*Brief Report*

# Establishment of a new cell line for high-throughput evaluation of chemokine CCR5 receptor antagonists

**Lili Ji[1], Yuchen Sheng[2], Li Chen[3,*], Zhengtao Wang[1,*]**

[1] *Key Laboratory of Standardization of Chinese Medicines of Ministry of Education, Shanghai Key Laboratory of Complex Prescription, Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine, Shanghai, China;*
[2] *The Department of Pharmacology, Shanghai Institute of Pharmaceutical Industry, Shanghai, China;*
[3] *Shanghai Target Drug Pharmaceutical Co.,Ltd., Shanghai, China.*

**ABSTRACT: Chemokine receptors belong to a superfamily of proteins that signal through coupled heterotrimeric G proteins. Chemokine receptor CCR5 is the major co-receptor essential for HIV entry into host cells, and now chemokine CCR5 receptor has become an important target in searching for anti-HIV drugs. Here, we describe the establishment of a human embryonic kidney (HEK) 293/CCR5-HA cell line stably expressing CCR5 receptor with influenza hemagglutinin (HA) tag at the N termini on the membrane surface of HEK293 cells. Plasmid pcDNA3.0-CCR5-HA was transfected into HEK293 cells, and monoclonal HEK293 cell lines expressing CCR5 receptor were generated under G418 selection. The expression of functional CCR5 receptor was tested by GTPγS assay, and the results showed about 5 monoclonal HEK293 cell lines expressed functional CCR5 receptor, and of which No.7 monoclonal cell line is the best. The FACS analysis results further confirmed that CCR5 receptor was positive in 96.89% of No.7 monoclonal HEK293/CCR5-HA cell line. Further results showed that RANTES significantly stimulated GTPγS binding in the dose-dependent manner, and CCR5 antagonist Sch-351125 inhibited RANTES-stimulated GTPγS binding in the dose-dependent manner in No.7 monoclonal HEK293/CCR5-HA cell line. Our results suggest that the established HEK293/CCR5-HA cell line is suitable for high-throughput screening and is feasible to identify CCR5 receptor antagonists.**

***Keywords:*** Chemokine, HEK293 cell, CCR5 receptor, Drug screening

******To whom correspondence should be addressed:*
Dr. Zhengtao Wang, Key Laboratory of Standardization of Chinese Medicines of Ministry of Education, Shanghai Key Laboratory of Complex Prescription, Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine, 1200 Cai Lun Road, Shanghai 201203, China;
e-mail: wangzht@hotmail.com
Dr. Li Chen, Shanghai Target Drug Pharmaceutical Co.,Ltd., Shanghai 200233, China;
e-mail: chenli8888@hotmail.com

## 1. Introduction

The incidence of acquired immune deficiency syndrome (AIDS) continues to increase in the world, and about 60 million people are estimated to have AIDS. In China, there are nearly 1 million people infected with human immunodeficiency virus (HIV), and this disease has been spreading aggressively ever since. An asymptomatic period precedes AIDS in which the immune system becomes progressively compromised and unable to fight opportunistic infections and certain cancers (*1*). The primary cell surface receptor for HIV infection is the CD4 glycoprotein, but only CD4 is not sufficient for the infection of HIV. It is now clearly established that chemokine receptors can act in concert with CD4 to enable the entry of HIV and simian immunodeficiency virus (SIV) into target cells (*2-5*).

Chemokines, a family of structurally related low molecular mass cytokines, are crucial to the development of lymphoid tissues and the migration of leukocytes (*6*). Chemokines are distinguished based on the relative position of conserved residues into four subfamilies, designated CC, CXC, C and CX3C chemokines, and chemokines exert their biological effects by binding to chemokine receptors, which belong to the seven-transmembrane G protein coupled receptor family (*7*).

Chemokine CCR5 receptor has recently sparked substantial interests because of its role as the co-receptor for the infection of M-tropic HIV strains (*8*). HIV binds to cellular membrane CD4 through

its envelope protein gp120. This binding induces a conformational change in gp120, and then exposes the binding sites of CCR5 receptor. This process leads to the formation of a hairpin configuration with the conformational change of another HIV protein gp41, and subsequently leads to the membrane fusion (*9-11*). One of the approaches to the blockade of this process is through interference with the binding to CCR5 receptor, therefore, its ligands such as regulated on activation normal T-cell expressed and secreted (RANTES), macrophage inflammatory protein (MIP)-1α, and MIP-1β are able to block infection of M-tropic HIV strains (*12*). Several $NH_2$-terminal modifications of RANTES have also been described to block the infection by HIV-1 such as Met-RANTES, aminooxypentane (AOP)-RANTES, *etc* (*13,14*). Recently the small molecule CCR5 receptor antagonists have been proved to be useful in blockade of the infection of HIV-1. There are many companies and institutes apply themselves to developing and searching the CCR5 receptor antagonists, and they get some progress in this field such as TAK-779 and its derivates, WO200066551 and its derivates, a serial of Sch-C derivates, *etc* (*15-17*). Therefore, CCR5 receptor antagonists, the next therapeutic class for treatment of HIV infection (*18*), have the potential to significantly alter the landscape of HIV treatment by providing a new class of drug that targets a host cellular receptor rather than a viral enzyme.

The present study describes the establishment of a stable HEK293/CCR5-HA cell line, which would be an ideal system for high-throughput screening of CCR5 receptor antagonist.

## 2. Materials and Methods

### 2.1. *Cells, chemicals, and plasmids*

HEK293 cells were obtained from the American Type Culture Collection (ATCC) and maintained in MEM supplemented with 10% fetal bovine serum (FBS). Recombinant human RANTES was from BD Biosciences Pharmingen, San Jose, CA, USA. Mouse mAb 12CA5 against HA epitope was obtained from Boehringer Mannheim, and FITC-conjugated goat anti-mouse IgG was from Tago, Burlingame, CA, USA. A plasmid for expressing CCR5-HA fusion protein (pcDNA3-CCR5-HA) was kindly provided by Prof. Gang Pei, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, China (*19,20*). Sch-351125 was gifts from Prof. Dawei Ma, Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences, China. Lipofectamine™ 2000 was from Invitrogen, Carlsbad, CA, USA. [$^{35}$S]GTPγS was from GE Healthcare Bio-Sciences, Piscataway, NJ, USA, and all other reagents unless indicated were from Sigma-Aldrich, St Louis, MO, USA.

### 2.2. *Transfection and cell line establishment*

HEK293 cells were transfected with the recombinant plasmid pcDNA3-CCR5-HA using Lipofectamine™ 2000 according to the manufacture's instructions. Forty-eight hours post transfection transfected HEK293 cells were trypsinized and persistently cultured in MEM medium containing 0.5 mg/mL G418 for 2-3 weeks. Every resistant "cell island" was trypsinized and seeded to the 96-well plate by the limiting dilution in MEM medium containing G418 to generate signal cell clones. The G418 resistant monoclonal cell clones that grew well were selected for further analysis.

### 2.3. *[$^{35}$S]GTPγS binding assay*

The assay was carried out as described (*20*). Cells were lysed in 5 mM Tris-HCl, pH 7.5, 5 mM EDTA, 5 mM EGTA at 4°C. The membrane pellet resulted from a 30,000 × g centrifugation was resuspended, and aliquots containing 10 μg protein were incubated at 30°C for 1 h in 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 mM $MgCl_2$, 100 mM NaCl, 40 μM GDP, 0.5 nM [$^{35}$S]GTPγS (1,200 Ci/mmol) in the presence or absence of the agonists in a total volume of 100 μL. The reaction was terminated by adding cold PBS and filtered through GF/C filters, which were counted in a liquid scintillation spectrophotometer. Data were means of duplicate samples. Basal binding was determined in the absence of agonists, and nonspecific binding was obtained in the presence of 10 μM GTPγS. The percentage of stimulated [$^{35}$S]GTPγS binding was calculated as $100 \times (cpm_{sample} - cpm_{nonspecific}) / (cpm_{basal} - cpm_{nonspecific})$.

### 2.4. *Flow Cytometry*

Cells were incubated with 5 μg/mL anti-HA antibody in PBS containing 2% BSA at 4°C for 1 h. The presence of CCR5 chemokine receptors on the cell surface was detected by incubation with FITC-conjugated goat anti-mouse IgG. The cells were analyzed on a FACSCalibur flow cytometer. Basal cell fluorescence intensity was determined with cells stained with the secondary antibody alone.

### 2.5. *Statistical analysis*

Data were presented as mean ± standard deviation and analyzed by the Student's *t* test using SigmaPlot 8.0. $P < 0.05$ was considered statistically significant.

## 3. Results and Discussion

### 3.1. *Establishment of stable cell lines expressing CCR5-HA fusion protein*

The application of stable and reliable *in vitro* cell model

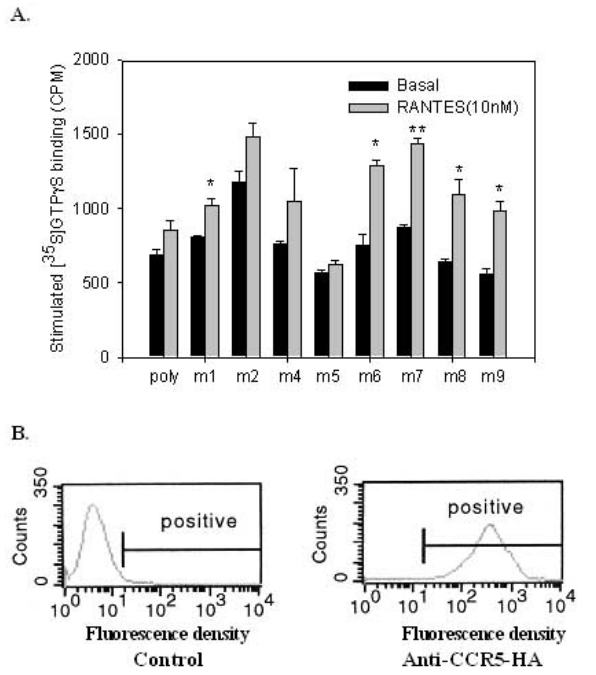


**Figure 1. Establishment of the monoclonal HEK293/CCR5-HA cell line.** (A) RANTES (10 nM) stimulated GTPγS binding in the non-selected polyclonal (poly) and selected monoclonal cell lines (m1, m2, m4, m5, m6, m7, m8, m9). Data were mean ± SD performed in duplicate. * $p < 0.05$, ** $p < 0.01$ compared with absence of RANTES. (B) FACS analysis of the expression of CCR5-HA on the membrane surface of No.7 monoclonal cell line. Cells were incubated with anti-HA antibody first, and then stained with FITC-conjugated secondary antibody and detected with a FACS scan analyzer.

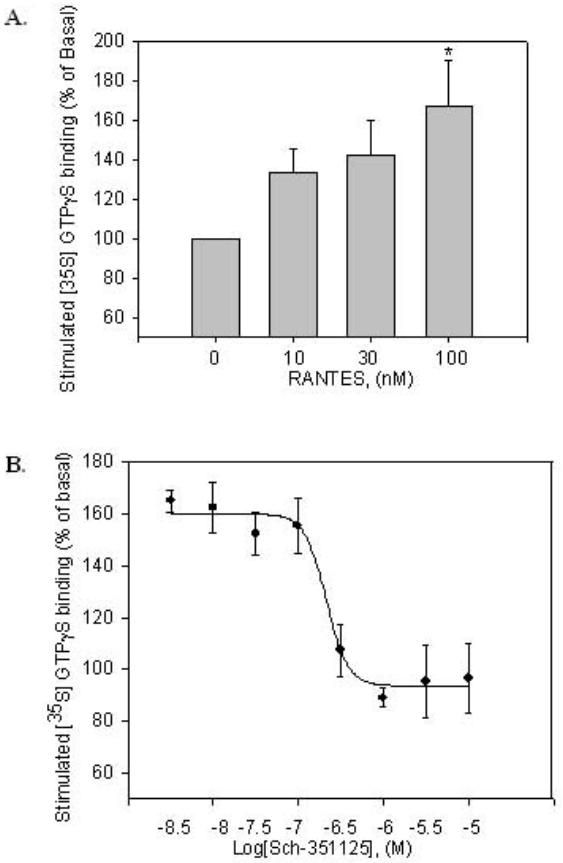


**Figure 2. Functional analysis of monoclonal No.7 HEK293/CCR5-HA cells.** (A) RANTES stimulated [$^{35}$S]GTPγS binding. Data were mean ± SD of two experiments performed in duplicate. * $p < 0.05$ compared with absence of RANTES. (B) CCR5 receptor antagonist Sch-351125 inhibited RANTES-stimulated [$^{35}$S]GTPγS binding in No.7 HEK293/CC5 cells. Data were mean ± SD of two experiments performed in duplicate.

is a prerequisite for the successful start of screening the CCR5 receptor antagonists. Since CCR5 receptor has no specific ligand, and since the ligands for CCR5 such as RANTES, MIP-1α, and MIP-1β are also the ligands for CCR1, CCR3, *etc*., the establishment of the particular CCR5 expression cell lines is of most importance. To establish cell lines stably expressing CCR5-HA fusion protein, HEK293 cells were transfected with the plasmid pcDNA3-CCR5-HA and colonies were selected in the presence of G418 sulfate and formed some visually distinct "cell islands". Independent and G418-resistant cell islands were trypsinized and cloned by limiting dilution. After selection, eight G418 resistant clones were obtained in HEK293 cells transfected with pcDNA3-CCR5-HA. The [$^{35}$S]GTPγS binding assay has been widely applied to determine the agonist-dependent of PTX-sensitive Gi/o proteins mediated by G-protein coupled receptors (GPCRs) (*21*). It has been reported that CCR5 receptor functionally couples to membrane-associated inhibitory G proteins (*20*), therefore, in this study we use [$^{35}$S]GTPγS binding assay to fast observe whether the selected monoclonal cell lines express the functional CCR5 receptor. As shown in Figure 1A, there were five monoclonal cell lines expressing functional CCR5 receptor, and of which No.7 is the best. We further used FACS analysis to confirm the membrane surface expression of CCR5-HA in No.7 HEK293/CCR5-HA cells. The results showed that CCR5 was positive in 96.89% of HEK293/CCR5-HA cells (No.7), while in 2.44% of HEK293 cell (Figure 1B). All these results indicate that No.7 monoclonal cell line stably expresses chemokine CCR5 receptor on HEK293 cell membrane surface.

### 3.2. *Functional analysis in HEK293/CCR5-HA cell line*

As shown in Figure 2A, CCR5 receptor agonist RANTES stimulated GTPγS binding in No.7 HEK293/CCR5-HA cells in the dose-dependent manner. In contrast, CCR5 receptor specific antagonist Sch-351125 inhibited RANTES-stimulated GTPγS binding in a dose-dependent manner (Figure 2B).

In summary, this established HEK293/CCR5-HA cell line would be most valuable as a tool for high-throughput screening the antagonists of chemokine CCR5 receptor and *in vitro* evaluation of therapeutic potential in the anti-HIV therapy.

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