_{EX} of 488 nm and λ_{EM} of 540 nm, upon addition of 30 nM RANTES, MIP1α, or MIP1β (R&D Systems, Minneapolis, MN) and/or serially diluted CCR5 mAbs. Five micromolar of ionophore inomycin was used for each experiment to control for maximal Ca^{2+} release. After addition of compounds, change in fluorescence was monitored for a period of 5 min and maximal increase in fluorescent signal was recorded. Calculation of EC_{50} and IC_{50} values were determined using GraphPad Prism v3.02 (GraphPad Software, Inc.).

2.7. Antiviral assays

Cell-based surrogate antiviral assay CCF assays were performed as described before (Ji et al., 2006). Single-cycle antiviral assays were performed as follows. Pseudotyped NL-Bal viruses were produced by cotransfecting 293FT cells with pNL4-3Δenv (HIV pNL4-3 genomic construct with a deletion within the env gene) and pcDNA3.1/NL-BAL *env* [pcDNA3.1 plasmid containing NL-Bal *env* gene (obtained from Roche Welwyn)].

The supernatants containing pseudotyped viruses were stored at −80 °C in aliquots. Test antibodies were serially diluted in 96-well plates. The equivalent of 1.5×10^5 RLU of viruses and 2.5×10^4 JC53-BL cells were added to each well. After 3 day incubation at 37 °C, 50 μl of Steady-Glo Luciferase Assay System was added and the assay plates were read on a Luminometer (Luminsokan, Thermo Electron Corporation, Waltham, MA).

The PhenoSense assay was performed by Monogram Biosciences (formerly Virologic) (Petropoulos et al., 2000). The PhenoSense assay is performed similar to the single-cycle assay except multiple pseudotyped viruses that carries envelope proteins from various HIV isolates were used. U87.CD4.CCR5 cells was used as the reporter cell. For antiviral assays in peripheral blood mononuclear cells (PBMC), human PBMC were isolated from buffy-coats (obtained from the Stanford Blood Center) by a Ficoll-Paque centrifugation. PBMC were treated with 2 μg/ml Phytohemagglutinin (Invitrogen, Carlsbad, CA) for 24 h at 37 °C, then with 5 Units/ml human IL-2 (Roche Applied Sciences, Indianapolis, IN) for a minimum of 48 h prior to the assay. In a 96 well round bottom plate, 1×10^5 PBMC were infected with the HIV-1 JR-CSF (kindly provided by Dr. Irvin Chen, UCLA) in the presence of serially diluted CCR5 antibody. Plates were incubated for 6 days at 37 °C. Virus production was measured at the end of infection by using p24 ELISA (PerkinElmer) according to the manufacturer's instruction. For all antiviral assays, IC_{50} was determined using the sigmoidal dose–response model with one binding site in Microsoft XLFit.

2.8. In vitro drug interaction analysis

CCR5 mAbs were serially diluted and added to the cell–cell fusion system alone or in combinations at fixed ratio of 2:1. Percent inhibition data from 3 independent experiments were averaged and analyzed for mode of interactions by using the combination index (CI) method as described by Chou and Talalay (1984). CI analysis is a commonly used tool for characterizing drug–drug interactions, it provides qualitative information on the nature of drug interaction and the extent of drug interaction. CI was calculated according to the following equation: $CI = (C_{A,x}/IC_{x,A}) + (C_{B,x}/IC_{x,B})$, $C_{A,x}$ and $C_{B,x}$ are the concentrations of drug A and drug B used in combination to achieve x% drug effect. $IC_{x,A}$ and $IC_{x,B}$, are the concentrations for single agents to achieve the same effect. $CI < 1$ indicate synergy, $CI = 1$ indicate additive effects, and $CI > 1$ indicate antagonism.

3. Results

3.1. Identification of mouse anti-human CCR5 monoclonal antibodies

About 26,400 hybridoma lines were screened for CCR5-specific monoclonal antibody production by using cell-based ELISA and more than 300 positive lines were identified. Among which, several mAbs showed potent inhibitory effects in CCF assays. Four mAbs, ROAb13 (IgG_{2a}), ROAb18 (IgG_{2a}), ROAb12 (IgG_{2b}), and ROAb14 (IgG_{2b}) with the highest potency were selected for further characterization. The specific binding of these mAbs to CCR5 were confirmed by FACS analysis. As shown in Fig. 1, no binding of mAbs to the parental CHO cells were observed by comparing to the isotype control (green). All four mAbs ROAb12, ROAb13, ROAb14, and ROAb18 showed potent binding to the CHO-CCR5 cells (red). These data indicate that the binding of these mAbs were specific to CCR5.

To further demonstrate the specificity of these CCR5 mAbs, the mAbs were examined in both CCR5-mediated and CXCR4-mediated CCF assays. All CCR5 mAbs, including the control antibody 2D7, inhibited CCR5-dependent cell–cell fusion (Fig. 2). ROAb12, ROAb14, and ROAb18, exhibited higher potency (IC_{50} range: 0.2–0.51 μg/ml) than 2D7 (IC_{50} = 0.73 μg/ml) in the CCR5-dependent CCF assays. While ROAb13 (IC_{50} = 2.1 μg/ml) exhibited weaker potency than 2D7 in this assay (Table 1). None of these CCR5 mAbs showed any inhibitory effects in the CXCR4-dependent CCF assays (Fig. 2B). CCR5 is one of the CC chemokine receptors that function as chemotactic signaling molecules in response to chemokines. There are significant sequence similarities among CC chemokine receptor CCR1, CCR2, CCR3, CCR4, CCR5, and CCR6. CCR5 shares the highest amino acid sequence identity with CCR2 (63.1%). To investigate if these CCR5 mAbs bind to other CC chemokine receptors, a selectivity study was carried out. CXC chemokine receptor CXCR4, although low in identity to CCR5 (28%), is the other main coreceptor for HIV, therefore was also included in the selectivity study for the CCR5 mAbs. As shown in Fig. 2C and D, CCR5 mAb ROAb13 and ROAb14 inhibited the binding of RANTES to CCR5; however, they did not inhibit the binding of the corresponding ligands to other chemokine receptors. It is noteworthy that the inhibition by ROAb13 only reached ∼80% while the inhibition by ROAb14 reached ∼97%. CCR5 mAbs ROAb12, ROAb18, and 2D7 showed similar results to ROAb14 (data not shown). In addition, MAb ROAb14 was also screened for binding to 74 receptors/ion channels included in a high-throughput pharmacology panel (CEREP, Celle L'Evescault, France) and no significant binding of the novel mAbs to any tested receptors/channels was found at 10 μg/ml concentrations (data not shown). These data demonstrated that the 4 novel CCR5 mAbs are highly CCR5 specific.

3.2. Potent antiviral activities of CCR5 mAbs

The 4 CCR5 mAbs that showed potent inhibitory effects in CCR5-dependent CCF assays were tested in single-cycle antiviral assays using pseudotyped HIV viruses (Table 1). ROAb12, ROAb14, and ROAb18 potently inhibited HIV NL-Bal infection with an IC_{50} range of 0.16–0.18 μg/ml and IC_{90} range of 1.8–2.73 μg/ml, about 10-fold more potent than mAb 2D7 (IC_{50} = 1.7 μg/ml). ROAb13 showed about 3-fold lower potency than 2D7 in the antiviral assay (IC_{50} = 4.33 μg/ml). The IC_{90}/IC_{50} ratios for these mAbs are in the range of 8–10.5 in the CCF assay and 7.3–16 in the antiviral assay, with an exception for ROAb13 whose 90% inhibition level could not be achieved at the highest concentration tested (20 μg/ml). In general, the antiviral potency for all tested CCR5 mAbs determined in the

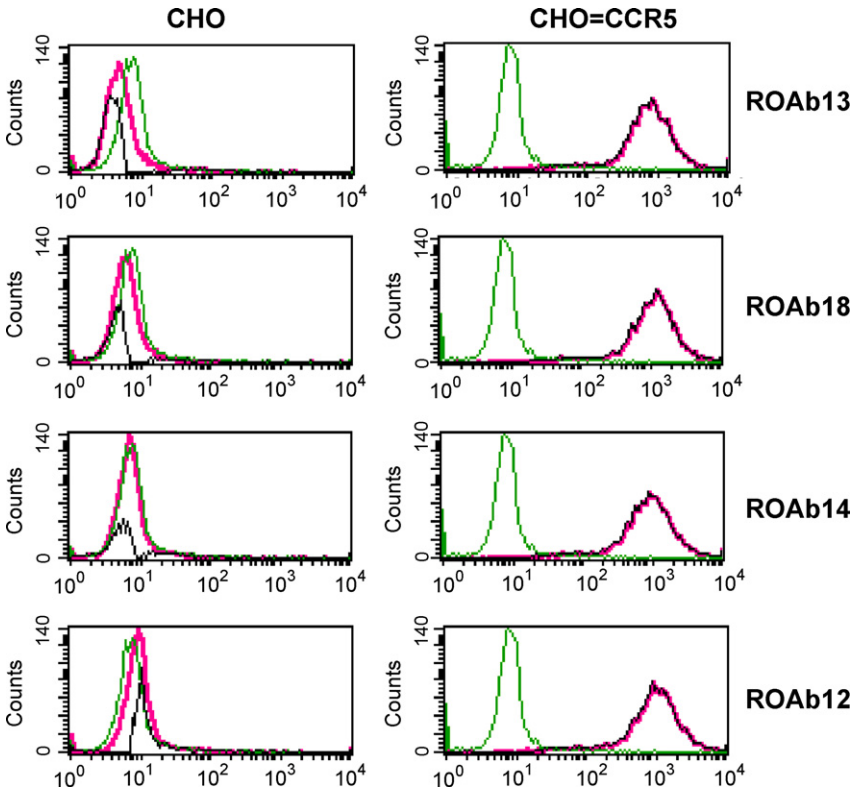


Fig. 1. Specific binding of CCR5 mAbs by FACS analysis. Histograms for each cell and primary mAb are shown. The fluorescence curves for the isotype control, CCR5 mAbs, and the isotype-subtracted CCR5 mAbs are shown in green, red, and black, respectively.

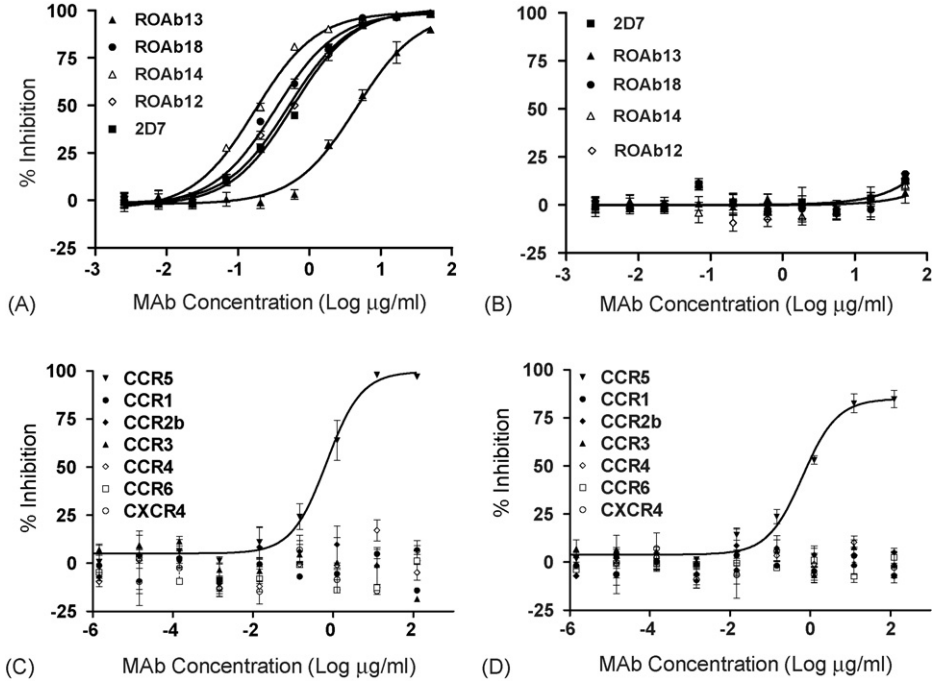


Fig. 2. CCR5-dependent antiviral effects of CCR5 antibodies. The inhibitory effects of CCR5 antibodies were determined in the CCR5-dependent (A) and CXCR4-dependent (B) CCF assays. The inhibitory effects of ROAb14 (C) and ROAb13 (D) on chemokine binding to CCR1 through CCR6 and CXCR4 were examined in ^{125}I-labeled chemokine ligand binding assays as described in Section 2. RANTES was used in the CCR5 binding assay.

Table 1
Potent anti-HIV entry activities of the CCR5 mAbs

CCR5 mAb	CCF assay			Single-cycle antiviral			PBMC antiviral		
	IC_{50}	IC_{90}	IC_{90}/IC_{50}	IC_{50}	IC_{90}	IC_{90}/IC_{50}	IC_{50}	IC_{90}	IC_{90}/IC_{50}
Maraviroc	0.96 ± 0.24	8.82 ± 1.62	9.2	1.12 ± 0.23	10.43 ± 2.68	9.3	0.18 ± 0.07	1.71 ± 0.57	9.5
2D7	0.73 ± 0.18	5.87 ± 0.93	8.0	1.70 ± 0.57	12.40 ± 4.95	7.3	0.34 ± 0.075	19.40 ± 13.4	56.7
ROAb13	2.10 ± 0.37	28.7	13.7	4.33 ± 1.7	>20	–	0.03 ± 0.032	3.16 ± 1.7	96.0
ROAb18	0.33 ± 0.13	3.12 ± 0.89	9.45	0.17 ± 0.03	2.73 ± 0.74	16.1	0.02 ± 0.009	1.21 ± 0.74	60.5
ROAb14	0.20 ± 0.07	2.11 ± 0.46	10.5	0.16 ± 0.04	1.80 ± 0.36	11.3	0.03 ± 0.017	1.15 ± 0.35	44.2
ROAb12	0.51 ± 0.14	4.66 ± 1.12	9.1	0.18 ± 0.04	2.10 ± 0.47	11.7	0.04 ± 0.031	1.92 ± 1.5	43.6

Data are from 3 or more independent experiments; IC_{50} and IC_{90} units are μg/ml for the antibodies and are nM for maraviroc.

CCF assays are similar to those determined in the antiviral assays (Table 1). These CCR5 mAbs were also tested in antiviral assays using PBMC cultures and the HIV-1 isolate JR-CSF. Much more potent antiviral activities were observed in the PBMC antiviral assays than in the CCF or single-cycle assays. All 4 CCR5 mAbs identified in this study markedly inhibited JR-CSF replication in PBMC with an IC_{50} range of 0.02–0.04 μg/ml and an IC_{90} range of 1.15–3.16 μg/ml. The IC_{50} and IC_{90} for the control mAb 2D7 (0.34 and 19.4 μg/ml, respectively) were again about 10-fold higher than these novel CCR5 mAbs. Interestingly, mAb ROAb13 showed similar antiviral potency to the other 3 novel CCR5 mAbs in the PBMC antiviral assays.

Although the IC50 values for these mAbs determined in the PBMC antiviral assays were about 5–7-fold lower than that determined in CCF or single-cycle assays, the IC_{90}s were similar among all assays, except for ROAb13 (Table 1). A CCR5 antagonist, maraviroc, was used as control in these assays, and similar potency was observed for maraviroc among these three assays. In addition, none of these CCR5 mAbs showed any cytotoxicity at concentrations up to 20 μg/ml to the cells used in the CCF, single-cycle, and PBMC assays (data not shown).

3.3. Donor-independent antiviral activities of CCR5 mAb ROAb14

HIV infection and replication efficiency within an infected individual is dependent on many factors, including but not limited to the virus fitness, tropism, virulence, host cell populations and immune response. Accordingly, responses of HIV-infected individuals to CCR5 inhibitors also may vary from person to person. PBMCs are the primary host cells for HIV in circulation. In order to determine if the CCR5 mAbs are effective against HIV infection in different patients, inhibitory effects of mAb ROAb13 and ROAb14 were determined in antiviral assays using PBMCs from 6 different donors. As shown in Table 2, potent antiviral activities of ROAb13 and ROAb14 were observed in antiviral assays using PBMCs from any of the 6 donors, with small variation in IC_{50}s and IC_{90}s among these PBMC sources. The small-molecule antagonist control maraviroc also showed tight IC_{50} and IC_{90} ranges (Table 2). The mean IC_{50} and IC_{90} from the 6 PBMC sources were very similar to the averaged IC_{50} and IC_{90} values generated from pooled PBMCs (Table 1). Again, ROAb13 showed similar potency to ROAb14 in all PBMC antiviral assays. Because CCR5 expression level may affect the antiviral responses of PBMCs, CCR5 expression on PBMCs from each donor was measured by FACS analysis using antibody 2D7. There was small variation in CCR5 expression levels among these activated PBMC samples from the 6 donors (MFI range: 16.7–60.9). Furthermore, no correlation between CCR5 expression and susceptibility to CCR5 mAb inhibition was found. These results suggest that CCR5 mAb ROAb13 and ROAb14 are highly potent in inhibiting HIV replication in PBMCs derived from multiple donors.

3.4. Broad-spectrum antiviral activities of CCR5 mAbs

As described above, potent antiviral activities were observed for the novel CCR5 mAbs in 3 different assays against 3 different subtype B R5 HIV-1 strains: 92US715 (used in CCF assay), NL-Bal (single-cycle antiviral assay), and JR-CSF (viral replication in PBMCs). To determine whether these mAbs are active against other common HIV-1 subtypes (clades), a panel of 30 HIV-1 isolates representing all common subtypes was tested in the PhenoSense assays for the antiviral activities of the mAbs. Two of the isolates were clade B dual-tropic (R5/X4) viruses, and the rest are R5 viruses. As shown in Table 3, all 4 CCR5 mAbs were active against all HIV isolates. Antibody ROAb12, ROAb14, and ROAb18 showed the most potent antiviral activities against all isolates, with a median IC_{90} of 3.4, 1.4, and 1.3 μg/ml, respectively. MAb ROAb13 showed about 5-fold less potent antiviral activities (median IC_{90} = 9.7 μg/ml) than the other 3 mAbs. In addition, some variations in IC_{50} and IC_{90} values for the mAbs

Table 2
Antiviral effects of mAbs in PBMCs from multiple donors

PBMC source	Maraviroc		ROAb13		ROAb14		$CCR5^+$
	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}	MFI
Donor P499	0.37	4.63	0.013	0.831	0.014	1.011	44.5
Donor P500	0.49	5.11	0.026	2.796	0.038	1.065	36.6
Donor P503	0.52	5.66	0.012	1.182	0.011	0.869	31.5
Donor Y974	0.43	4.49	0.018	3.532	0.009	0.674	16.7
Donor Y975	0.67	6.95	0.104	4.004	0.052	1.746	26.3
Donor Y976	0.54	5.82	0.034	5.159	0.021	1.372	60.9
GeoMean	0.49	5.38	0.025	2.42	0.020	1.07	33.3
S.D.	0.10	0.9	0.035	1.7	0.017	0.38	15.4

JR-CSF virus was used in the PBMC antiviral assays; IC_{50} and IC_{90} units are μg/ml for antibodies and are nM for maraviroc.

Table 3
Broad and potent antiviral activities of CCR5 mAbs

HIV virus		IC_{50} (μg/ml)				IC_{90} μg/ml)			
Subtype	No.	ROAb12	ROAb13	ROAb14	ROAb18	ROAb12	ROAb13	ROAb14	ROAb18
R5/X4	2	0.036	0.063	0.042	0.039	0.241	0.521	0.272	0.232
Subtype A	3	0.055	0.080	0.074	0.065	0.458	1.386	0.467	0.407
Subtype AE	3	0.065	0.144	0.060	0.072	0.762	0.660	0.630	0.589
Subtype B	5	0.200	2.922	0.175	0.163	7.496	>30	2.938	2.549
Subtype C	3	0.076	0.251	0.080	0.068	0.895	8.3	0.555	0.543
Subtype D	3	0.122	0.723	0.113	0.101	3.090	25.9	1.545	1.374
Subtype F	3	0.151	0.942	0.152	0.128	5.277	18.8	2.432	2.283
Subtype G	3	0.239	0.271	0.159	0.141	8.738	12.3	2.589	2.462
Median (range)		0.118 (0.04–0.24)	0.674 (0.06–2.92)	0.107 (0.04–0.18)	0.097 (0.04–0.17)	3.37 (0.24–8.8)	9.69 (0.52–>30)	1.43 (0.27–2.9)	1.31 (0.23–2.55)

Data were generated in the PhenoSense assay using an indicator cell line that expresses CCR5 but not CXCR4. This explains the full inhibitory activity of the CCR5 mAbs on the two dual tropic (R5/X4) viruses.

among different HIV subtypes were also observed. Due to small number of isolates from each subtype (5 for subtype B and 3 for other subtypes), the differences may not be significant. Furthermore, all mAbs are active against the 2 R5/X4 subtype B viruses. This is because the reporter cell used in the PhenoSense assays expresses coreceptor CCR5 but not CXCR4, so that the infection of these R5/X4 viruses is CCR5-dependent.

3.5. Effects of CCR5 mAbs on chemokine binding and function

These mAbs were also tested for their ability to inhibit the binding of the three natural ligands RANTES, MIP1α, and MIP1β to CCR5. As shown in Table 4, all 4 novel mAbs inhibited the ligand binding with an IC_{50} range of 0.6–4 μg/ml. Similar levels of binding inhibition were observed for the 3 ligands, although the potency of ROAb12 was about 2-fold higher than the other 3 mAbs. The commercial mAb 2D7 showed greater inhibition than the 4 novel mAbs, with an IC_{50} range of 0.16–0.57 μg/ml. Although ROAb13 inhibited ligand binding to CCR5, the inhibition only reach 77–82%. In contrast, mAb ROAb14 inhibited ligand binding by 95–100%. All other mAbs also showed greater than 95% inhibition of ligand binding (data not shown). Similarly, the effects of these CCR5 mAbs on ligand-triggered calcium flux (FLIPR assay) were also examined. Dose responses of the 3 ligands were performed (Fig. 3A), and 100 ng/ml concentrations of each of the ligands was used in all FLIPR assays. MAbs ROAb12, ROAb14, ROAb18, and 2D7 showed similar level of inhibition on calcium flux triggered by the three ligands. MAb ROAb13 only exhibited partial inhibition (∼40% inhibition at the highest tested concentration of 20 μg/ml) in RANTES FLIPR, and it was inactive in MIP1α, and MIP1β FLIPR assays (Table 4 and Fig. 3B–D).

3.6. The CCR5 mAbs are fully active against viruses resistant to other HIV entry inhibitors

In the PhenoSense assays, CCR5 mAbs were also tested against 4 ENF-resistant subtype B isolates showing an average of 140-fold resistance to the fusion inhibitor ENF. The CCR5 mAbs ROAb12, ROAb13, ROAb14, and ROAb18 were very potent on these ENF-resistant viruses, with an average IC_{50} of 0.1, 0.46, 0.12, and 0.1 μg/ml, respectively (Table 5). These values are identical to the mean IC_{50}s (0.12, 0.67, 0.11, and 0.1 μg/ml, respectively) of these mAbs against all wild-type viruses tested in the PhenoSense assays (Table 3). These CCR5 mAbs were also assayed for their potency in inhibiting a virus resistant to CCR5 antagonist Maraviroc (MVC, formerly named UK427,857). This virus (CC1/85_MVCres) was obtained by passaging the CC1/85 virus in PBMCs in the presence of increasing concentrations of MVC. Sequence analysis revealed that CC1/85-MVCres carries the two mutations A316T or I323V (numbering from HxB2) in V3 loop that had been reported to be responsible for the resistance of CC1/85 virus to MVC (Mosley et al., Abstract #598, 13th CROI, February 5–8, Denver, CO). CC1/85-MVCres was no longer responsive to MVC at up to 20 μM concentrations, whereas the parental virus or the no-drug control passaging CC1/85 virus CC1/85_NDC was

Table 4
MAb effects on CCR5 ligand binding and function

Assays	CCR5 mAb IC_{50} (μg/ml)				
	ROAb13	ROAb18	ROAb14	ROAb12	2D7
RANTES binding	2.6 ± 0.12	1.6 ± 0.1	1.4 ± 0.11	0.7 ± 0.08	0.57 ± 0.13
MIP1α binding	2.4 ± 0.16	1.5 ± 0.15	1.2 ± 0.17	0.6 ± 0.09	0.16 ± 0.06
MIP1β binding	4 ± 0.13	1.9 ± 0.13	1.9 ± 0.12	0.9 ± 0.12	0.2 ± 0.14
RANTES FLIPR	Partial inhibition	2.1 ± 0.22	2.4 ± 0.42	1.8 ± 0.33	2.5 ± 0.31
MIP1α FLIPR	Inactive	2.6 ± 0.31	4.5 ± 0.47	2.2 ± 0.38	2.9 ± 0.37
MIP1β FLIPR	Inactive	1.5 ± 0.14	1.7 ± 0.28	1.1 ± 0.13	1.6 ± 0.2

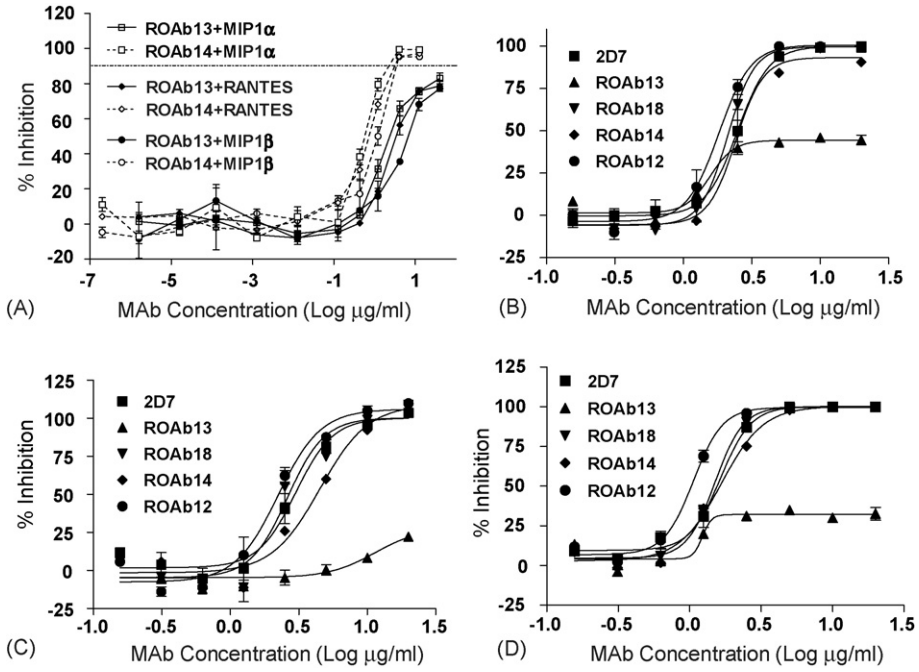


Fig. 3. Effects of CCR5 mAbs on binding and cell activation by CCR5 natural ligands. (A) Inhibition of RANTES, MIP1α, and MIP1β binding to CHO-CCR5 cells by mAbs ROAb13 and ROAb14. (B–D) CHO-CCR5 cells were incubated with 30 nM of RANTES (B), MIP1α (C), and MIP1β (D), and various doses of mAb ROAb13 and ROAb14, calcium flux was measures by FLIPR.

Table 5
Novel mAbs are fully active against fusion inhibitor-resistant viruses

Virus	ENF IC_{50} (μM)	Resistance (fold)	IC_{50} (μg/ml) for CCR5 mAbs			
			ROAb12	ROAb13	ROAb14	ROAb18
04-153817	19.2	165	0.079	0.276	0.083	0.077
04-153818	2.2	28	0.094	0.316	0.107	0.112
04-153820	27.7	305	0.099	0.295	0.114	0.079
04-153822	5.5	61	0.133	0.964	0.163	0.141
Mean		140	0.101	0.463	0.117	0.102

The IC_{50} values for all mAbs were from 2 or more independent PhenoSense assays. ENF-resistant viruses are listed as Monogram Biosciences accession numbers.

sensitive to MVC at concentrations less than 1 nM, suggesting a resistance of greater than 20,000-fold. However, the two representative novel mAbs ROAb13 and ROAb14 showed identical antiviral potency against CC1/85_MVCres and CC1/85_NDC at both 50% and 90% inhibition levels. Furthermore, ROAb13 showed identical potency to ROAb14 against both control and MVC-resistant CC1/85 viruses. NNRTI efavirenz was used as a control, and it was almost equally potent against CC1/85_NDC and CC1/85_MVCres viruses. CC1/85 has been used for resistance studies for many CCR5 inhibitors because it is one of the lease sensitive viruses to CCR5 inhibitors. By comparing the PBMC antiviral IC_{50} data from Table 6 and Table 1, ROAb13, ROAb14, and 2D7 are all relatively less potent against CC1/85, with an IC_{50} shift of 3×, 11×, and 23×, respectively.

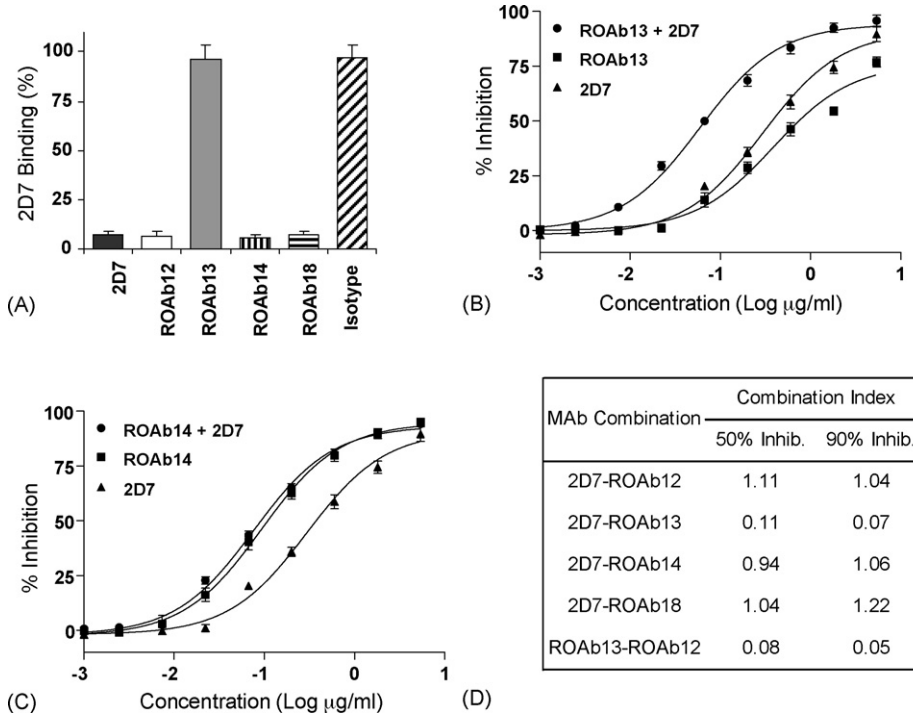
3.7. Combinational antiviral effects of CCR5 mAbs

The four extracellular domains of CCR5 may provide multiple epitopes. MAbs recognizing different sites on CCR5 may act synergistically in inhibiting viral entry. The combinational effects of each of the 4 novel mAbs with 2D7 were tested in the CCF assays at a ratio of 2:1. As shown in Fig. 4B, markedly increased potency of 2D7-ROAb13 combination in

Table 6
CCR5 mAbs are active against MVC-resistant virus

Inhibitor	CC1/85_NDC		CC1/85_MVCres	
	IC_{50} (μg/ml)	IC_{90} (μg/ml)	IC_{50} (μg/ml)	IC_{90} (μg/ml)
Efavirenz	0.07 nM	0.75 nM	0.13 nM	1.18 nM
Maraviroc	0.5 nM	4.3 nM	>20 μM	>20 μM
ROAb13	0.09	2.65	0.28	1.75
ROAb14	0.33	2.66	0.31	1.82
2D7	7.83	>20	3.47	>10

HIV virus CC1/85 was passaged in PBMCs in the absence or presence of increasing concentrations of CCR5 antagonist Maraviroc (MVC). The no drug control virus CC1/85_NDC and the MVR-resistant virus CC1/85_MVRres at passage 14 were tested for their responses to efavirenz, MVC, and CCR5 mAbs 2D7, ROAb13, and ROAb14 in PBMC antiviral assays as described in Section 2. The IC_{50} and IC_{90} values from 2 or more independent experiments were summarized in the table.



MAb Combination	Combination Index	
	50% Inhib.	90% Inhib.
2D7-ROAb12	1.11	1.04
2D7-ROAb13	0.11	0.07
2D7-ROAb14	0.94	1.06
2D7-ROAb18	1.04	1.22
ROAb13-ROAb12	0.08	0.05

Fig. 4. Inhibition of cell–cell fusion by CCR5 mAbs in combination. (A) Binding competition of novel CCR5 mAbs with 2D7. CHO-CCR5 cells were pre-incubated with 10 μg/ml of 2D7, ROAb12, ROAb13, ROAb14, ROAb18 or mouse IgG isotype control on ice for 45 min, followed by the addition of 5 μg/ml of labeled 2D7. After further incubation for 30 min, cells were subject to FACS analysis. Mean fluorescence intensity (MFI) for each treatment was normalized against isotype control group (set as 100%) and shown as bars. (B) and (C) are dose response curves of CCR5 mAbs in combination or alone in CCF assays. All mAbs are dosed from 2 ng/ml to 5.4 μg/ml. Results are mean values from 3 independent experiments. The ratio for all mAb combinations is 2:1 (ROAb12:2D7, ROAb13:2D7, ROAb14:2D7, ROAb18:2D7, and ROAb13:ROAb12). The combination indexes (CI) for all combinations are shown in (D).

comparison with 2D7 and ROAb13 alone at every dose points was observed (dose–response curve shift). However, when 2D7 and ROAb14 were combined, no significant dose response curve shift was found (Fig. 4C). The combination index (CI) for 2D7-ROAb13 at 50 and 90% inhibition levels were calculated as 0.11 and 0.07, respectively; and the CI for 2D7-ROAb14 at 50 and 90% inhibition levels were calculated as 0.94 and 1.06, respectively. A CI smaller than 1 indicates synergism; a CI equals 1 indicates additive effect; and a CI greater than 1 indicates antagonism. Therefore, a very strong synergy was found between 2D7 and ROAb13, but only additive effect was observed between 2D7 and ROAb14. Similarly, ROAb12 and ROAb18 also showed additive effects in combination with 2D7. However, when ROAb13 and ROAb12 were dosed together, a strong synergistic effect was also found (Fig. 4D). In order to understand the mechanism of these interactions, a binding competition experiment was performed. As shown in Fig. 4A, ROAb12, ROAb14, ROAb18, and 2D7 itself competed equally well with labeled 2D7 for binding to CCR5. In contrast, ROAb13 did not compete with 2D7 for CCR5 binding. These results suggest that ROAb13 binds to different epitopes from the other mAbs, and the other 3 novel mAbs and 2D7 may recognize similar regions of CCR5. This explains why ROAb13 is synergistic with 2D7 but not the other 3 mAbs. This was also confirmed by the finding that ROAb13 was also highly synergistic with ROAb12 in inhibiting CCR5-mediated cell–cell fusion (Fig. 4D).

3.8. CCR5 mAbs do not possess agonist activity

Anti-CCR5 mAbs are large molecules that bind to large surface areas of CCR5 with their binding regions overlapping with the binding sites of the natural ligands. It has been shown that CCR5 mAb MC-6 induces signaling without promoting receptor internalization (Blanpain et al., 2002). Therefore, it is necessary to determine if these mAbs possess agonist activity. CHO-CCR5 cells that express G16α protein for efficient signal pathway coupling were incubated with various amounts of CCR5 mAbs or MIP1α and calcium flux was measured by FLIPR. Calcium ionophore ionomycin was used as the maximum calcium flux control. As shown in Fig. 5A, MIP1α induced calcium flux almost to the level of that by ionomycin in a dose-dependent manner. None of the CCR5 mAbs induced calcium influx in CHO-CCR5 cells, suggesting these mAbs do not activate CCR5. Since activation of CCR5 also results in the activation of a variety of signal pathways including MAP kinases (Ottonello et al., 2005; Rodriguez-Frade et al., 1999; Wong and Fish, 1998), to verify the above finding the MAb-treated cells were also analyzed for CCR5-mediated MAP kinase activation. Although both RANTES and MIP1α were shown to be able to activate MAP kinases in CCR5-expressing cells, it appears that they use different routes. MIP1α activates MAP kinases through CCR5, but RANTES activates MAP kinases independent of CCR5, through interaction with proteoglycans on the cell surface (Chang et al., 2002; Roscic-Mrkic et al., 2003).

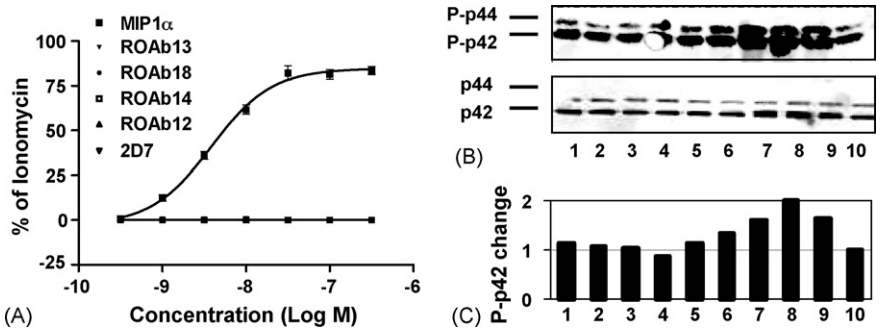


Fig. 5. CCR5 antibodies exhibited no agonist activity. (A) FLIPR assay using CHO-CCR5 cells treated with 100 nM MIP1α, or 20 μg/ml CCR5 mAbs. (B) CHO-CCR5 cells were treated with vehicle, CCR5 mAbs (20 μg/ml), or MIP1α (100 nM). Whole cell extracts were subject to PAGE and the unphosphorylated and phosphorylated MAP kinases p42 and p44 were detected by western blots. (C) Western blots were scanned and the densities of the phosphorylated p42 (P-p42) from various treatment were divided by the density from vehicle control to reflect fold changes in P-p42 upon treatment. In both Fig. 3B and C, lanes 1–3 represent ROAb14 treatment for 7, 15, and 30 min, respectively. Lanes 4–6 represent ROAb12 treatment for 7, 15, and 30 min, respectively. Lanes 7–9 represent MIP1α treatment for 7, 15, and 30 min, respectively. Lane 10 represents vehicle treatment for 30 min.

Therefore, MIP1α instead of RANTES was used as the positive control in the MAP kinase activation studies. The results showed that MIP1α induced MAP kinase p42 and p44 activation. The phosphorylated p42 increased 1.6-fold at 7 min post-exposure to MIP1α, and peaked at 15 min (2-fold). However, no increase in phosphorylated p42 or p44 kinases was observed when CHO-CCR5 cells were exposed to CCR5 mAbs ROAb14 or ROAb12 for various time periods (Fig. 5B and C). These results further confirmed that these mAbs do not possess CCR5 agonist activity.

3.9. CCR5 mAbs do not internalize the receptor

CCR5 is activated by chemokine ligands RANTES, MIP1α, and MIP1β, and the activated receptor can undergo internalization. It has been reported that the mouse anti-hCCR5 mAbs were able to down-regulate cell surface CCR5 (Barassi et al., 2005; Blanpain et al., 2002). To assess whether these novel CCR5 mAbs trigger internalization of the receptor, various CCR5 mAbs were incubated with CHO-CCR5 cells and cell surface receptors were monitored by FACS. CCR5 ligand RANTES was used in this study as a positive control. As shown in Fig. 6, about 40% of cell surface CCR5 was internalized after 2 h incubation of the cells with 100 nM of RANTES at 37 °C. However, under the same condition, none of the CCR5 mAbs internalized the receptors at concentrations as high as 10 μg/ml. The reason for the incomplete internalization of CCR5 by saturating amount of RANTES is unclear. It may be explained by the lack of sufficient cell proteins or machinery to internalize high levels of cell surface CCR5. Because the down-regulation of CCR5 by certain antibodies occurred only at 48 h post-exposure to anti-CCR5 antibodies (Barassi et al., 2005), the effect of CCR5 mAbs on cell surface CCR5 was monitored up to 48 h. No down-regulation of CCR5 after incubation of CHO-CCR5 cells with various CCR5 mAbs for 1, 24, or 48 h (data not shown) could be observed, suggesting that none of the novel CCR5 mAbs trigger the internalization of CCR5 even after extended exposure.

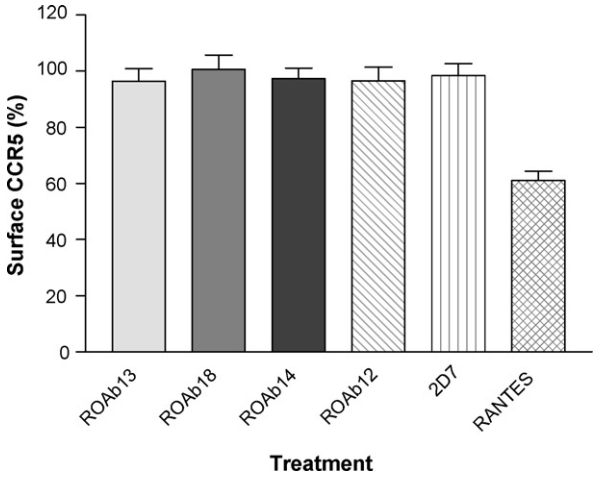


Fig. 6. CCR5 antibodies do not trigger CCR5 internalization. CHO-CCR5 cells were pre-incubated with PE-labeled CCR5 antibodies or 100 nM RANTES at 37 °C for 2 h, then PE-labeled 2D7 was added to RANTES-treated cells and all cells were further incubated on ice for another 30 min before FACS analysis. The mean fluorescence intensity (MFI) values from the standard FACS group were divided by those from the 37 °C pre-incubated groups and the results were represented as percent of CCR5 receptors remained on the surface.

4. Discussion

Because CCR5 exists in multiple conformation states and its exodomains are highly flexible, mAbs recognizing many different conformational epitopes can be generated. Published evidence suggests that mAbs binding to different epitopes showed differential inhibitory effects on ligands binding and viral infection (Atchison et al., 1996; Olson et al., 1999). Although many anti-human CCR5 mAbs have been reported, very few of them exhibited potent antiviral activities. Two of the most potent antibodies, PRO 140 and mAb004, are in phase I clinical trial. Another potent CCR5 mAb 2D7 is in preclinical development. In the present study, we described 4 novel mouse anti-human CCR5 mAbs that showed broad and potent antiviral activities. By comparison to PRO 140 and mAb004, the novel mAb ROAb14 exhibited equivalent or better antiviral activities and clade coverage. ROAb14 showed a very small IC_{90} range of 0.27–2.94 μg/ml (median IC_{90} = 1.4 μg/ml) against 29 HIV isolates from all common clades. This spectrum of activity is superior to the IC_{90} ranges of mAb004 (0.11–189 μg/ml) (Roschke et al., Abstract #H-213,

44th ICAAC, October 30–November 2, Washington, DC) and PRO 140 (0.54–16 μg/ml) (Trkola et al., 2001).

CCR5 is the receptor for chemokines RANTES, MIP1α, and MIP1β, it constitutes one of the important components of the complex immune response network against invading pathogens. Although homozygous CCR5Δ32 individuals appear physiologically normal, studies using CCR5-knockout (KO) mice suggested that CCR5 deficiency exacerbates host infection by a variety of pathogens, including influenza A virus, herpes simplex virus type 2, bacterium Chlamydia trachomatis, West Nile, and non-cytolytic lymphocytic choriomeningitis virus (Ank et al., 2005; Barr et al., 2005; Dawson et al., 2000; de Lemos et al., 2005; Glass et al., 2005). The impaired activation and trafficking of natural killer cells and $CD8^+$ cells to the sites of infection in these CCR5-KO mice may be responsible for the reduced immune response against these pathogens. Recently, a link between CCR5 deficiency and increased West Nile virus infection was also found (Glass et al., 2006). Therefore, CCR5 inhibitors with differential antiviral and anti-cell activation activities may be preferable as new anti-HIV drugs. MAb ROAb13 only partially inhibited (40% maximal) RANTES-triggered calcium flux, and it was inactive against cell activation by MIP1α and MIP1β (Fig. 3). Moreover, our unpublished data suggest that ROAb13 is inactive against chemotaxis mediated by all three CCR5 ligands at concentrations up to 10 μg/ml. Although ROAb13 is weak or inactive in inhibiting ligand functions, it does inhibit ligand binding to CCR5. The reason for this discrepancy is not completely clear. One possible explanation is the incomplete inhibition of ligand binding by ROAb13 (Fig. 2D). It may only need a few ligand–receptor interactions per cell to trigger cell signaling and activation. ROAb13 inhibited ligand binding only up to 82% at the highest tested concentrations, while all other tested mAbs demonstrated greater than 95% inhibition.

It was observed that all 5 CCR5 mAbs are 5–14-fold more potent (IC_{50}) in inhibiting viral replication in PMBCs than in inhibiting single-cycle HIV-cell fusion or gp160/CCR5-mediated cell–cell fusion, although similar activities were observed for all mAbs in the CCF assay and single-cycle assay (Table 1). The reason for this is not completely clear. One possible explanation is that the PBMCs express very low levels of CCR5 and the indicator cells used in the CCF or single-cycle assays are engineered to overexpress CCR5. Although enriched distribution of CCR5 and CD4 molecules in the lipid rafts on cell surface may facilitate fusion event, very low expression of surface CCR5 is believed to slow down virus-cell fusion kinetics (Platt et al., 2005). It has been shown that HIV-cell fusion kinetics is associated with the expression level of cell surface CCR5 (Platt et al., 2005; Reeves et al., 2002). Moreover, HIV infection in cells expressing CCR5 at basal level is more susceptible to entry inhibitors TAK-779 and ENF (Platt et al., 2005). This observation could help explain the low IC_{50} values of CCR5 mAbs in the PBMC antiviral assays. The other difference between the PBMC antiviral assay and single-cycle antiviral assay or CCF assay is that multiple rounds of HIV infection occur in the PBMC antiviral assay while a single round of infection/fusion happens in the other assays. It is likely that further reduction of viral production happens after each additional round of infection cycle in the presence of CCR5 inhibitors. The virus–cell ratios used in the single-cycle and PBMC antiviral assays may not be the same, this could also be an potential cause of the IC_{50} differences in these assays. Since PBMCs are isolated from human donors, the antiviral data obtained from PBMC assay system is likely a better indicator of the true in vivo drug potency than the assays utilizing engineered indicator cells.

Drug resistance is common to all known antiviral agents, and it is one of the main attributes to the failure of drug response in treated patients. Combination of 3 or more antiviral drugs with different mechanism of actions may partly overcome this problem. HIV viruses resistant to one class of drugs may still be sensitive to other classes of drugs. Drug resistance has been seen for the first HIV entry inhibitor ENF, and all small-molecule CCR5 antagonists in clinical trials. In the current study, we demonstrated that all novel CCR5 mAbs are not only fully active against viruses resistant to the fusion inhibitor ENF but also fully active against HIV viruses resistant to the CCR5 antagonist MVC. Resistance to ENF is mainly caused by mutations in gp41 especially HR1 region that interacts with ENF. Because it is the surface envelope protein gp120 that interact with CCR5, this explains the full susceptibility of ENF-resistant viruses to CCR5 mAbs. MVC resistance is most likely due to mutations in gp120 primarily the V3 loop that directly makes contact with the coreceptor CCR5. Since CCR5 mAbs bind to large areas on the surface of CCR5 and they may induce conformational changes different from those induced by the deeply bound CCR5 antagonists, MVC-resistant viruses may be unable to use mAb-bound CCR5 although they can still use MVC-bound CCR5 efficiently. These results suggest that CCR5 mAbs are potentially effective in HIV-infected patients pre-exposed to other anti-HIV drugs including entry inhibitors and small-molecule CCR5 inhibitors.

Although mAbs have several disadvantages as drugs, such as inconvenience of i.v. administration, potential allergic reactions and the development of neutralizing anti-antibodies, they do offer several advantages over other types of drugs. MAbs are highly target-specific, thus minimizing non-target associated side effects or toxicity; mAbs have very long plasma half-lives, allowing for biweekly or even monthly dosing; mAbs use different route of administration, metabolism, and clearance, thus are unlikely to interfere with the small-molecule drugs. In addition, almost all small molecule CCR5 antagonists showed potent inhibitory effects on CCR5 natural ligand binding and functions, while it is possible to identify CCR5 mAbs with differential antiviral and anti-chemotaxis activities. Furthermore, because all antagonists compete for binding to the same pocket, no favorable combinations between two antagonists are expected. However, mAbs recognizing different epitopes on CCR5 may not interfere with each other's binding thus may exert synergistic antiviral activities as demonstrated in the current study and as reported before (Lee et al., 1999).

In summary, 4 novel mouse anti-human CCR5 mAbs are described. All of them demonstrated potent antiviral activities against lab-adapted HIV-1 strains and clinical isolates representing all common clades. These mAbs are highly potent in PBMCs from multiple donors. All mAbs are active against viruses resis-

tant to other entry inhibitors including small-molecule CCR5 antagonists. Furthermore, strong synergistic antiviral effects were observed between some of the mAbs.

Acknowledgements

The authors would like to acknowledge Dr. Chris J. Petropoulos and Monogram Biosciences for providing the PhenoSense antiviral data used in this article. We also would like to thank Drs. John C. Kappes and Xiaoyun Wu and Tranzyme Inc. for providing JC53-BL cells through the NIH AIDS Research Reagents and Reference Program, and Dr. Irvin Chen for providing the JR-CSF virus.

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