研究成果の刊行に関する一覧表

主任研究者：京都大学ウイルス研究所　松岡　雅雄
分担研究者：京都大学大学院薬学研究科　藤井　信孝
分担研究者：京都大学大学院薬学研究科　大石　真也

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Tamamura, H.; Tsutsumi, H.; Masuno H.; Fujii N.	Development of low molecular weight CXCR4 antagonists by exploratory structural tuning of cyclic tetra- and pentapeptide- scaffolds towards the treatment of HIV infection, Cancer Metastasis and Rheumatoid arthritis.	*Curr. Med. Chem.*	14(1)	93-102	2007
Sasaki, Y.; Shigenaga, A.; Fujii, N.; Otaka, A.	Synthesis of (*Z*)-alkene-containing cis-proline dipeptide mimetics using samarium(II) diiodide (SmI_2)-mediated reductive alkylation reaction.	*Tetrahedron*	63(9)	2000-2008	2007
Ueda, S.; Oishi, S.; Wang, Z.; Araki, T.; Tamamura, H.; Cluzeau, J; Ohno, H.; Kusano, S.; Nakashima, H.; Trent, J. O.; Peiper, S. C.; Fujii, N.	Structure-activity relationships of cyclic Peptide-based chemokine receptor CXCR4 antagonists: disclosing the importance of side-chain and backbone functionalities.	*J. Med. Chem.*	50(2)	192-198	2007
Kato-Takagaki, K; Suzuki, N.; Yokoyama, F.; Takaki, S.; Umezawa, K.; Higo, J.; Mochizuki, M.; Kikkawa, Y.; Oishi, S.; Utani, A.; Nomizu, M.	Cyclic peptide analysis of the biologically active loop region in the laminin α3 Chain LG4 module de-monstrates the importance of peptide conformation on biological activity.	*Biochemistry*	46(7)	1952-1960	2007
Narumi, T.; Niida, A.; Tomita, K.; Oishi, S.; Otaka, A.; Ohno, H.; Fujii, N.	A novel one-pot reaction involving organocopper-mediated reduction/ transmetalation/asymmetric alkylation, leading to the diastereoselective synthesis of functionalized (Z)-fluoroalkene dipeptide isosteres.	*Chem. Commun.*	(45)	4720-4722	2006
Baba, M.; Hong, S.-B.; Sharma, N.; Warren, M. B.; Nickerson, M. L.; Iwamatsu, A.; Esposito, D.; Gillette, W. K.; Hopkins, R. F., III; Hartley, J. L.; Furihata, M.; Oishi, S.; Zhen, W.; Burke, T. R., Jr.; Linehan, W. M.; Schmidt, L. S.; Zbar, B.	Folliculin encoded by the BHD gene interacts with a binding protein, FNIP1, and AMPK, and is involved in AMPK and mTOR signaling.	*Proc. Natl. Acad. Sci.*	103 (42)	15552 -15557	2006
Otaka, A.; Fujii, N.	Development of anti-viral fusion inhibiting peptides.	*Kagaku Kogyo*	57(10)	797-801	2006
Tomita, K.; Niida, A.; Oishi, S.; Ohno, H.; Cluzeau, J.; Navenot, J.-M.; Wang, Z.; Peiper, S. C.; Fujii, N.	Structure-activity relation- ship study on small peptidic GPR54 agonists.	*Bioorg. Med. Chem.*	14(22)	7595-7603	2006
Tamamura, H.; Tsutsumi, H.; Fujii, N.	The chemokine receptor CXCR4 as a therapeutic target for several diseases.	*Mini-Rev. Med. Chem.*	6(9)	989-995	2006

Tsuda, M.; Terada, T.; Irie, M.; Katsura, T.; Niida, A.; Tomita, K.; Fujii, N.; Inui, K.	Transport characteristics of a novel peptide transporter 1 substrate, antihypotensive drug midodrine, and its amino acid derivatives.	*J. Pharmacol. Exp. Ther.*	318(1)	455-60	2006
Menu, E.; Asosingh, K.; Indraccolo, S.; De Raeve, H.; Riet, V. I.; Valckenborgh, V. E.; Vande B., V. I.; Fujii, N.; Tamamura, H.; Camp, V. B.; Vanderkerken, K.	The involvement of stromal derived factor 1α in homing and progression of multiple myeloma in the 5TMM model.	*Haematologica*	91(5)	605-612	2006
Kasyanov, A.; Tamamura, H.; Fujii, N.; Xiong H.	HIV-1 gp120 enhances giant depolarizing potentials via chemokine receptor CXCR4 in neonatal rat hippocampus.	*Eur. J. Neurosci.*	23(5)	1120-1128	2006
Tamamura, H.; Tsutsumi, H.; Masuno, H.; Mizokami, S.; Hiramatsu, K.; Wang, Z.; Trent, J. O.; Nakashima, H.; Yamamoto, N.; Peiper, S. C.; Fujii, N.	Development of linear type of low molecular weight CXCR4 antagonists based on T140 analogs.	*Org. Biomol. Chem.*	4(12)	2354-2357	2006
Hanaoka, H.; Mukai, T.; Tamamura, H.; Mori, T.; Ishino, S.; Ogawa, K.; Iida, Y.; Doi, R.; Fujii, N.; Saji, H.	Development of a ^{111}In-labeled peptide derivative targeting a chemokine receptor, CXCR4, for imaging tumors.	*Nucl. Med. Biol.*	33(4)	489-494	2006
Sasaki, Y.; Niida, A.; Tsuji, T.; Shigenaga, Ak.; Fujii, N.; Otaka, A.	Stereoselective synthesis of (Z)-alkene-containing proline dipeptide mimetics.	*J. Org. Chem.*	71(13)	4969-4979	2006
Tamamura, H.; Ojida, A.; Ogawa, T.; Tsutsumi, H.; Masuno, H.; Nakashima, H.; Yamamoto, N.; Hamachi, I.; Fujii, N.	Identification of a new class of low molecular weight antagonists against the chemokine receptor CXCR4 having the dipicolylamine-zinc(II) complex structure.	*J. Med. Chem.*	49(11)	3412-3415	2006
Niida, A.; Mizumoto, M.; Narumi, T.; Inokuchi, E.; Oishi, S.; Ohno, H.; Otaka, A.; Kitaura, K.; Fujii, N.	Synthesis of (Z)-alkene and (*E*)-fluoroalkene-containing diketopiperazine mimetics utilizing organo-copper-mediated reduction-alkylation and diastereo- selectivity examination using DFT calculations.	*J. Org. Chem.*	71(11)	4118-4129	2006
Niida, A.; Tanigaki, H.; Inokuchi, E.; Sasaki, Y.; Oishi, S.; Ohno, H.; Tamamura, H.; Wang, Z.; Peiper, S. C.; Kitaura, K.; Otaka, A.; Fujii, N.	Stereoselective synthesis of 3,6-di-substituted-3,6-dihydropyridin-2-ones as potential diketopiperazine mimetics using organo-copper-mediated anti-SN2' reactions and their use in the preparation of low-molecule CXCR4 antagonists.	*J. Org. Chem.*	71(10)	3942-3951	2006
Avniel, S.; Arik, Z.; Maly, A.; Sagie, Assa; Basst, H. B.; Yahana, M. D.; Weiss, I. D.; Pal, B.; Wald, O.; Ad-El, D.; Fujii, N.; Arenzana- Seisdedos, F.; Jung, S.; Galun, E.; Gur, E.; Peled, A.	Involvement of the CXCL12/CXCR4 pathway in the recovery of skin following burns.	*J. Invest. Dermatol.*	126(2)	468-476	2006
Ohno, H.; Kadoh, Y.; Fujii, N.; Tanaka, T.	Potassium carbonate-promoted stereospecific 5-endo-trig cyclization of unactivated allenes in the absence of any transition metals.	*Org. Lett.*	8(5)	947-950	2006

Niida, A.; Tomita, K.; Mizumoto, M.; Tanigaki, H.; Terada, T.; Oishi, S.; Otaka, A.; Inui, K.; Fujii, N.	Unequivocal synthesis of (*Z*)-alkene and (*E*)-fluoroalkene dipeptide isosteres to probe structural requirements of the peptide transporter PEPT1.	*Org. Lett.*	8(4)	613-616	2006
Oishi, S.; Miyamoto, K.; Niida, A.; Yamamoto, M.; Ajito, K.; Tamamura, H.; Otaka, Akira; Kuroda, Y.; Asai, A.; Fujii, N.	Application of tri- and tetrasubstituted alkene dipeptide mimetics to conformational studies of cyclic RGD peptides.	*Tetrahedron*	62(7)	1416-1424	2006
Dewan, M. Z.; Uchihara, J.; Terashima, K.; Honda, M.; Sata, T.; Ito, M.; Fujii, N.; Uozumi, K.; Tsukasaki, K.; Tomonaga, M.; Kubuki, Y.; Okayama, A.; Toi, M.; Mori, N.; Yamamoto, N.	Efficient intervention of growth and infiltration of primary adult T-cell leukemia cells by an HIV protease inhibitor, ritonavir.	*Blood*	107(2)	716-724	2006
Ohta, Y.; Itoh, S.; Shigenaga, A.; Shintaku, S.; Fujii, N.; Otaka, A.	Cysteine-derived S-protected oxazolidinones: Potential chemical devices for the preparation of peptide thioesters.	*Org. Lett.*	8(3)	467-470	2006
Niida, A.; Wang, Z.; Tomita, K.; Oishi, S.; Tamamura, H.; Otaka, A.; Navenot, J.-M.; Broach, J. R.; Peiper, S. C.; Fujii, N.	Design and synthesis of down-sized metastin (45-54) analogs with maintenance of high GPR54 agonistic activity.	*Bioorg. Med. Chem. Lett.*	16(1)	134-137	2006

A novel colorimetric assay for CXCR4 and CCR5 tropic human immunodeficiency viruses

Keiko Kajiwara, Eiichi Kodama* and Masao Matsuoka

Laboratory of Virus Immunology, Institute for Virus Research, Kyoto University, Shogoin, Sakyo-ku, Kyoto, Japan

*Corresponding author: Tel: +81 75 751 3986; Fax: +81 75 751 3986; E-mail: ekodama@virus.kyoto-u.ac.jp

The majority of HIV isolated from infected patients uses CCR5 as a coreceptor (R5-HIV). Although R5-HIV fails to replicate efficiently in human transformed T-cell lines, HIV using CXCR4 (X4-HIV) can replicate well in such cell lines. Therefore, most of screening systems using the T-cell lines detect only X4-HIV replication. Here we report a new assay to monitor the replication of R5- as well as X4-HIV. An MTT assay using CD4-, CXCR4-, and CCR5-transduced human glioma NP-2 cells (NCK45 cells) was established and then compared with the representative assays including multinuclear activation of a galactosidase indicator assay (MAGI assay). The antiviral activities of not only an adsorption inhibitor and reverse transcriptase inhibitors but also a Tat antagonist in the NCK45 cells, were comparable to those obtained from the MTT assay using MT-4 cells or the MAGI assay. However, the activity of protease inhibitors (PIs) was underestimated, even though expressions of major multidrug resistant genes involved in efflux of PIs were comparable in MT-2, NP-2, and NCK45 cells. After cultivation of more than 6 months, NCK45 cells remained susceptible to HIV infection since NCK45 cells consistently expressed CD4, CXCR4, and CCR5. On the other hand, MAGI cells lost the CD4 expression during culture. Thus, this assay system can stably detect the replication of both X4- and R5-HIV, indicating that it should be useful for the evaluation of HIV replication and drug susceptibility.

Keywords: CCR5, CXCR4, human immunodeficiency virus, MTT Assay

Introduction

Human immunodeficiency virus (HIV) infects the host cells by targeting CD4 molecules on the cell surface and the seven transmembrane-spanning G protein-coupled coreceptors, for example, CXCR4 (X4) and CCR5 (R5), via the V3 loop of gp120 (Wu *et al.*, 1996; Berger *et al.*, 1999). HIV using R5 as a coreceptor (R5-HIV) has been isolated from patients in the all stages, whereas X4-HIV is isolated from patients during the later stages of disease progression towards acquired immunodeficiency syndrome (AIDS). Transformed T-cells expressing CXCR4 on their surface, such as MT-2, H9, and Molt4 cells, are highly susceptible to X4-HIV infection, but are less susceptible, or not susceptable at all, to R5-HIV. Therefore, a novel cell line allowing R5-HIV replication is required to evaluate drug susceptibility of R5-HIV, the major isolate in patients.

Multinuclear activation of a galactosidase indicator assay (MAGI assay; Kimpton & Emerman, 1992) and p24 enzyme-linked immunosorbent assay (ELISA) with peripheral blood mononuclear cells (PBMCs) are frequently used for the evaluation of R5-HIV replication (Schupbach *et al.*, 2000), although these assay systems have several problems. In the MAGI assay, susceptibility to HIV infection gradually declines in HeLa CD4/CCR5/ LTR-β-galactosidase (MAGI/CCR5) cells after extensive passages and tedious counting is required to determine infectivity. In the p24 ELISA, the sample preparation is complicated and the cost of each assay is high. Moreover, expression levels of CD4, CXCR4, and CCR5 on freshly isolated PBMCs differ over a wide range depending on cell subsets and donors (Ostrowski *et al.*, 1998; Lee *et al.*, 1999), and intracellular drug metabolisms in PBMCs are generally different from donor to donor, resulting in difficulties to obtain reproducible data (Ghezzi *et al.*, 2001).

Recently, a novel reporter assay using CCR5-transduced Molt4 (MOCHA) cells has been reported (Miyake *et al.*, 2003). This assay system detects Tat expression using the activity of long terminal repeat (LTR), which transcribes secretary alkaline phosphatase (SEAP) gene and provides a simple procedure. However, MOCHA cells express various HIV coreceptors, for example, CCR3, that may influence the infectivity or activity of coreceptor antagonists (Dejucq, 2000). Another assay using CXCR4- and CCR5- transduced

U87 cells has been established previously (Princen *et al.*, 2004). In this assay, tedious p24 ELISA was applied to evaluate the anti-HIV activities. Moreover, U87 cells expressed various G-proteins coupled receptors that act as coreceptors not only for HIV-1 and 2, but also simian immunodeficiency viruses (Shimizu *et al.*, 2000).

Here we developed an assay system for monitoring not only X4- but also R5-HIV replication and drug susceptibility using an MTT method, which was found to be simple and reproducible. This system can provide a convenient and stable method to screen a wide rage of anti-HIV compounds.

Materials and methods

Reagents

An adsorption inhibitor, dextran sulphate (DS5000; molecular weight 5,000), reverse transcriptase inhibitors (RTIs), 3′-azido-2′,3′-dideoxythymidine (AZT) and 2′,3′-dideoxycytidine (ddC) were purchased from Sigma (St. Louis, MO, USA). A Tat antagonist, Ro5-3335 (Hsu *et al.*, 1991), and a CXCR4 antagonist, AMD3100 (Schols *et al.*, 1997), were a kind gift from S Shigeta (Fukushima Medical University, Fukushima, Japan). A CXCR4 antagonist, T-140 (Fujii & Tamamura, 2001), and the following protease inhibitors (PIs): VX-478 and nelfinavir (Livington *et al.*, 1995; Witvrouw *et al.*, 2004), were kindly provided by N Fujii (Kyoto University, Kyoto, Japan). A CCR5 antagonist, TAK-779 (Baba *et al.*, 1999), was obtained from the AIDS Research and Reference Program, Division of AIDS, National Institute of Allergy and Infectious Diseases (Bethesda, MD, USA). Mouse anti-human CXCR4 monoclonal antibody (MAb; 12G5) and mouse anti-human CCR5 MAb (2D7) were purchased from BD Biosciences Clonetech (San Jose, CA, USA). Phycoerythin cyanine-5 (PC5)-conjugated mouse anti-human CD4 was purchased from Immunotech (Marseille, France). Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG and isotype-matched control MAb were purchased from Jackson ImmunoResearch Laboratory (West Grove, PA, USA). 1-(4,5-Dimethylthiazol-2yl)-3,5-dephenylformazan (MTT) was also purchased from Sigma.

Cells

MT-2 and MT-4 cells were propagated and maintained in RPMI1680 medium (Sigma) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin G and 50 μg/ml of streptomycin. NP-2 cells derived from a human glioma were kindly provided by H Hoshino (Gunma University, Maebashi, Japan; Kanbe *et al.*, 1999; Soda *et al.*, 1999). NP-2 cells were propagated and maintained in Dulbecco's modified Eagle medium (DMEM; Sigma) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin G and 50 μg/ml of streptomycin. HeLa CD4/CCR5/LTR-β-galactosidase (MAGI/CCR5) cells were obtained from M Emerman through the AIDS Research and Reference Program and were propagated in DMEM supplemented with 10% FCS, 0.2 mg/ml G418 disulphate (Nacalai Tesque, Kyoto, Japan), 0.2 mg/ml hygromycin B (Calbiochem, La Jolla, CA, USA) and 10 μg/ml puromycin (Sigma).

PBMCs were obtained from healthy HIV-seronegative donors and stimulated for 3 days with 20 U/ml of IL-2 (Shionogi & Co., Ltd, Osaka, Japan) and 0.5 μg/ml of phytohaemagglutinin (PHA; Sigma) as previously described (Kodama *et al.*, 2001).

Establishment of NCK45 cells

A lentiviral transfer vector (an HIV-1-based vector), pHR, was generously gifted by I Verma (Salk Institute, La Jolla, CA, USA), and modified for the expression of HIV-1 receptors. A packaging plasmid (pCMVΔ8/9) and an envelope-coding vector (pVSV-G) were a generous gift from H Miyoshi (RIKEN, Tsukuba, Japan).

First, we constructed pBC-CD4/CXR4-IG and introduced the genes for CD4, the internal ribosomal entry site (IRES), CXCR4, IRES, and neomycin phosphotranspherase ($G418^r$) under control of a cytomegalovirus promoter, to express HIV receptors with $G418^r$ simultaneously (as shown in Figure 1A). The pBC-CD4/CXCR4-IG plasmid was cotransfected with pCMVΔ8/9 and pVSV-G into 293T cells using FuGENE 6 transfection reagent (Roche Diagnostics, Indianapolis, IN, USA). After 48 h of transfection, culture supernatants were harvested and placed onto NP-2 cells. The transduced NP-2 cells were subjected to G418 selection, and designated as NCK cells. Similarly, an expression vector (pBC-CCR5-IH) encoding for the chemokine receptor, CCR5, along with hygromycin B phosphotranspherase ($Hygro^r$) was cotransfected with pCMVΔ8/9 and pVSV-G, and the culture supernatants were added to the NCK cells as described above. The transduced NCK cells were then subjected to both G418 and hygromycin B selection, and designated as NCK45 cells. NCK45 cells were further cultured in the presence of 0.5 mg/ml of G418 and 0.2 mg/ml of hygromycin B.

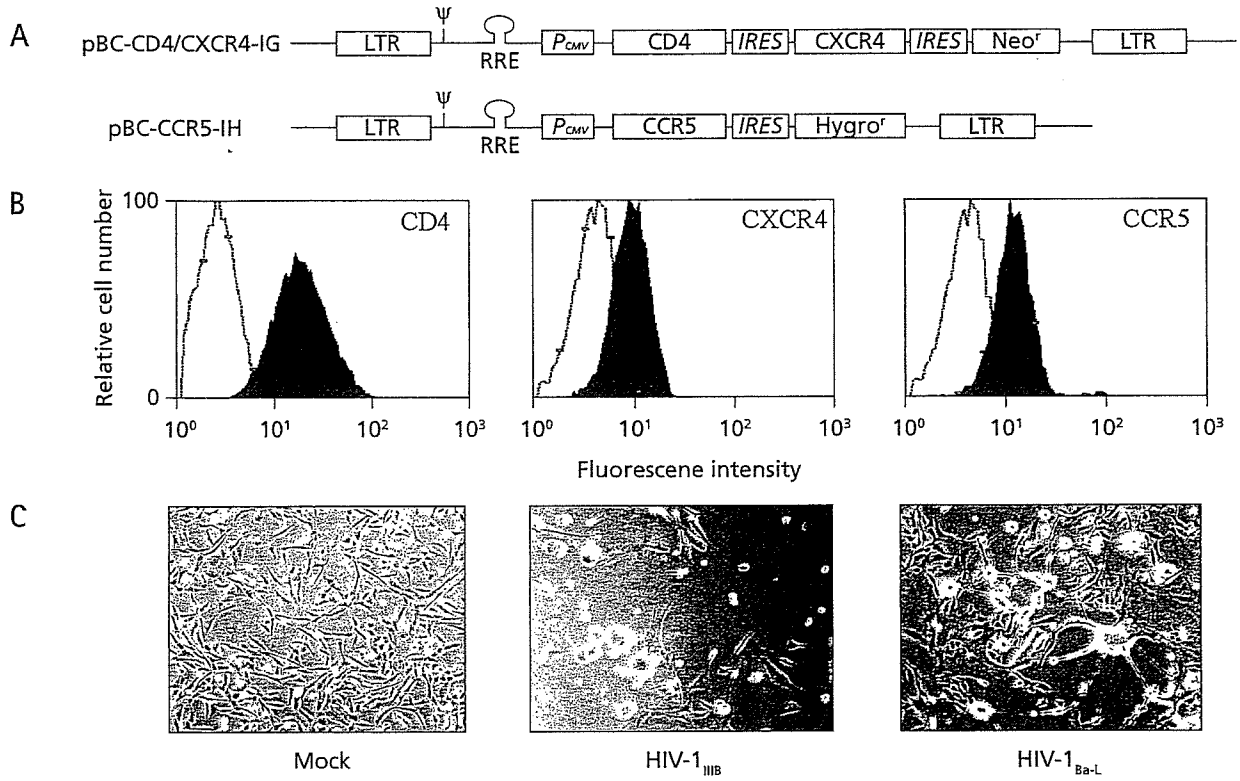
Viruses

$HIV\text{-}1_{IIIB}$ (T cell-tropic or X4 strain), $HIV\text{-}1_{Ba\text{-}L}$ (macrophage-tropic or R5 strain), $HIV\text{-}2_{EHO}$ (dual-tropic), and $HIV\text{-}2_{ROD}$ (X4) were propagated in NCK45 cells. The virus titres were determined using both NCK45 and MAGI/CCR5 cells. The viruses were stored at –80°C until use.

Flow cytometric analysis

The cells were digested with trypsin and then washed with phosphate-buffered saline (PBS) containing 1% FCS. The cells (10^6 cells) were incubated with anti-CXCR4,

Figure 1. Establishment of CD4, CXCR4, and CCR5 transduced NP-2 cells



(A) Schema for vector constructs used in this study. To simultaneously express CD4, CXCR4, and the selection marker neomycin phosphotransferase (G418^r) under the control of a single cytomagalovirus immediate-early promoter (P_{CMV}), two internal ribosomal entry site (IRES) sequences were inserted (pBC-CD4/CXCR4-IG). Expression of CCR5 and hygromycin B phosphotransferase (Hygro^r) was also controlled with a single cytomagalovirus (CMV) promoter and one inserted IRES sequence (pBC-CCR5-IH). (B) Surface expression of CD4, CXCR4 and CCR5 was analysed by a flow cytometer. The white histograms represent staining by the isotype-matched control MAbs. The gray histograms represent staining by the specific CD4, CXCR4 or CCR5 MAbs. (C) Microscopic view of NCK45 cells. Virus induced cytopathic effects (CPE) were observed after 48 h of virus inoculations. NCK45 cells infected with mock, HIV-1_{IIIB}, and HIV-1_{Ba-L} were shown. LTR, long terminal repeat; MAbs, monoclonal antibodies; RRE, Rev responsive element; P_{CMV}, CMV immediate-early promoter; ψ, packaging signal.

anti-CCR5, PC5-conjugated anti-CD4, or isotype-matched control monoclonal antibodies (MAbs) for 30 min on ice. These cells, except for cells incubated with anti-CD4 MAbs, were washed three times with PBS containing 1% FCS, and incubated with the secondary antibody fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratory) for 30 min on ice. Then, all cells were washed three times with PBS containing 1% FCS and for each sample, cells were fixed with 1% formaldehyde, and analysed on an EPICS XL flow cytometer (Beckman Coulter, Miami Lakes, FL, USA).

HIV titration

One hundred microlitres of NCK45 cells (0.5×10^4 cells/ml) were seeded in flat-bottom 96-well microtitre plates. Serial dilutions (100 µl) in 0.5 log increments of HIV-1_{IIIB} or HIV-1_{Ba-L} were added to cells and incubated at 37°C. After 7 days of virus infection, the infectivity and the viability of HIV-inoculated cells were examined by microscopic observation. The MTT method is described below (Figure 3).

MTT Assay

The MTT assay using MT-4 cells was performed as previously described (Kodama *et al.*, 2001). The MTT assay using NCK45 cells was also performed as previously described, but with some modifications. Briefly, 100 µl of culture medium containing an appropriate concentration of compound was added in triplicate to the wells of the flat bottom 96-well microtitre plates; serial 10-fold dilutions were directly made in the plate. The NCK45 cells (0.5×10^3 cells/well) were mixed with HIV-1_{IIIB} or HIV-1_{Ba-L} at a titre of 100× the 50% tissue culture infectious dose

(100 $TCID_{50}$) in the presence and absence of various concentrations of the compounds in the plate. The last row of wells was used as the untreated control and 100 µl of culture medium was added to the rest of the wells. At the end of the incubation period, 20 µl of the MTT solution (7.5 mg/ml in PBS) was added to each well of the plate. The plates were then incubated at 37°C for 3 h. After incubation, 120 µl of medium was removed from each well. To dissolve the formazan crystals, 100 µl of isopropanol containing 4% Triton X-100 and 0.4% concentrated HCl was added to each well, and thoroughly mixed. After confirming microscopically that the formazan crystals were completely dissolved, the absorbance of the wells was measured using a plate reader (Model 3550, Bio-Rad Laboratories, San Jose, CA, USA) at a wavelength of 595 nm.

The 50% cytotoxic concentration (CC_{50}) of the compound was defined as the concentration that reduced the absorbance of mock-infected cells by 50% of the control. The 50% effective concentration (EC_{50}) indicated from the viral induced cytotoxicity was defined as the concentration that protected 50% of the cells against virus-induced cytopathic effect (CPE), as previously described (Kodama *et al.*, 1996).

MAGI assay

As previously described, anti-HIV activities were determined by the MAGI assay (Kodama *et al.*, 2001). Briefly, the MAGI/CCR5 cells (10^4 cells/well) were seeded in flat bottom 96-well microtitre plates. On the following day, the cells were inoculated with HIV-1 or HIV-2 (60 MAGI U/well, producing 60 blue cells after 48 h of incubation) and cultured in the presence of various concentrations of drugs in fresh medium. Forty-eight hours after viral exposure, all the blue cells stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) in each well were counted.

Reverse transcription (RT)-PCR

Total RNA was extracted from NP-2, NCK45, and MT-2 cells (10^5 cells) using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Complimentary DNA was synthesized from 1 µg of total RNA and then subjected to PCR using the RNA LA PCR Kit Ver.1.1 (TaKaRa Bio Inc, Shiga, Japan) following the manufacturer's protocol. The primer sequences used for amplification of multidrug resistance (MDR) and the related genes are listed in Table 2.

Results

Establishment of NCK45 cells

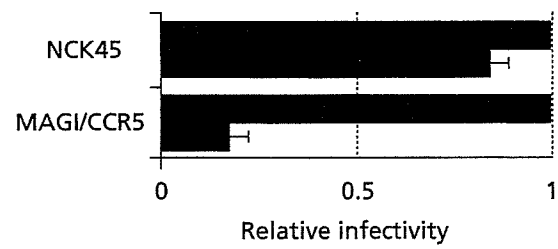
NP-2 cells simultaneously transduced with two recombinant lentiviruses expressing CD4, CXCR4, and CCR5 were designated as NCK45 cells. We first confirmed the expression of these molecules on their surface with flow cytometric analysis. NCK45 cells were found to express CD4, CXC4R, and CCR5 as shown in Figure 1B. After infection with HIV-1_{IIIB} or HIV-$1_{Ba\text{-}L}$, a massive CPE was observed (Figure 1C), indicating that these cells were highly susceptible to both X4- and R5-HIV-1 infection. NCK45 cells are also susceptible to HIV-2_{EHO} and HIV-2_{ROD} (data not shown). Since NCK45 cells allowed efficient replication of various HIV strains used in this study, NCK45 cells were used for viral preparation.

We compared susceptibility to HIV-1 infection using long-cultured NCK45 cells with MAGI/CCR5 cells using aliquots of HIV-1 virus stock from the same lot (Figure 2). Even after serial passage for 6 months, the infectivity to NCK45 cells remained high (85%), whereas the infectivity to MAGI/CCR5 cells severely decreased (17%). Flow cytometric analysis also revealed that the expression of these receptors was constant on NCK45 cells (data not shown). For long-term culture, expression of CD4 gradually declined on MAGI/CCR5 cells, whereas those of CXCR4 and CCR5 did not. These results indicated that simultaneous expression of HIV receptors and the selection markers, $G418^r$ and $Hygro^r$ by inserted IRES fragments, prevented gene silencing, ensuring the continuous expression of HIV receptors.

Optimization of the MTT method

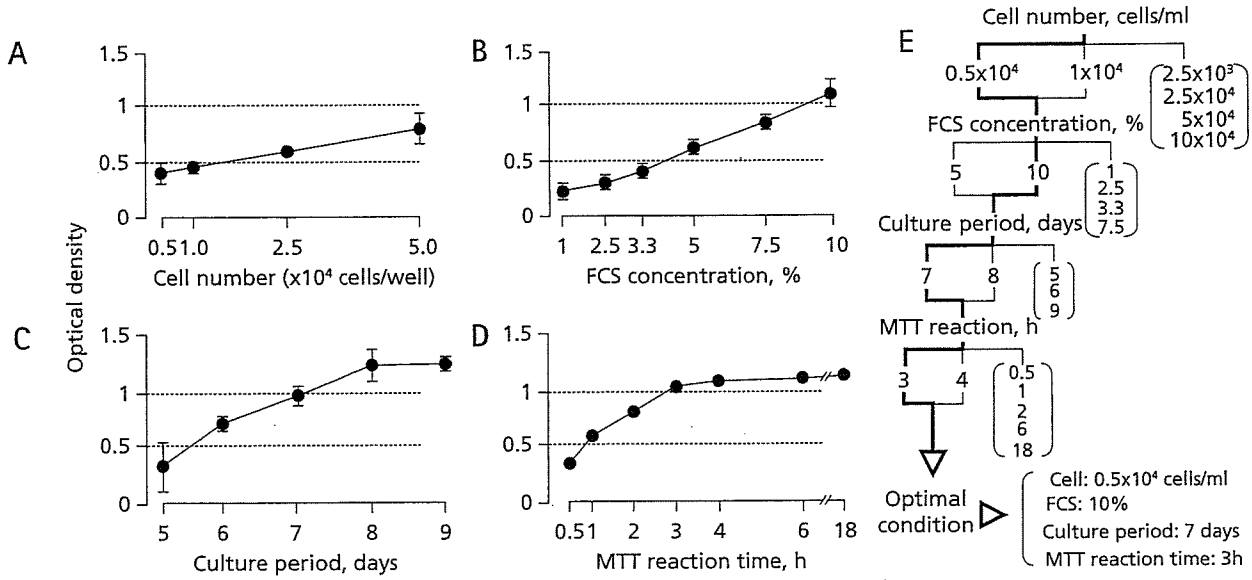
To evaluate the activity of anti-HIV agents using NCK45 cells, we determined the appropriate conditions for the MTT assay in the flowchart as shown in Figure 3E. In this optimization, we decided that the optimal optical density (OD) values were close to 1.0, given that Pauwels *et al.* (1988) reported that OD values of formazan resulting from conversion of MTT must be over 0.4 to obtain reproducible data. As shown in Figure 3A, the optimal cell numbers are 0.5 or 1×10^4 cells/ml, since greater cell numbers resulted in an overgrowth of the cells. Next, we

Figure 2. Comparison of infectivity in NCK45 and MAGI/CCR5 cells after long-term culture



Susceptibility to HIV infection (HIV-1_{IIIB}) at 0 months was used as a control (gray bar). The relative susceptibility after 6 months is shown by the black bar.

Figure 3. Effect of various conditions on the MTT optical density



(A) NCK45 cells were seeded at the various cell numbers in 96-well plates and cultured for 7 days. (B) NCK45 cells containing various concentrations of fetal calf serum (FCS) were tested on day 7. (C) Effect of the culture period on the MTT optical density. NCK45 cells (0.5×10^4 cells/ml) were cultured for the indicated periods. (D) Effect of the MTT incubation periods on the optical density. NCK45 cells were seeded at the 0.5×10^4 cells/ml and measured at OD 595 nm. All data shown as mean ±SD for each point is the result of 6 separate wells. (E) Flow chart of MTT optimization.

determined optimal concentrations of FCS. In the low FCS concentrations (<7.5%), OD values were unstable and low (Figure 3B). Cultivation over 7 days resulted in sufficient OD values (Figure 3C). Finally, the MTT reaction time was determined. The OD values reached 1.0 and increased little more after 3 h (Figure 3D), whereas the background increased after 6 h (data not shown). Thus, the optimal conditions were established to be: cell number= 0.5×10^4 cells/ml, FCS concentration=10%, culture period= 7 days and MTT reaction time= 3 h, as shown in Figure 3E.

Anti-HIV activity in NCK45 cells

The activity of various anti-HIV agents was examined in NCK45 cells. We tested various types of anti-HIV drugs: an adsorption inhibitor (DS5000), RTIs (AZT and ddC), PIs (VX-478 and nelfinavir), a Tat antagonist (Ro5-3335), CXCR4 antagonists (T-140 and AMD3100) and a CCR5 antagonist (TAK-779; Table 1). The activities of DS5000, AZT, ddC, and AMD3100 were similar when MT-4/MTT, MAGI, and NCK45/MTT were used for assays, whereas the activities of T-140 and the PIs were different among the assays. The activity of T-140 was 0.5, 6.0, and 29 nM for MT-4/MTT, MAGI, and NCK45/MTT, respectively. To address the incompatibility, the influence of CXCR4 expression levels on the cell's surface was examined with the flow cytometer. Results showed that MT-4 cells expressed high level of CXCR4, whereas MAGI and NCK45 cells moderately expressed it (data not shown). These results indicate that expression levels were not correlated with activity in our assay. Interestingly, Ghezzi *et al.* (2001) also reported similar results. Moreover, another CXCR4 antagonist, AMD3100, showed comparable activity in these assays. These results indicate that some properties of T-140, for example, stability of a peptide-based relatively large molecule, rather than those of NCK45 cells are associated with the differential activity. Since the activity of PIs was underestimated in NCK45 cells even under multi-round infection of HIV (VX-478=4.1 nM and 522 nM; nelfinavir=29 nM and >1,000 nM, in MT-4/MTT and NCK45/MTT, respectively), further experiments were performed as described below.

Expression of a MDR gene in NCK45 cells

To clarify why the activities of the PIs (VX-478 and nelfinavir) were underestimated in NCK45 cells, we analysed the gene expression of major multidrug-resistant phosphoglycoproteins (MDR-p-gps) by RT-PCR. MDR-p-gps are cell surface transporters belonging to the ATP-binding cassette family and are ATP-dependent efflux

Table 1. Anti-HIV activity in NCK45 cells with MTT methods

		EC_{50}, nM						
		HIV-1				HIV-2		
Compound	Target	MT-4/MTT IIIB	MAGI-CCR5 IIIB	NCK45 IIIB	NCK45 Ba-L	NCK45 EHO	NCK45 ROD	CC_{50} NCK45, nM
DS5000	gp120	127 ±39	135 ±25	164 ±50	530 ±36	52 ±8	327 ±194	>10,000
AZT	RT	2.8 ±0.4	31 ±13	39 ±14	34 ±8	49 ±15	175 ±69	>1,000
ddC	RT	145 ±28	402 ±162	292 ±139	460 ±0	174 ±27	244 ±78	>10,000
T-140	CXCR4	0.5 ±0.1	6.0 ±0.6	29 ±7	>100	35 ±4	10 ±6	>100
AMD3100	CXCR4	0.3 ±0.1	0.4±0.03	0.4±0.1	>100	ND	ND	>100
TAK-779	CCR5	>100	>100	>100	32 ±18	>100	>100	>100
Ro5-3335	Tat	ND	3,340 ±892	2,507 ±437	3,537 ±965	ND	ND	>10,000
VX-478	Protease	4.1 ±1.2	ND	522 ±155	512 ±68	ND	ND	>1,000
nelfinavir	Protease	29 ±8	ND	>1,000	>1,000	ND	ND	>1,000

AZT, 3'-Azido-2',3'-dideoxythymidine; CC_{50}, 50% cytotoxic concentration; ddC, 2',3'-Dideoxycytidine; EC_{50}, 50% antiviral effective concentration; ND; Not determined. Data represent mean values of at least three independent experiments (±SD).

pumps that prevent the accumulation of drugs in cells. In previous reports, the expression of MDR-p-gps in the blood brain barrier has been shown to prevent the entry of PIs into the central nervous system (Cordon-Cardo *et al.*, 1989; Kim *et al.*, 1998; Lee *et al.*, 1998; Edwards *et al.*, 2005). It is likely that NP-2 cells express MDR-p-gps, since NP-2 cells are derived from a glioma. We performed RT-PCR to examine the expression of MDRs, multidrug resistance-associated proteins (MRPs), and a breast cancer resistant protein (BCRP) gene in NP-2, NCK45, and MT-2 cells. In MT-2 cells, PIs were well evaluated in Yoshimura *et al.*, (1999). MT-2 cells expressed all MDR-p-gps except for MDR-3, MRP-3, and MRP-6, whereas NP-2 and NCK45 cells expressed all MDR-p-gps except for MDR-3, MRP-2, MRP-3 and MRP-6 (Table 2). We further examined the possibility of MDR induction with G418 and hygromycin selection; however, there were no differences in gene expression between NP-2 and NCK45 cells (data not shown). These results suggested that the major MDR-p-gps tested were not involved in the loss of PI activity.

Discussion

In this study, we established a new cell line, NCK45, to monitor the replication and their drug susceptibilities of both X4- and R5-HIV. NCK45 cells simultaneously expressed CD4, CXCR4, and CCR5 on their cell surface and allowed the use of the MTT method. The MAGI assay and p24 ELISA are frequently used as representative assays; however, these have some disadvantages. Since the MAGI assay uses the detection of intracellular Tat expression by LTR-driven β-galactosidase, it cannot evaluate drug activities that inhibit the later replication steps, for example, protease cleavage or assembly. Moreover, after prolonged passages, the levels of HIV receptors expression decreased, which resulted in reduced HIV infectivity. To stably express HIV receptors, the receptors and a drug resistant marker were simultaneously expressed by IRES. The HIV based vector may also prevent gene silencing, since it is preferentially integrated into actively transcribed genes and local hot spots of the host genome (Schroder *et al.*, 2002). Although gene silencing status was not analysed, NCK45 cells may escape from gene silencing. The p24 ELISA using PBMCs is useful to monitor the replication of various HIV strains. However, expression levels of HIV receptors and intracellular drug metabolisms vary between individuals, indicating that it is difficult to obtain reproducible data. Moreover, PBMCs must be stimulated with IL-2 and PHA for 3 days. We first tried to establish an MTT assay using PM-1/CCR5 or CEM/CCR5 cells. However, after HIV infection, these cells displayed persistent infections within 2 weeks of culture (data not shown).

Considering that PBMCs and MOCHA cells express various coreceptors (Dejucq *et al.*, 1999; Dejucq, 2000), it may be difficult to estimate the exact coreceptor antagonisms. In contrast, NP-2 cells have been shown not to express any coreceptors for HIV (Shimizu *et al.*, 1999; Soda *et al.*, 1999; Liu *et al.*, 2000; Shimizu *et al.*, 2000), suggesting that data obtained from NCK45 cells showed accurate coreceptor antagonisms. Indeed, coreceptor dependency of clinical isolates were easily evaluated in NP-2 derived cells (Soda *et al.*, 1999), indicating that NCK45 cells expressing other coreceptors may enable the evaluation of various coreceptor antagonisms or agonisms.

In human T-cell leukaemia virus-I (HTLV-I) transformed cells, for example, MT-4 cells, no antiviral activities of Tat antagonist were observed even at subtoxic concentrations. It is believed that, since HIV transcription

Table 2. Primer sequence used for RT-PCR and expression of MDR-p-gps in the cells

Gene	Primer sequence	MT-2	NCK45
MDR-1 (AF016535)*	5′-CCTGGAGGTGAAGAAGGGCCAGAC-3′(3287-3310)† 5′-TTAGGCAGTGACTCGATGAAGGCA-3′ (3581-3604)	+‡	±
MDR-3 (M23234)	5′-CAATGTATGTTGAAAAATTGTATGGA-3′ (2782-2807) 5′-GGTTGTCTTTCAAACAGCATGAATAA-3′ (3051-3076)	–	–
MRP-1 (L05628)	5′-CAGCACAGAGCAGGAGCAGGATG-3′ (2806-2828) 5′-CAATAGTTGGAAGCCAGCGCGGA-3′ (3158-3180)	+	+
MRP-2 (NM_000392)	5′-CATTGATGGAGTAGATATTGCTT-3′ (4178-4200) 5′-TGGATTTCCGAAGCAGAGCCCTG-3′ (4445-4467)	+	–
MRP-3 (AF009670)	5′-GCAGGCCTGGACTTCCAGTGCTCA-3′ (4310-4333) 5′-ATCAAACTCAGCTACTACTCCTT-3′ (4566-4588)	–	–
MRP-4 (NM_005845)	5′-CAACTTAAAGAAACCATTGAAGA-3′ (3575-3597) 5′-AAACCATTATCTTGTCGCTGTCA-3′ (3832-3854)	++	++
MRP-5 (U83661)	5′-GTATCCTTCACGATCAAACCTAA-3′ (3756-3778) 5′-AATACATTCTTTCATGTGTGTCCT-3′ (4032-4055)	+	±
MRP-6 (NM_001171)	5′-CCAGCTGCAGTACAAGTGTGCTGA-3′ (4190-4213) 5′-TTGTCCATGACCAGAACCCGGGCACA-3′ (4419-4444)	–	–
ABCT1 (AF261092)	5′-AGATGAAGAAAGCTGCTGCTCGAGCTGT-3′ (283-310) 5′-TGGCCCGCTCTGTGTCGACTTCCATCA-3′ (577-603)	+	±
ABCG2 (AY289766)	5′-CAAATGCTGTCCTTTTGCTCCTGAA-3′ (855-879) 5′-TAGCTGATCTCCTTGAAGACTGTGAT-3′ (1274-1299)	+	±

*Genbank accession number. †Position of primer sequence. ‡Comparison of MDR gene expression intensity in MT-2 and NCK45 cells confirmed by RT-PCR. ++, gene expression more than control (GAPDH). ±, gene expression equal with control; –, gene expression less than control or not detected.

in MT-4 cells is highly activated by Tax, which is a trans-activator of HTLV-I, inhibitory effect of Tat antagonist is compensated by a Tax-mediated transcriptional pathway (Witvrouw *et al.*, 1992). One of the obstacles appears to be the ability to perform a high throughput screening for anti-HIV transcriptional inhibitors including Tat antagonists. Taken together, the MTT assay using NCK45 cells seems to be useful especially for monitoring antagonisms of coreceptors and Tat. However, the culture periods with NCK45 cells for assays are relatively long (7 days) to obtain efficient OD value. In this respect, we plan to introduce the reporter gene (for example, β-galactosidase) to allow for a more rapid evaluation.

At present, the mechanism for the reduction of PI activity in NCK45 cells remains unknown. MDR genes contribute to various drug efflux not only for PIs, but also nucleoside/nucleotide RTIs (Chow *et al.*, 1993; Schuetz *et al.*, 1999; Villalba *et al.*, 1999). However, the effects of RTIs were well evaluated in NCK45 cells. It is possible that unknown influx or efflux systems for PIs, polymorphisms or mutations in the MDR genes, and/or some altered processing of MDR gene product(s), influence the PI activities (Chaillou *et al.*, 2002; Zhu *et al.*, 2004).

In conclusion, we have established a novel MTT assay for the screening of anti-R5- and X4-HIV activity using NCK45 cells. This simple and inexpensive assay should be useful for both a clinical phenotype assay and high-throughput screening of anti-HIV compounds.

Acknowledgements

We thank M Ikeuchi for his excellent technical assistance. This work was supported in part by a grant from the Ministry of Health and Welfare of Japan (E.K. & M.M) for the Promotion of AIDS Research, a grant from the Japan Health Science Foundation (E.K.) for the Research for Health Science Focusing on Drug Innovation, and a grant from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (E.K.). K.K. is supported by the 21st Century COE Program of the Ministry of Education, Culture, Sports, Science, and Technology.

References

Baba M, Nishimura O, Kanzaki N, Okamoto M, Sawada H, Iizawa Y, Shiraishi M, Aramaki Y, Okonogi K, Ogawa Y, Meguro K & Fujino M (1999) A small-molecule, nonpeptide CCR5 antagonist with highly potent and selective anti-HIV-1 activity. *Proceedings of*

the National Academy of Sciences of the United States of America **96**:5698–5703.

Berger EA, Murphy PM & Farber JM (1999) Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease. *Annual Review of Immunology* **17**:657–700.

Chaillou S, Durant J, Garraffo R, Georgenthum E, Roptin C, Dunais B, Mondain V, Roger PM & Dellamonica P (2002) Intracellular concentration of protease inhibitors in HIV-1-infected patients: correlation with MDR-1 gene expression and low dose of ritonavir. *HIV Clinical Trials* **3**:493–501.

Chow YK, Hirsch MS, Merrill DP, Bechtel LJ, Eron JJ, Kaplan JC & D'Aquila RT (1993) Use of evolutionary limitations of HIV-1 multidrug resistance to optimize therapy. *Nature* **361**:650–654.

Cordon-Cardo C, O'Brien JP, Casals D, Rittman-Grauer L, Biedler JL, Melamed MR & Bertino JR (1989) Multidrug-resistance gene (P-glycoprotein) is expressed by endothelial cells at blood-brain barrier sites. *Proceedings of the National Academy of Sciences of the United States of America* **86**:695–698.

Dejucq N (2000) HIV-1 replication in CD4+ T cell lines: the effects of adaptation on co-receptor use, tropism, and accessory gene function. *Journal of Leukocyte Biology* **68**:331–337.

Dejucq N, Simmons G & Clapham PR (1999) Expanded tropism of primary human immunodeficiency virus type 1 R5 strains to CD4 (+) T-cell lines determined by the capacity to exploit low concentrations of CCR5. *Journal of Virology* **73**:7842–7847.

Edwards JE, Alcorn J, Savolainen J, Anderson BD & McNamara PJ (2005) Role of P-glycoprotein in distribution of nelfinavir across the blood-mammary tissue barrier and blood-brain barrier. *Antimicrobial Agents and Chemotherapy* **49**:1626–1628.

Fujii N & Tamamura H (2001) Peptide-lead CXCR4 antagonists with high anti-HIV activity. *Current Opinion in Investigational Drugs* **2**:1198–1202.

Ghezzi S, Menzo S, Brambilla A, Bordignon PP, Lorini AL, Clementi M, Poli G & Vicenzi E (2001) Inhibition of R5X4 dualtropic HIV-1 primary isolates by single chemokine co-receptor ligands. *Virology* **280**:253–261.

Hsu MC, Schutt AD, Holly M, Slice LW, Sherman MI, Richman DD, Potash MJ & Volsky DJ (1991) Inhibition of HIV replication in acute and chronic infections *in vitro* by a Tat antagonist. *Science* **254**:1799–1802.

Kanbe K, Shimizu N, Soda Y, Takagishi K & Hoshino H (1999) A CXC chemokine receptor, CXCR5/BLR1, is a novel and specific coreceptor for human immunodeficiency virus type 2. *Virology* **265**:264–273.

Kim RB, Fromm MF, Wandel C, Leake B, Wood AJ, Roden DM & Wilkinson GR (1998) The drug transporter P-glycoprotein limits oral absorption and brain entry of HIV-1 protease inhibitors. *Journal of Clinical Investigation* **101**:289–294.

Kimpton J & Emerman M (1992) Detection of replication-competent and pseudotyped human immunodeficiency virus with a sensitive cell line on the basis of activation of an integrated beta-galactosidase gene. *Journal of Virology* **66**:2232–2239.

Kodama E, Igarashi A, Mori S, Hashimoto K, Suzuki T, De Clercq E & Shigeta S (1996) Evaluation of antiherpetic compounds using a gastric cancer cell line: pronounced activity of BVDU against herpes simplex virus replication. *Microbiology and Immunology* **40**:359–363.

Kodama EI, Kohgo S, Kitano K, Machida H, Gatanaga H, Shigeta S, Matsuoka M, Ohrui H & Mitsuya H (2001) 4'-Ethynyl nucleoside analogs: potent inhibitors of multidrug-resistant human immunodeficiency virus variants *in vitro*. *Antimicrobial Agents and Chemotherapy* **45**:1539–1546.

Lee B, Sharron M, Montaner LJ, Weissman D & Doms RW (1999) Quantification of CD4, CCR5, and CXCR4 levels on lymphocyte subsets, dendritic cells, and differentially conditioned monocyte-derived macrophages. *Proceedings of the National Academy of Sciences of the United States of America* **96**:5215–5220.

Lee CG, Gottesman MM, Cardarelli CO, Ramachandra M, Jeang KT, Ambudkar SV, Pastan I & Dey S (1998) HIV-1 protease inhibitors are substrates for the MDR1 multidrug transporter. *Biochemistry* **37**:3594–3601.

Liu HY, Soda Y, Shimizu N, Haraguchi Y, Jinno A, Takeuchi Y & Hoshino H (2000) CD4-Dependent and CD4-independent utilization of coreceptors by human immunodeficiency viruses type 2 and simian immunodeficiency viruses. *Virology* **278**:276–288.

Livington DJ, Pazhanisamy S, Porter DJ, Partaledis JA, Tung RD & Painter GR (1995) Weak binding of VX-478 to human plasma proteins and implications for anti-human immunodeficiency virus therapy. *Journal of Infectious Diseases* **172**:1238–1245.

Miyake H, Iizawa Y & Baba M (2003) Novel reporter T-cell line highly susceptible to both CCR5- and CXCR4-using human immunodeficiency virus type 1 and its application to drug susceptibility tests. *Journal of Clinical Microbiology* **41**:2515–2521.

Ostrowski MA, Justement SJ, Catanzaro A, Hallahan CA, Ehler LA, Mizell SB, Kumar PN, Mican JA, Chun TW & Fauci AS (1998) Expression of chemokine receptors CXCR4 and CCR5 in HIV-1-infected and uninfected individuals. *Journal of Immunology* **161**:3195–3201.

Pauwels R, Balzarini J, Baba M, Snoeck R, Schols D, Herdewijn P, Desmyter J & De Clercq E (1988) Rapid and automated tetrazolium-based colorimetric assay for the detection of anti-HIV compounds. *Journal of Virological Methods* **20**:309–321.

Princen K, Hatse S, Vermeire K, De Clercq E & Schols D (2004) Establishment of a novel CCR5 and CXCR4 expressing CD4+ cell line which is highly sensitive to HIV and suitable for high-throughput evaluation of CCR5 and CXCR4 antagonists. *Retrovirology* **1**:2.

Schols D, Struyf S, Van Damme J, Este JA, Henson G & De Clercq E (1997) Inhibition of T-tropic HIV strains by selective antagonization of the chemokine receptor CXCR4. *Journal of Experimental Medicine* **186**:1383–1388.

Schroder AR, Shinn P, Chen H, Berry C, Ecker JR & Bushman F (2002) HIV-1 integration in the human genome favors active genes and local hotspots. *Cell* **110**:521–529.

Schuetz JD, Connelly MC, Sun D, Paibir SG, Flynn PM, Srinivas RV, Kumar A & Fridland A (1999) MRP4: A previously unidentified factor in resistance to nucleoside-based antiviral drugs. *Nature Medicine* **5**:1048–1051.

Schupbach J, Tomasik Z, Nadal D, Ledergerber B, Flepp M, Opravil M & Boni J (2000) Use of HIV-1 p24 as a sensitive, precise and inexpensive marker for infection, disease progression and treatment failure. *International Journal of Antimicrobial Agents* **16**:441–445.

Shimizu N, Haraguchi Y, Takeuchi Y, Soda Y, Kanbe K & Hoshino H (1999) Changes in and discrepancies between cell tropisms and coreceptor uses of human immunodeficiency virus type 1 induced by single point mutations at the V3 tip of the env protein. *Virology* **259**:324–333.

Shimizu N, Soda Y, Kanbe K, Liu HY, Mukai R, Kitamura T & Hoshino H (2000) A putative G protein-coupled receptor, RDC1, is a novel coreceptor for human and simian immunodeficiency viruses. *Journal of Virology* **74**:619–626.

Soda Y, Shimizu N, Jinno A, Liu HY, Kanbe K, Kitamura T & Hoshino H (1999) Establishment of a new system for determination of coreceptor usages of HIV based on the human glioma NP-2 cell line. *Biochemical and Biophysical Research Communications* **258**:313–321.

Villalba N, Gomez-Cano M, Holguin A & Soriano V (1999) Multiple drug resistance genotype causing failure of antiretroviral treatment in an HIV-infected patient heavily exposed to

nucleoside analogues. *European Journal of Clinical Microbiology & Infectious Diseases* **18**:372–375.

Witvrouw M, Pannecouque C, Switzer WM, Folks TM, De Clercq E & Heneine W (2004) Susceptibility of HIV-2, SIV and SHIV to various anti-HIV-1 compounds: implications for treatment and postexposure prophylaxis. *Antiviral Therapy* **9**:57-65.

Witvrouw M, Pauwels R, Vandamme AM, Schols D, Reymen D, Yamamoto N, Desmyter J & De Clercq E (1992) Cell type-specific anti-human immunodeficiency virus type 1 activity of the transactivation inhibitor Ro5-3335. *Antimicrobial Agents and Chemotherapy* **36**:2628–2633.

Wu L, Gerard NP, Wyatt R, Choe H, Parolin C, Ruffing N, Borsetti A, Cardoso AA, Desjardin E, Newman W, Gerard C & Sodroski J (1996) CD4-induced interaction of primary HIV-1 gp120 glycoproteins with the chemokine receptor CCR-5. *Nature* **384**:179–183.

Yoshimura K, Kato R, Yusa K, Kavlick MF, Maroun V, Nguyen A, Mimoto T, Ueno T, Shintani M, Falloon J, Masur H, Hayashi H, Erickson J & Mitsuya H (1999) JE-2147: a dipeptide protease inhibitor (PI) that potently inhibits multi-PI-resistant HIV-1. *Proceedings of the National Academy of Sciences of the United States of America* **96**:8675–8680

Zhu D, Taguchi-Nakamura H, Goto M, Odawara T, Nakamura T, Yamada H, Kotaki H, Sugiura W, Iwamoto A & Kitamura Y (2004) Influence of single-nucleotide polymorphisms in the multidrug resistance-1 gene on the cellular export of nelfinavir and its clinical implication for highly active antiretroviral therapy. *Antiviral Therapy* **9**:929–935.

Received 30 March 2006, accepted 6 June 2006

Structural Basis for the Interaction of CCR5 with a Small Molecule, Functionally Selective CCR5 Agonist

Yuji Saita,[1]* Eiichi Kodama,† Masaya Orita,‡ Mitsuhiro Kondo,§ Takahiro Miyazaki,§ Kenji Sudo,§ Keiko Kajiwara,† Masao Matsuoka,† and Yasuaki Shimizu§

The chemokine receptor CCR5 is an attractive target for HIV-1 drug development, as individuals whose cells lack surface CCR5 expression are highly resistant to HIV-1 infection. CCR5 ligands, such as CCL5/RANTES, effectively inhibit HIV-1 infection by competing for binding opportunities to the CCR5 and inducing its internalization. However, the inherent proinflammatory activity of the chemotactic response of CCR5 ligands has limited their clinical use. In this study, we found that a novel small molecule, functionally selective CCR5 agonist, 2,2-dichloro-1-(triphenylphosphonio)vinyl formamide perchlorate (YM-370749), down-modulates CCR5 from the cell surface without inducing a chemotactic response and inhibits HIV-1 replication. In molecular docking studies of YM-370749 and a three-dimensional model of CCR5 based on the rhodopsin crystal structure as well as binding and functional studies using various CCR5 mutants, the amino acid residues necessary for interaction with YM-370749 were marked. These results provide a structural basis for understanding the activation mechanism of CCR5 and for designing functionally selective agonists as a novel class of anti-HIV-1 agents. *The Journal of Immunology,* **2006, 177: 3116–3122.**

The introduction of highly active antiretroviral therapy (HAART),[2] comprising two nucleoside/nucleotide reverse-transcriptase inhibitors plus a nonnucleoside reverse-transcriptase inhibitor or a protease inhibitor, has markedly decreased HIV-associated morbidity and mortality (1). However, there are still several problems with HAART. First, complicated regimens with large numbers of pills reduce long-term adherence to HAART. Second, adverse drug-associated effects require treatment modification or cessation (2). Finally, incomplete eradication of HIV-1 favors the emergence of multidrug-resistant strains (3). Therefore, the discovery of novel anti-HIV-1 agents with new mechanisms of action is still needed to increase the efficacy with which HIV replication is inhibited and delay the emergence of drug-resistant variants.

The β-chemokine receptor CCR5 belongs to the rhodopsin-type, G protein-coupled receptor (GPCR) superfamily (4) and is a major coreceptor for HIV-1 (5–10). Because individuals who are homozygous for the *CCR5Δ32* mutation do not express CCR5 on the cell surface and are highly resistant to macrophage-tropic HIV-1 infection (11, 12), CCR5 seems to be an attractive target for anti-HIV-1 drug development (13). In fact, low molecular weight CCR5 antagonists inhibited HIV-1 replication by disturbing the HIV adsorption step (14). Alternatively, CCR5 agonists such as CCL5/RANTES also inhibited HIV replication by two mechanisms: disturbing HIV adsorption to CCR5 and inducing CCR5 internalization (15, 16). Although peptide agonists for CCR5 are candidates for a new class of anti-HIV agent, they are not orally available and, moreover, have undesirable proinflammatory activities that are likely to induce adverse side effects (17).

In this study, we show that a small molecule compound, 2,2-dichloro-1-(triphenylphosphonio)vinyl formamide perchlorate (YM-370749), did not stimulate undesirable chemotactic activity but induced internalization of CCR5 from the cell surface. These results suggest that CCR5 active states induced by YM-370749 are different from those induced by CCL5. Our molecular model of the YM-370749–CCR5 complex provides a structural basis for understanding the activation mechanism of CCR5 as well as for designing a novel anti-HIV-1 agent.

Materials and Methods

Wild-type and mutant CCR5 expression

The murine pre-B cell line B300-19 was cultured in RPMI 1640 medium containing 10% FBS, 50 μM 2-mercaptethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin. The expression vector pEF-BOS-Neo (18), carrying full-length CCR5 cDNA, was transfected into B300-19 cells by electroporation, and G418-resistant stable transformants were isolated. CCR5 mutants were generated by PCR using primers bearing the relevant specific mutations.

Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) elevation assay

Cells were incubated with 5 μM fura-2 acetoxymethyl ester (Dojindo Laboratories) in HEPES-buffered salt solution (20 mM HEPES (pH 7.4), 140 mM NaCl, 4 mM KCl, 1 mM K_2HPO_4, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, and 0.05% BSA) at 37°C for 45 min. After washing, $[Ca^{2+}]_i$ was analyzed using CAF-110 (Jasco). Pertussis toxin (Alexis Biochemicals) was used at a concentration of 100 ng/ml and added 17 h before the agonists. The CCR5-selective antagonist SCH-351125 (19) was synthesized at Astellas Pharma and added 2 min before the agonists.

[^{35}S]GTPγS-binding assay

Cell membranes (5 μg/ml protein) were incubated at 25°C for 1.5 h with 150 pM [^{35}S]GTPγS (Amersham Biosciences), 5 mg/ml wheat germ agglutinin scintillation proximity assay beads, and 2 μM GDP in the presence

*Molecular Medicine Research Laboratories, Institute for Drug Discovery Research, Astellas Pharma Inc., Ibaraki, Japan; †Laboratory of Virus Immunology, Research Center for AIDS, Institute for Virus Research, Kyoto University, Kyoto, Japan; ‡Lead Discovery Research Laboratories, Institute for Drug Discovery Research, Astellas Pharma Inc., Ibaraki, Japan; and §Pharmacology Research Laboratories, Institute for Drug Discovery Research, Astellas Pharma Inc., Osaka, Japan

Received for publication October 26, 2005. Accepted for publication June 16, 2006.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Address correspondence and reprint requests to Dr. Yuji Saita, Molecular Medicine Research Laboratories, Institute for Drug Discovery Research, Astellas Pharama Inc., 21 Miyukigaoka, Tsukuba, Ibaraki 305-8585, Japan. E-mail address: yuuji.saita@jp.astellas.com

[2] Abbreviations used in this paper: HAART, highly active antiretroviral therapy; B300-19/CCR5, B300-19 cells expressing CCR5; βGal, β-galactosidase; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; EGFP, enhanced GFP; FluV-A, influenza virus type A; GPCR, G protein-coupled receptor; LTR, long-terminal repeat; PHAM, PHA M; TM, transmembrane; YM-370749, 2,2-dichloro-1-(triphenylphosphonio)vinyl formamide perchlorate; WT, wild type.

0022-1767/06/$02.00

or absence of various concentrations of unlabelled ligands in incubation buffer (20 mM HEPES-NaOH (pH 7.05), 100 mM NaCl, 5 mM $MgCl_2$, and 0.2% (w/v) BSA). Radioactivity was counted using a TopCount scintillation counter (Packard Biosciences).

Flow cytometric analysis of CCR5 internalization

Cells were incubated for 3 h with different concentrations of CCL5 (PeproTech) or YM-370749 (Sigma-Aldrich Library of Rare Chemicals), immediately placed on ice, and washed with ice-cold FACS buffer (PBS, 1% FBS, and 0.1% NaN_3). The cells were stained with anti-CCR5 Ab (2D7; BD Pharmingen) or an isotype-matched control Ab, placed on ice for 30 min, washed twice with FACS buffer, and analyzed for levels of surface expression of CCR5 using a flow cytometer (FACSCalibur; BD Biosciences). Data analysis was conducted using CellQuest software (BD Pharmingen). Relative fluorescence intensity was calculated as follows: [mean channel fluorescence (ligands) − mean channel fluorescence (negative control)]/[mean channel fluorescence (medium) − mean channel fluorescence (negative control)].

Fluorescence microscopy

A PCR-synthesized β-arrestin-2 cDNA insert deleted from the stop codon was fused to the enhanced GFP (EGFP; BD Clontech) in the pEF-BOS-Neo expression vector. FuGENE 6 transfection reagent (Roche) caused the transient expression of CCR5, β-arrestin-2-EGFP, and G protein receptor kinase 2 in COS-1 cells. The cells were incubated in DMEM containing 0.1% (w/v) BSA for 12 h before treatment with 100 nM CCL5 or 30 μM YM-370749 for 1 h at 37°C. After being washed with ice-cold PBS, the cells were fixed in PBS containing 3% paraformaldehyde for 30 min. The samples were analyzed using confocal laser-scanning microscopy and a LSM5 PASCAL system (Zeiss).

Chemotaxis assay

Chemotaxis assays were performed using 96-well chemotaxis chambers (Neuro Probe) with 5-μm pore, polyvinylpyrrolidone-free, polycarbonate filters for 3 h at 37°C in a humidified 5% CO_2 incubator. Cells suspended at 5×10^6 cells/ml in RPMI 1640 supplemented with 0.1% (w/v) BSA were applied to the upper wells, with various concentrations of CCL5 or YM-370749 in the lower wells. Cell numbers migrating to the lower chambers were quantified by bioluminescent assay (ATP-Lite; PerkinElmer).

Antiviral assays

PBMC were stimulated for 3 days with PHA M (PHAM; 10 μg/ml; Sigma-Aldrich). PHAM-PBMC (10^6 cells/ml) were infected with HIV-1_{BaL} (R5 strain) or HIV-1_{HXB2} (X4 strain) equivalent to 1 ng/ml p24 in the presence of various concentrations of YM-370749. After 7 days incubation, the amounts of p24 Ag produced by the cells into the culture medium were determined by an enzyme immunoassay kit (Cellular Products).

CD4, CCR5, and long-terminal repeat (LTR)-controlled β-galactosidase (βGal) expression vectors were transfected into human glioma-derived NP-2 cells (NP-2/CD4/CCR5-LTR-βGal cells). NP-2/CD4/CCR5-LTR-βGal cells were infected with HIV-1_{BaL} or four clinical isolates (401, 409, 411, and 415) as described previously (20) and cultured in the presence of various concentrations of YM-370749. Forty-eight hours after viral exposure, all of the cells stained blue with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside were counted. Cytotoxicity was determined by the MTT method as previously described (21).

Anti-viral activity against influenza virus type A (FluV-A) (Ishikawa/7/82 (H3N2); Rational Drug Design Laboratories) and HSV-1 (KOS strain; Rational Drug Design Laboratories) were determined using plaque-reduction assays (22). Madin-Darby canine kidney cells for anti-FluV-A assay and Vero cells for anti-HSV-1 assay were grown in Eagle's MEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. The culture medium was removed and replaced with a maintenance medium consisting of Eagle's MEM supplemented with 2% FBS and antibiotics, and viruses (100 PFU) were added. After a 1-h virus adsorption period, the cells were washed and filled with maintenance medium containing 0.8% methylcellulose. After a 4-day incubation for anti-influenza virus assay and a 3-day incubation for anti-HSV-1 assay, the number of plaques was counted. YM-370749 was included throughout the assay.

YM-370749 and CCL5 binding studies

Cell membranes (5 μg/ml protein) were incubated at 25°C for 3 h with 50 pM ^{125}I-labeled CCL3 (PerkinElmer) and 5 mg/ml wheat germ agglutinin scintillation proximity assay beads (Amersham Biosciences) in the presence or absence of various concentrations of unlabeled ligands in incubation buffer containing 50 mM HEPES (pH 7.4), 5 mM $MgCl_2$, 1 mM

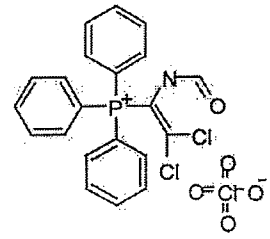


FIGURE 1. Two-dimensional structure of YM-370749. Molecular weight, 501; purity, 99% as determined by HPLC.

$CaCl_2$, and 0.1% (w/v) BSA. Radioactivity was counted using a TopCount scintillation counter (Packard Biosciences). Nonspecific binding was determined in the presence of 50 nM CCL3. Absolute inhibition constant (K_i) values were calculated from the IC_{50} using the equation of Cheng and Prusoff (23).

Molecular modeling and docking study

We created a three-dimensional model of the human CCR5 based on the sequence alignment and the crystal structure of bovine rhodopsin (24) using the MOE software package (Chemical Computing Group). The docking of YM-370749 with this model was performed using the GOLD program (25).

Results

YM-370749 is a functionally selective CCR5 agonist

To identify small molecule CCR5 agonists that induced CCR5 internalization, but not chemotaxis, in CCR5-expressing B300-19

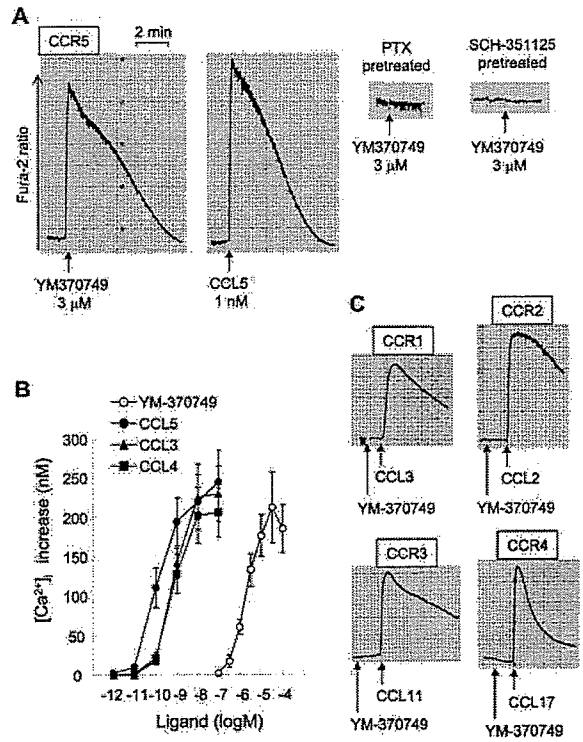


FIGURE 2. Increase in CCR5-mediated $[Ca^{2+}]_i$ caused by YM-370749. *A*, $[Ca^{2+}]_i$ increase in B300-19/CCR5 cells. YM-370749 or CCL5 were added to the B300-19/CCR5 cells, and $[Ca^{2+}]_i$ was measured using fura-2 fluorometry. Pertussis toxin (PTX; 100 ng/ml) and SCH-351125 (1 μM) were added 17 h and 2 min, respectively, before the addition of YM-370749. *B*, Concentration dependence of the initial transient increase in $[Ca^{2+}]_i$. Values are the mean ± SE of five separate experiments. *C*, $[Ca^{2+}]_i$ increase in B300-19/CCR1, CCR2, CCR3, or CCR4 cells. YM-370749 (30 μM) and the natural ligands (1 nM) were added.

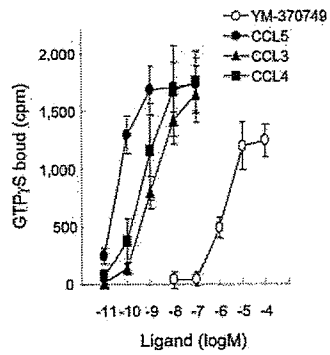


FIGURE 3. Concentration dependence of GTPγS binding. Values are the mean ± SE of three separate experiments.

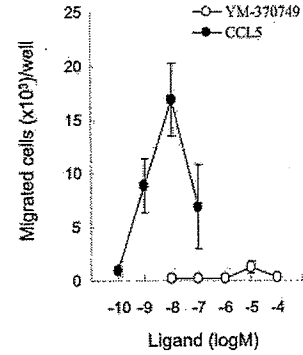


FIGURE 5. Chemotactic effects of CCL5 and YM-370749 on B300-19/CCR5 cells. Values are the mean ± SE of four separate experiments.

(B300-19/CCR5) cells, we conducted a large-scale, high-throughput screening of in-house chemical libraries by measuring $[Ca^{2+}]_i$ and discovered that YM-370749 (Fig. 1) had the required properties. A low molecular weight compound, YM-370749, elevated $[Ca^{2+}]_i$ in B300-19/CCR5 cells, which was also the case with the natural CCR5 ligand CCL5 (Fig. 2*A*). The $[Ca^{2+}]_i$ increase elicited by YM-370749 was completely inhibited by pretreatment with 100 ng/ml pertussis toxin, indicating that this compound signaled through a G_i protein-coupled receptor, and it was also inhibited by the CCR5-selective antagonist SCH-351125 (Fig. 2*A*). The increase of $[Ca^{2+}]_i$ by YM-370749 was concentration-dependent with an EC_{50} of 2.1 μM (Fig. 2*B*). This compound at 30 μM had no effect against CCR1-, CCR2-, CCR3-, or CCR4-expressing cells (Fig. 2*C*). YM-370749 also induced GTPγS binding to B300-19/CCR5 cell membranes in a concentration-dependent manner with an EC_{50} of 1.4 μM (Fig. 3).

It is well known that chemokines induce the internalization of their receptors. We analyzed the effects of YM-370749 on CCR5 internalization in B300-19/CCR5 cells. The cell surface CCR5 was measured by FACS using the CCR5-specific mAb 2D7. As shown in Fig. 4, *A* and *B*, YM-370749 induced CCR5 internalization in a concentration-dependent manner. YM-370749 did not affect the cell surface CCR5 level when the experiments were done at 4°C, which suggests that YM-370749 did not compete for 2D7 binding to CCR5. Next, YM-370749-induced CCR5 internalization was assessed by fluorescent microscopy using COS-1 cells transiently expressing CCR5, β-arrestin-2-EGFP, and G protein receptor kinase 2 (Fig. 4, *C–F*). It has been reported that β-arrestin-2 mediates CCR5 internalization (26) and that activation of CCR5 by chemokines results in the colocalization of the receptor and β-arrestin-2 (27). In the absence of ligands, the fluorescence was detected mainly at the cell surface (Fig. 4*C*). As is the case for CCL5, the addition of YM-370749 induced a profound redistribution of the staining that is consistent with intracellular accumulation of the receptor (Fig. 4, *D* and *E*). The effect of YM-370749 was inhibited by pretreating the cells with the CCR5 antagonist SCH-351125 (Fig. 4*F*).

In contrast to CCL5, YM-370749 did not stimulate significant chemotaxis in B300-19/CCR5 cells at any of the concentrations tested (Fig. 5). Pretreating the cells with YM-370749 was shown to

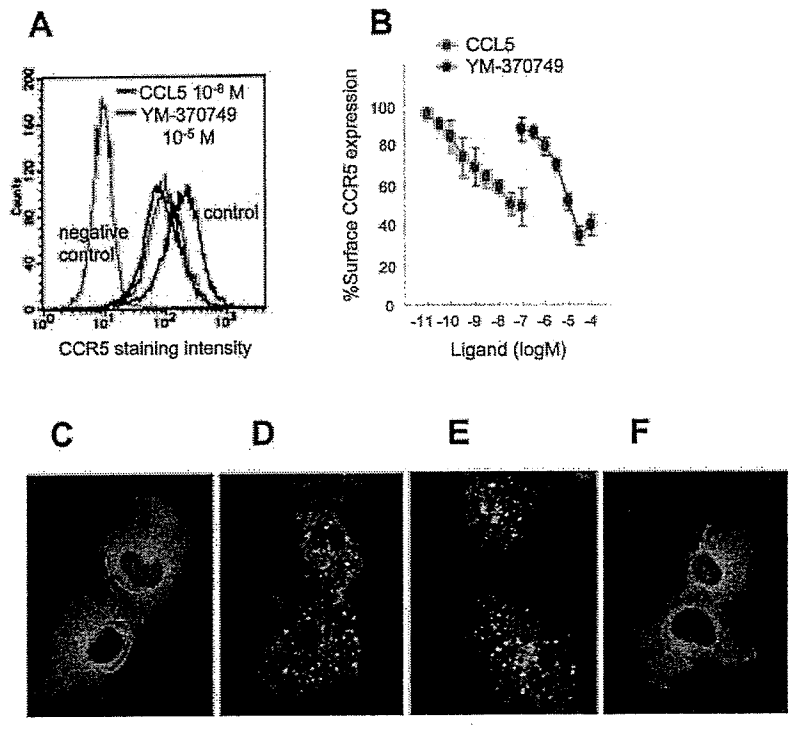


FIGURE 4. Down-modulation of CCR5 from the cell surface. *A*, Detection of cell surface CCR5 on B300-19/CCR5 cells using FACS. An isotype-matched mAb was used as the negative control. *B*, Concentration dependence of CCR5 down-modulation in B300-19/CCR5 cells. Values are the mean ± SE of three separate experiments. *C–F*, Fluorescence detection of β-arrestin-2-EGFP using confocal laser scanning microscopy. *C*, Untreated control. *D*, Cells treated with 100 nM CCL5. *E*, Cells treated with 30 μM YM-370749. *F*, Cells pretreated with 1 μM SCH-351125 for 15 min before the addition of 30 μM YM-370749.

Table I. *Anti-viral activities of YM-370749*[a]

Virus Type	Assay	IC_{50} (μM)	CC_{50}[b] (μM)
HIV-1 *BaL*	p24 ELISA	0.20	2.2
HIV-1 *HXB2*	p24 ELISA	>2.0	
HIV-1 *BaL*	β-Galactosidase	1.0 ± 0.05	16 ± 3.6
HIV-1 *401*	β-Galactosidase	1.1 ± 0.04	
HIV-1 *409*	β-Galactosidase	1.5 ± 0.15	
HIV-1 *411*	β-Galactosidase	0.74 ± 0.06	
HIV-1 *415*	β-Galactosidase	0.50 ± 0.08	
FluV-A *Ishikawa782*	Plaque reduction	>30	>30
HSV-1 *KOS*	Plaque reduction	>30	>30

[a] PBM were stimulated with PHAM (10 μg/ml) for 3 days and then infected with R5 strain BaL or X4 strain HXB2 in the presence of various concentrations of YM-370749. Viral production was determined 7 days after the infection by measuring p24 in culture supernatants using ELISA kit. The data are derived from a single assay. NP-2/CD4/CCR5-LTR-βGal cells were exposed to either HIV-1_{BaL} or four clinical isolates (401, 409, 411, and 415) in the presence of various concentrations of YM-370749. Viral infection was confirmed 2 days after exposure to the virus by counting the cells stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. Values are the mean ± SE of three separate experiments. Madin-Darby canine kidney and Vero cells were infected with FluV-A and HSV-1, respectively. The virus was washed away after 1 h. After incubation for 4 days for the anti-FluV-A assay and incubation for 3 days for the anti-HSV-1 assay, the number of plaques was counted. YM-370749 was included throughout the assay.

[b] Cytotoxic concentrations of a compound that reduces the number of cells by 50%.

inhibit CCL5-induced chemotaxis (data not shown). We determined whether YM-370749 had any potential cytostatic or cytotoxic effects in the chemotaxis assay. The cells were incubated in RPMI 1640 medium supplemented with 0.1% BSA for 3 h in the presence or absence of 10^{-4} M YM-370749 under the same conditions as those used in chemotaxis assay. The cells were then cultured in RPMI 1640 supplemented with 10% FBS for 24 h before determination of the viable cell number. YM-370749 did not affect the cell growth. Taken together, these results suggest that YM-370749 is a functionally selective CCR5 agonist.

YM-370749 selectively inhibits the replication of R5 strain HIV-1

We examined the antiviral activities of YM-370749 against several HIV-1 strains including BaL, HXB2, four clinical isolates (401, 409, 411, and 415), FluV-A, and HSV-1. In the p24 ELISA using PHAM-PBMC, YM-370749 selectively inhibited the replication of the R5 strain HIV-1_{BaL} (IC_{50} = 0.20 μM), which uses CCR5 as a coreceptor. It did not, however, inhibit the X4 strain HIV-1_{HxB2}, which uses CXCR4 as a coreceptor (Table I). In the βGal assay, which does not allow the infection of X4 strain viruses (20), YM-370749 inhibited the infection of NP-2/CD4/CCR5-LTR-βGal cells by HIV-1_{BaL} and the four clinical isolates with similar potency (IC_{50} = 0.50–1.5 μM). As expected, at concentrations up to 30 μM YM-370749 did not exhibit any antiviral activity against FluV-A or HSV-1. These results suggest that the anti-HIV-1 activity of YM-370749 is specifically represented as interaction with CCR5.

Molecular model of the YM-370749–CCR5 complex

Structural analysis of the molecular complex between YM-370749 and human CCR5 will provide useful information for understanding the mechanism of CCR5 activation and the future drug design of a novel anti-HIV agent. We initially compared the binding affinity of YM-370749, CCL5, and CCL3 for both human and rhesus monkey CCR5. Rhesus CCR5 differs from human CCR5 by eight substitutions of the 352 total amino acid residues. We found that CCL5 and CCL3 bound to both human and rhesus monkey CCR5 with the same affinity, whereas YM-370749 bound to rhesus CCR5 11 times more weakly than to human CCR5 (Table II). To determine which amino acids were involved in YM-370749 binding, we created eight human CCR5 mutants, each containing one of the eight amino acid substitutions found in rhesus monkey CCR5: I9T, N13D, M49I, I52V, F78L, V130I, K171R, or I198M. The expression level of these mutated receptors on the B300-19 cell surface, as determined by FACS analysis, was comparable to that of wild-type (WT) CCR5, and a global conformational change caused by these mutations is unlikely given that that all mutants are equally recognized by four different mAbs specific to CCR5 (data not

Table II. *Binding affinities of YM-370749, CCL5, and CCL3 for human WT, rhesus WT, and various mutant human CCR5 receptors*

	YM-370749[a]		CCL5[a]		CCL3[a]	
Mutants	K_i (μM)	Ratio	K_i (pM)	Ratio	K_i (pM)	Ratio
Human WT	2.7 ± 0.16	1.0	48 ± 7.3	1.0	200 ± 14	1.0
Rhesus WT	29 ± 2.7[b]	11	21 ± 2.5	0.44	180 ± 16	0.9
Human I9T	2.7 ± 0.29	1.0	18 ± 4.8	0.37	240 ± 23	1.2
Human N13D	4.1 ± 0.46	1.5	52 ± 42	1.1	210 ± 17	1.1
Human M49I	3.0 ± 0.10	1.1	18 ± 5.3	0.38	230 ± 4.2	1.2
Human I52V	2.1 ± 0.21	0.79	24 ± 7.9	0.50	200 ± 13	1.0
Human F78L	2.6 ± 0.23	0.99	18 ± 6.5	0.37	190 ± 5.1	0.95
Human V130I	3.2 ± 0.17	1.2	12 ± 3.4	0.25	220 ± 13	1.1
Human K171R	4.6 ± 0.67	1.7	42 ± 5.3	0.87	190 ± 15	0.95
Human I198M	19 ± 2.5[b]	7.0	49 ± 14	1.0	220 ± 4.1	1.1

[a] K_i values are given for YM-370749, CCL5, and CCL3 inhibition of ^{125}I-labeled CCL3 binding to B300-19/CCR5 cell membranes. K_i values represent the mean ± SE of three experiments. The ratio given is relative to the K_i for human wild-type CCR5.

[b] Significant differences from human WT CCR5 with $p < 0.01$ (Dunnet's multiple comparison test).

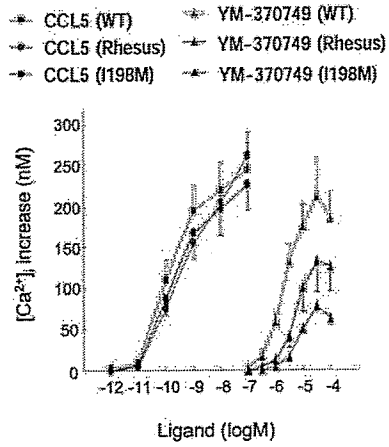


FIGURE 6. Comparison of $[Ca^{2+}]_i$ increase by YM-370749 in B300-19/human CCR5, rhesus CCR5, and human CCR5-I198M cells. Concentration dependence of an initial transient increase in $[Ca^{2+}]_i$ induced by YM-370749 and CCL5 in B300-19/human CCR5 (WT), B300-19/rhesus CCR5 (Rhesus), and B300-19/human-I198M cells (I198M) are shown. Values are the mean ±SE of five separate experiments.

shown). The binding affinities of CCL5 and CCL3 for all of the mutants were similar to or slightly higher than that of WT CCR5. The binding affinities of YM-370749 for these mutants were also comparable to that of the WT receptor, with the exception of CCR5-I198M; the affinity of YM-370749 for CCR5-I198M was 19 μM as compared with 2.7 μM for WT CCR5, which was a significant 7.0-fold reduction (Table II). We next examined the $[Ca^{2+}]_i$ elevation activity of YM-370749 in B300-19/CCR5, B300-19/CCR5-I198M, and B300-19/rhesus CCR5 cells (Fig. 6). The $[Ca^{2+}]_i$ elevation activity of CCL5 in B300-19/CCR5 cells was almost similar to those in B300-19/CCR5-I198M, and B300-19/rhesus CCR5 cells. However, YM-370749 could not reach the same maximum efficacy in B300-19/CCR5-I198M (62% elevation) or B300-19/rhesus CCR5 cells (37% elevation) as in B300-19/CCR5 cells, and it was less potent in B300-19/CCR5-I198M

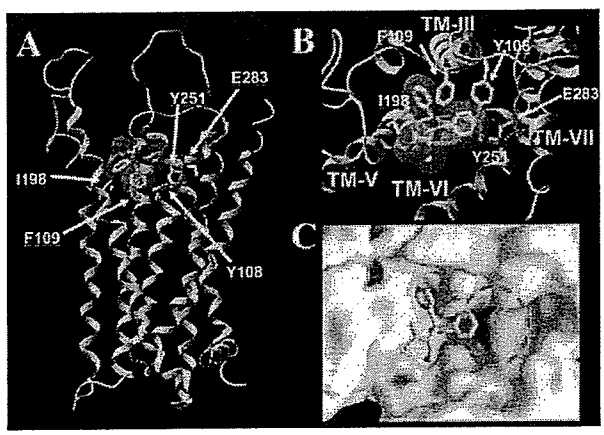


FIGURE 7. Structural model of the human CCR5-YM-370749 complex. *A*, Ribbon diagram. Arrows indicate the side chains of the key residues Tyr^{108} (Y108), Phe^{109} (F109), Ile^{198} (I198), Tyr^{251} (Y251), and Glu^{283} (E283). YM-370749 is shown as a stick model with the carbon atoms colored yellow and the van der Waals surface colored light green. *B*, Close-up view along the helical axes from the extracellular side. *C*, Surface representation of the binding pocket of human CCR5. The surface is colored according to the local electrostatic potential (56) (negative in red and positive in blue). The dashed red circle indicates the negatively charged region around Glu^{283}.

(EC_{50} = 4.7 μM) and B300-19/rhesus CCR5 cells (EC_{50} = 6.2 μM) than in B300-19/CCR5 cells (EC_{50} = 2.1 μM). These results implied that the Ile^{198} in CCR5 is an important residue for the interaction with YM-370749.

To further investigate the molecular interaction between YM-370749 and CCR5, we conducted a docking study with YM-370749 and a three-dimensional model of CCR5, which was based on the rhodopsin crystal structure. As shown in Fig. 7*A*, the proposed binding site for YM-370749 is located in a pocket surrounded by the human CCR5 transmembrane (TM) helices III, V, VI, and VII. Each of the three hydrophobic benzene groups of YM-370749 seem to contribute to the van der Waals interactions with the side chains of the hydrophobic residues, contacting Tyr^{108} and Phe^{109} in TM-III, Ile^{198} in TM-V, and Tyr^{251} in TM-VI (Fig. 7*B*). The importance of Ile^{198} was supported by the CCR5 mutational analysis described above. The docking study also pointed to an electrostatic interaction between the partially positively charged phenyl ring in YM-370749 and the negatively charged carboxyl group of Glu^{283} in TM-VII (Fig. 7*C*).

Discussion

To date, small molecule agonists to GPCR for small peptide ligands (<20 amino acid residues) such as cholecystokinin (28), angiotensin (29), somatostatin (30), and opioid (31), were reported. However, small molecule agonists to GPCR for high molecular weight protein ligands, such as chemokines (66, 69, and 68 amino acid residues for CCL3, CCL4, and CCL5, respectively) have not been reported. In this study, we showed that YM-370749 exerted several agonistic activities to CCR5, including intracellular Ca^{2+} elevation, GTPγS binding, and receptor down-modulation (Figs. 2–4). These agonistic activities by YM-370749 were mediated directly through CCR5, as evidenced by the observations that YM-370749 did not show any activity in B300-19/CCR1–4, and the agonistic activities of YM-370749 in B300-19/CCR5 such as $[Ca^{2+}]_i$ elevation and receptor down-modulation were completely inhibited by the CCR5-selective antagonist SCH-351125 (Figs. 2*A* and 4*F*). This is the first report regarding a small molecule agonist to GPCR for large peptides like chemokine.

Interestingly, in contrast to CCL5, YM-370749 did not stimulate undesirable chemotaxis. The fact that YM-370749 induced pertussis toxin-sensitive Ca^{2+} elevation, but not chemotaxis, indicated that the level of G_i protein activation was not sufficient for chemotaxis. These results suggest that YM-370749 and CCL5 stabilized different active conformations of CCR5, which then coupled with different intracellular signaling cascades. YM-370749 has been reported to be a kind of active state selective agonist (32) for mAbs against CCR5 (33), calcitonin ligands (34), and cholecystokinin analog (35). Because chemokine receptor activation stimulates various intracellular signaling events, including those involving the Jak/signaling transducer and transcription activator (36), PI3K (37), Syk (38), and Pyk2 (39), it will be interesting to see whether YM-370749 could stimulate these signaling events as well.

CCR5 antagonists that act by inhibiting the binding of HIV to CCR5 could induce drug-resistant variants in vitro (40). If a functionally selective agonist like YM-370749 showed anti-HIV activity by inducing receptor internalization, the rapid emergence of drug-resistant HIV variants might be avoided. Additionally, it has been reported that the cell surface CCR5 density is critical for HIV-1 infection (41, 42) and that receptor internalization makes the greater contribution to antiviral activity by CCR5 ligands (43). Compounds that promote internalization of CCR5 without undesirable proinflammatory activity are ideal agents against HIV-1 infection. YM-370749 selectively inhibited the replication of R5

strain HIV-1 but did not inhibit the X4 strain HIV-1 (Table I). These results suggest that YM-370749 inhibited HIV-1 replication by inducing CCR5 internalization and/or inhibiting HIV binding. Although the extent of CCR5 internalization caused by YM-370749 in B300-19/CCR5 cells was modest (Fig. 4*B*), YM-370749 may induce CCR5 internalization more efficiently in PBM or NP-2 cells. Internalization of CCR5 has been reported to be affected by the expression levels of the cellular β-arrestins and G protein receptor kinase (26). Alternatively, the anti-HIV-1 activity might be attributable to the modest effect on CCR5 internalization. This is because the reduced expression level of CCR5 in *CCR5Δ32* heterozygotes is known to be responsible for the slower progression of HIV-1 infection (44). Alternatively, Reeves et al. (45) reported that cell susceptibility to R5 virus infection in other in vitro systems is unaffected by changes in CCR5 density >10-fold. Further study will be required to clarify whether the antiviral activity of YM-370749 is dependent on CCR5 internalization. The IC_{50} value (0.2 μM) obtained for the antiviral assay using PHAM-PBMC was lower than the IC_{50} values (0.5–1.5 μM) obtained for the antiviral assay using NP-2/CD4/CCR5-LTR-βGal cells and the EC_{50} values obtained for the functional assays using B300-19/CCR5 cells (2.1 and 1.4 μM for the $[Ca^{2+}]_i$ and GTPγS assays, respectively). Lengthy incubation periods with YM-370749 and/or a lower CCR5 density on the PBM cell surface compared with that on NP-2/CD4/CCR5-LTR-βGal cells and B300-19/CCR5 cells might cause a more effective down-modulation and/or occupation of CCR5 by YM-370749. However, cytotoxic (concentrations of 2.2 μM for PHAM-PBMC and 16 μM for NP-2/CD4/CCR5; see Table I), cytostatic, or other effects caused by YM-370749 might affect the antiviral activity. Further study will be required to clarify that the antiviral activity of YM-370749 is directly mediated through CCR5.

Structural analysis is useful for understanding the activation mechanism of CCR5 and for designing the novel anti-HIV-1 agent. The binding and $[Ca^{2+}]_i$ study using the I198M mutant clearly showed that Ile^{198} was involved in the interaction with YM-370749 (Table II and Fig. 6). Billick et al. (46) reported that I198M mutation abrogated HIV-1 entry inhibition by SCH-351125 and suggested that the region of CCR5 near Ile^{198} has an important influence on the conformational state of this receptor. It is tempting to speculate that interaction of YM-370749 with Ile^{198} induces a conformational change of CCR5 that leads to the activation.

Docking studies using YM-370749 and a three-dimensional model of CCR5 showed that the binding site of YM-370749 is located in a pocket surrounded by a transmembrane domain (Fig. 7, *A* and *B*). Considering that CCL5 interacts with the N-terminal extracellular domain and the second extracellular loop of CCR5 (47), our results suggest that the binding site of YM-370749 is quite different from that of CCL5, and it would explain the functional difference between YM-370749 and CCL5. The docking studies also pointed out that the binding of YM-370749 is mediated by an electrostatic interaction with Glu^{283} in TM-VII and by hydrophobic interactions with the Tyr^{108} and Phe^{109} in TM-III, Ile^{198} in TM-V, and Tyr^{251} in TM-VI on CCR5. Among the five amino acid residues at which we showed the interaction with YM-370749 in the docking studies, the Phe^{109} is not conserved in other chemokine receptor subtypes (Tyr in CCR1, His in CCR2 and CCR3, and Leu in CCR4). The Phe^{109} might therefore contribute to the chemokine receptor selectivity of YM-370749.

The Glu^{283} is well conserved among chemokine receptors and is involved in CCR5 antagonist binding (48, 49). Analogous glutamate residues within CCR1, CCR2, and CXCR4 have been shown to be important for small molecule antagonist binding (50–52). Our results suggest that YM-370749 also uses common electrostatic interactions between the antagonist and a common acidic amino acid in TM-VII of the receptors. The crystal structure of the transcription regulator BmrR complex with tetraphenylphosphonium, the structure of which is similar to that of YM-370749 (53), will explain that the positive charge on the phenyl ring in YM-370749 is due to the phosphorus atom and makes an electrostatic interaction with Glu^{283}.

Govaerts et al. (54, 55) has reported that mutations of Tyr^{108} and other aromatic residues located in CCR5 TM-II and TM-III differentially affected the functional response to various natural ligands without significantly altering their binding affinity and suggested that the amino acid residues were involved in the activation of the receptor. YM-370749 may exert its agonistic activity through the interaction with Tyr^{108}. Further studies will be needed to understand more precisely the molecular details of CCR5 active states induced by YM-370749.

In summary, YM-370749 is a small molecule, functionally selective agonist for the human CCR5 that can inhibit the replication of HIV-1. Insights into the molecular basis for the interaction of CCR5 with YM-370749 will provide structural basis for understanding the CCR5 activation mechanism and for designing ideal anti-HIV-1 agents that promote internalization of CCR5 without proinflammatory activity.

Acknowledgments

We thank Dr. Shigekazu Nagata (Osaka University, Osaka, Japan) for providing pEF-BOS and Dr. H. Hoshino (Gunma University, Maebashi, Japan) for providing the NP-2 cells derived from human glioma.

Disclosures

The authors have no financial conflict of interest.

References

1. Carpenter, C. C., M. A. Fischl, S. M. Hammer, M. S. Hirsch, D. M. Jacobsen, D. A. Katzenstein, J. S. Montaner, D. D. Richman, M. S. Saag, R. T. Schooley, et al. 1998. Antiretroviral therapy for HIV infection in 1998: updated recommendations of the International AIDS Society-USA panel. *J. Am. Med. Assoc.* 280: 78–86.
2. Montessori, V., N. Press, M. Harris, L. Akagi, and J. S. Montaner. 2004. Adverse effects of antiretroviral therapy for HIV infection. *Can. Med. Assoc. J.* 170: 229–238.
3. Siliciano, J. D., J. Kajdas, D. Finzi, T. C. Quinn, K. Chadwick, J. B. Margolick, C. Kovacs, S. J. Gange, and R. F. Siliciano. 2003. Long-term follow-up studies confirm the stability of the latent reservoir for HIV-1 in resting $CD4^+$ T cells. *Nat. Med.* 9: 727–728.
4. Strader, C. D., T. M. Fong, M. R. Tota, D. Underwood, and R. A. Dixon. 1994. Structure and function of G protein-coupled receptors. *Annu. Rev. Biochem.* 63: 101–132.
5. Alkhatib, G., C. Combadiere, C. C. Broder, Y. Feng, P. E. Kennedy, P. M. Murphy, and E. A. Berger. 1996. CC CKR5: a RANTES, MIP-1α, MIP-1β receptor as a fusion cofactor for macrophage-tropic HIV-1. *Science* 272: 1955–1958.
6. Deng, H., R. Liu, W. Ellmeier, S. Choe, D. Unutmaz, M. Burkhart, P. Di Marzio, S. Marmon, R. E. Sutton, C. M. Hill, et al. 1996. Identification of a major co-receptor for primary isolates of HIV-1. *Nature* 381: 661–666.
7. Choe, H., M. Farzan, Y. Sun, N. Sullivan, B. Rollins, P. D. Ponath, L. Wu, C. R. Mackay, G. LaRosa, W. Newman, et al. 1996. The β-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. *Cell* 85: 1135–1148.
8. Feng, Y., C. C. Broder, P. E. Kennedy, and E. A. Berger. 1996. HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science* 272: 872–877.
9. Doranz, B. J., J. Rucker, Y. Yi, R. J. Smyth, M. Samson, S. C. Peiper, M. Parmentier, R. G. Collman, and R. W. Doms. 1996. A dual-tropic primary HIV-1 isolate that uses fusin and the β-chemokine receptors CKR-5, CKR-3, and CKR-2b as fusion cofactors. *Cell* 85: 1149–1158.
10. Dragic, T., V. Litwin, G. P. Allaway, S. R. Martin, Y. Huang, K. A. Nagashima, C. Cayanan, P. J. Maddon, R. A. Koup, J. P. Moore, and W. A. Paxton. 1996. HIV-1 entry into $CD4^+$ cells is mediated by the chemokine receptor CC-CKR-5. *Nature* 381: 667–673.
11. Liu, R., W. A. Paxton, S. Choe, D. Ceradini, S. R. Martin, R. Horuk, M. E. MacDonald, H. Stuhlmann, R. A. Koup, and N. R. Landau. 1996. Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. *Cell* 86: 367–377.
12. Samson, M., F. Libert, B. J. Doranz, J. Rucker, C. Liesnard, C. M. Farber, S. Saragosti, C. Lapoumeroulie, J. Cognaux, C. Forceille, et al. 1996. Resistance

to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. *Nature* 382: 722–725.
13. Kazmierski, W., N. Bifulco, H. Yang, L. Boone, F. DeAnda, C. Watson, and T. Kenakin. 2003. Recent progress in discovery of small-molecule CCR5 chemokine receptor ligands as HIV-1 inhibitors. *Bioorg. Med. Chem.* 11: 2663–2676.
14. Tsamis, F., S. Gavrilov, F. Kajumo, C. Seibert, S. Kuhmann, T. Ketas, A. Trkola, A. Palani, J. W. Clader, J. R. Tagat, et al. 2003. Analysis of the mechanism by which the small-molecule CCR5 antagonists SCH-351125 and SCH-350581 inhibit human immunodeficiency virus type 1 entry. *J. Virol.* 77: 5201–5208.
15. Cocchi, F., A. L. DeVico, A. Garzino-Demo, S. K. Arya, R. C. Gallo, and P. Lusso. 1995. Identification of RANTES, MIP-1 α, and MIP-1 β as the major HIV-suppressive factors produced by $CD8^+$ T cells. *Science* 270: 1811–1815.
16. Mack, M., B. Luckow, P. J. Nelson, J. Cihak, G. Simmons, P. R. Clapham, N. Signoret, M. Marsh, M. Stangassinger, F. Borlat, et al. 1998. Aminooxypentane-RANTES induces CCR5 internalization but inhibits recycling: a novel inhibitory mechanism of HIV infectivity. *J. Exp. Med.* 187: 1215–1224.
17. Beck, L. A., S. Dalke, K. M. Leiferman, C. A. Bickel, R. Hamilton, H. Rosen, B. S. Bochner, and R. P. Schleimer. 1997. Cutaneous injection of RANTES causes eosinophil recruitment: comparison of nonallergic and allergic human subjects. *J. Immunol.* 159: 2962–2972.
18. Mizushima, S., and S. Nagata. 1990. pEF-BOS, a powerful mammalian expression vector. *Nucleic Acids Res.* 18: 11.
19. Strizki, J. M., S. Xu, N. E. Wagner, L. Wojcik, J. Liu, Y. Hou, M. Endres, A. Palani, S. Shapiro, J. W. Clader, et al. 2001. SCH-C (SCH 351125), an orally bioavailable, small molecule antagonist of the chemokine receptor CCR5, is a potent inhibitor of HIV-1 infection in vitro and in vivo. *Proc. Natl. Acad. Sci. USA* 98: 12718–12723.
20. Soda, Y., N. Shimizu, A. Jinno, H. Y. Liu, K. Kanbe, T. Kitamura, and H. Hoshino. 1999. Establishment of a new system for determination of coreceptor usages of HIV based on the human glioma NP-2 cell line. *Biochem. Biophys. Res. Commun.* 258: 313–321.
21. Kodama, E. I., S. Kohgo, K. Kitano, H. Machida, H. Gatanaga, S. Shigeta, M. Matsuoka, H. Ohrui, and H. Mitsuya. 2001. 4′-Ethynyl nucleoside analogs: potent inhibitors of multidrug-resistant human immunodeficiency virus variants in vitro. *Antimicrob. Agents Chemother.* 45: 1539–1546.
22. Suzutani, T., H. Machida, and T. Sakuma. 1988. Efficacies of antiherpesvirus nucleosides against two strains of herpes simplex virus type 1 in Vero and human embryo lung fibroblast cells. *Antimicrob. Agents Chemother.* 32: 1046–1052.
23. Cheng, Y., and W. H. Prusoff. 1973. Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 percent inhibition (I50) of an enzymatic reaction. *Biochem. Pharmacol.* 22: 3099–3108.
24. Palczewski, K., T. Kumasaka, T. Hori, C. A. Behnke, H. Motoshima, B. A. Fox, I. Le Trong, D. C. Teller, T. Okada, R. E. Stenkamp, et al. 2000. Crystal structure of rhodopsin: A G protein-coupled receptor. *Science* 289: 739–745.
25. Jones, G., P. Willett, and R. C. Glen. 1995. Molecular recognition of receptor sites using a genetic algorithm with a description of desolvation. *J. Mol. Biol.* 245: 43–53.
26. Aramori, I., S. S. Ferguson, P. D. Bieniasz, J. Zhang, B. Cullen, and M. G. Caron. 1997. Molecular mechanism of desensitization of the chemokine receptor CCR-5: receptor signaling and internalization are dissociable from its role as an HIV-1 co-receptor. [Published erratum appears in 1997 EMBO J. 16: 6055.] *EMBO J.* 16: 4606–4616.
27. Mueller, A., E. Kelly, and P. G. Strange. 2002. Pathways for internalization and recycling of the chemokine receptor CCR5. *Blood* 99: 785–791.
28. Aquino, C. J., D. R. Armour, J. M. Berman, L. S. Birkemo, R. A. Carr, D. K. Croom, M. Dezube, R. W. Dougherty, Jr., G. N. Ervin, M. K. Grizzle, et al. 1996. Discovery of 1,5-benzodiazepines with peripheral cholecystokinin (CCK-A) receptor agonist activity. 1. Optimization of the agonist "trigger." *J. Med. Chem.* 39: 562–569.
29. Perlman, S., H. T. Schambye, R. A. Rivero, W. J. Greenlee, S. A. Hjorth, and T. W. Schwartz. 1995. Nonpeptide angiotensin agonist. Functional and molecular interaction with the AT1 receptor. *J. Biol. Chem.* 270: 1493–1496.
30. Emery, P. T., N. B. Higgs, A. C. Warhurst, G. L. Carlson. and G. Warhurst. 2002. Anti-secretory properties of non-peptide somatostatin receptor agonists in isolated rat colon: luminal activity and possible interaction with P-glycoprotein. *Br. J. Pharmacol.* 135: 1443–1448.
31. Kaczor, A., and D. Matosiuk. 2002. Non-peptide opioid receptor ligands - recent advances. Part I — agonists. *Curr. Med. Chem.* 9: 1567–1589.
32. Kenakin, T. 2003. Ligand-selective receptor conformations revisited: the promise and the problem. *Trends Pharmacol. Sci.* 24: 346–354.
33. Blanpain, C., J. M. Vanderwinden, J. Cihak, V. Wittamer, E. Le Poul, H. Issafras, M. Stangassinger, G. Vassart, S. Marullo, D. Schlndorff, et al. 2002. Multiple active states and oligomerization of CCR5 revealed by functional properties of monoclonal antibodies. *Mol. Biol. Cell* 13: 723–737.
34. Watson, C., G. Chen, P. Irving, J. Way, W. J. Chen, and T. Kenakin. 2000. The use of stimulus-biased assay systems to detect agonist-specific receptor active states: implications for the trafficking of receptor stimulus by agonists. *Mol. Pharmacol.* 58: 1230–1238.
35. Roettger, B. F., D. Ghanekar, R. Rao, C. Toledo, J. Yingling, D. Pinon, and L. J. Miller. 1997. Antagonist-stimulated internalization of the G protein-coupled cholecystokinin receptor. *Mol. Pharmacol.* 51: 357–362.
36. Rodriguez-Frade, J. M., A. J. Vila-Coro, A. Martin, M. Nieto, F. Sanchez-Madrid, A. E. Proudfoot, T. N. Wells, A. C. Martinez, and M. Mellado. 1999. Similarities and differences in RANTES- and (AOP)-RANTES-triggered signals: implications for chemotaxis. *J. Cell Biol.* 144: 755–765.
37. Turner, L., S. G. Ward, and J. Westwick. 1995. RANTES-activated human T lymphocytes. A role for phosphoinositide 3-kinase. *J. Immunol.* 155: 2437–2444.
38. Ganju, R. K., S. A. Brubaker, R. D. Chernock, S. Avraham, and J. E. Groopman. 2000. β-Chemokine receptor CCR5 signals through SHP1, SHP2, and Syk. *J. Biol. Chem.* 275: 17263–17268.
39. Ganju, R. K., P. Dutt, L. Wu, W. Newman, H. Avraham, S. Avraham, and J. E. Groopman. 1998. β-Chemokine receptor CCR5 signals via the novel tyrosine kinase RAFTK. *Blood* 91: 791–797.
40. Trkola, A., S. E. Kuhmann, J. M. Strizki, E. Maxwell, T. Ketas, T. Morgan, P. Pugach, S. Xu, L. Wojcik, J. Tagat, et al. 2002. HIV-1 escape from a small molecule, CCR5-specific entry inhibitor does not involve CXCR4 use. *Proc. Natl. Acad. Sci. USA* 99: 395–400.
41. Platt, E. J., K. Wehrly, S. E. Kuhmann, B. Chesebro, and D. Kabat. 1998. Effects of CCR5 and CD4 cell surface concentrations on infections by macrophagetropic isolates of human immunodeficiency virus type 1. *J. Virol.* 72: 2855–2864.
42. Lin, Y. L., C. Mettling, P. Portales, J. Reynes, J. Clot, and P. Corbeau. 2002. Cell surface CCR5 density determines the postentry efficiency of R5 HIV-1 infection. *Proc. Natl. Acad. Sci. USA* 99: 15590–15595.
43. Pastore, C., G. R. Picchio, F. Galimi, R. Fish, O. Hartley, R. E. Offord, and D. E. Mosier. 2003. Two mechanisms for human immunodeficiency virus type 1 inhibition by N-terminal modifications of RANTES. *Antimicrob. Agents Chemother.* 47: 509–517.
44. Visco-Comandini, U., C. Hultgren, C. Brostrom, M. Birk, S. Kim, and M. Sallberg. 1998. Human immunodeficiency virus type 1 disease progression, CCR5 genotype, and specific immune response. *Clin. Diagn. Lab. Immunol.* 5: 463–466.
45. Reeves, J. D., S. A. Gallo, N. Ahmad, J. L. Miamidian, P. E. Harvey, M. Sharron, S. Pohlmann, J. N. Sfakianos, C. A. Derdeyn, R. Blumenthal, et al. 2002. Sensitivity of HIV-1 to entry inhibitors correlates with envelope/coreceptor affinity, receptor density, and fusion kinetics. *Proc. Natl. Acad. Sci. USA* 99: 16249–16254.
46. Billick, E., C. Seibert, P. Pugach, T. Ketas, A. Trkola, M. J. Endres, N. J. Murgolo, E. Coates, G. R. Reyes, B. M. Baroudy, et al. 2004. The differential sensitivity of human and rhesus macaque CCR5 to small-molecule inhibitors of human immunodeficiency virus type 1 entry is explained by a single amino acid difference and suggests a mechanism of action for these inhibitors. *J. Virol.* 78: 4134–4144.
47. Navenot, J. M., Z. X. Wang, J. O. Trent, J. L. Murray, Q. X. Hu, L. DeLeeuw, P. S. Moore, Y. Chang, and S. C. Peiper. 2001. Molecular anatomy of CCR5 engagement by physiologic and viral chemokines and HIV-1 envelope glycoproteins: differences in primary structural requirements for RANTES, MIP-1 α, and vMIP-II binding. *J. Mol. Biol.* 313: 1181–1193.
48. Castonguay, L. A., Y. Weng, W. Adolfsen, J. Di Salvo, R. Kilburn, C. G. Caldwell, B. L. Daugherty, P. E. Finke, J. J. Hale, C. L. Lynch, et al. 2003. Binding of 2-aryl-4-(piperidin-1-yl)butanamines and 1,3,4-trisubstituted pyrrolidines to human CCR5: a molecular modeling-guided mutagenesis study of the binding pocket. *Biochemistry* 42: 1544–1550.
49. Dragic, T., A. Trkola, D. A. Thompson, E. G. Cormier, F. A. Kajumo, E. Maxwell, S. W. Lin, W. Ying, S. O. Smith, T. P. Sakmar, and J. P. Moore. 2000. A binding pocket for a small molecule inhibitor of HIV-1 entry within the transmembrane helices of CCR5. *Proc. Natl. Acad. Sci. USA* 97: 5639–5644.
50. de Mendonca, F. L., P. C. da Fonseca, R. M. Phillips, J. W. Saldanha, T. J. Williams, and J. E. Pease. 2005. Site-directed mutagenesis of CC chemokine receptor 1 reveals the mechanism of action of UCB 35625, a small molecule chemokine receptor antagonist. *J. Biol. Chem.* 280: 4808–4816.
51. Mirzadegan, T., F. Diehl, B. Ebi, S. Bhakta, I. Polsky, D. McCarley, M. Mulkins, G. S. Weatherhead, J. M. Lapierre, J. Dankwardt, et al. 2000. Identification of the binding site for a novel class of CCR2b chemokine receptor antagonists: binding to a common chemokine receptor motif within the helical bundle. *J. Biol. Chem.* 275: 25562–25571.
52. Rosenkilde, M. M., L. O. Gerlach, J. S. Jakobsen, R. T. Skerlj, G. J. Bridger, and T. W. Schwartz. 2004. Molecular mechanism of AMD3100 antagonism in the CXCR4 receptor: transfer of binding site to the CXCR3 receptor. *J. Biol. Chem.* 279: 3033–3041.
53. Zheleznova, E. E., P. N. Markham, A. A. Neyfakh, and R. G. Brennan. 1999. Structural basis of multidrug recognition by BmrR, a transcription activator of a multidrug transporter. *Cell* 96: 353–362.
54. Govaerts, C., C. Blanpain, X. Deupi, S. Ballet, J. A. Ballesteros, S. J. Wodak, G. Vassart, L. Pardo, and M. Parmentier. 2001. The TXP motif in the second transmembrane helix of CCR5. A structural determinant of chemokine-induced activation. *J. Biol. Chem.* 276: 13217–13225.
55. Govaerts, C., A. Bondue, J. Y. Springael, M. Olivella, X. Deupi, E. Le Poul, S. J. Wodak, M. Parmentier, L. Pardo, and C. Blanpain. 2003. Activation of CCR5 by chemokines involves an aromatic cluster between transmembrane helices 2 and 3. *J. Biol. Chem.* 278: 1892–1903.
56. Nicholls, A., K. A. Sharp, and B. Honig. 1991. Protein folding and association: insights from the interfacial and thermodynamic properties of hydrocarbons. *Proteins* 11: 281–296.

Novel HIV-1 Integrase Inhibitors Derived from Quinolone Antibiotics

Motohide Sato,† Takahisa Motomura,† Hisateru Aramaki,† Takashi Matsuda,† Masaki Yamashita,† Yoshiharu Ito,† Hiroshi Kawakami,† Yuji Matsuzaki,† Wataru Watanabe,† Kazunobu Yamataka,† Satoru Ikeda,† Eiichi Kodama,‡ Masao Matsuoka,‡ and Hisashi Shinkai*,†

Central Pharmaceutical Research Institute, JT Inc., 1-1 Murasaki-cho, Takatsuki, Osaka, 569-1125, Japan, and Institute for Virus Research, Kyoto University, 53 Shogoin Kawaramachi, Sakyo-ku, Kyoto, 606-8507, Japan

Received January 6, 2006

Abstract: The viral enzyme integrase is essential for the replication of human immunodeficiency virus type 1 (HIV-1) and represents a remaining target for antiretroviral drugs. Here, we describe the modification of a quinolone antibiotic to produce the novel integrase inhibitor JTK-303 (GS 9137) that blocks strand transfer by the viral enzyme. It shares the core structure of quinolone antibiotics, exhibits an IC_{50} of 7.2 nM in the strand transfer assay, and shows an EC_{50} of 0.9 nM in an acute HIV-1 infection assay.

Human immunodeficiency virus type 1 (HIV-1) integrase, along with HIV-1 reverse transcriptase and HIV-1 protease, is an essential enzyme for retroviral replication and represents an important target for interrupting the viral replication cycle.[1] HIV-1 integrase first catalyzes removal of the terminal dinucleotide from each 3′-end of viral DNA (3′-processing) and subsequently mediates joining of the 3′-end of the viral DNA to host DNA (strand transfer).[2] Reverse transcriptase inhibitors and protease inhibitors have already made significant advances in antiretroviral therapy but cannot achieve complete suppression and risk producing resistant HIV-1.[3,4] On the other hand, despite numerous attempts to develop integrase inhibitors, only the diketo acid class of compounds is at an advanced stage of development and no integrase inhibitors have yet been approved for therapeutic use.[1,5−7] Here, we report that the core structure of quinolone antibiotics can be used as an alternative to the diketo acid class of HIV-1 integrase inhibitors and how this finding led to a novel quinolone integrase inhibitor, JTK-303 (GS 9137).

The diketo acid moiety (γ-ketone, enolizable α-ketone, and carboxylic acid) was believed to be essential for the inhibitory activity of this series of integrase inhibitors,[8] and the structures of diketotriazole **2**,[6] diketotetrazole **3**,[9] diketopyridine **4**,[10] and 7-carbonyl-8-hydroxy-(1,6)-naphthyridine **5** [7,11] were reported to be bioisosters of the diketo acid pharmacophore (Figure 1). The carboxylic acid could be replaced with not only acidic bioisosters, such as tetrazole and triazole, but also by a basic heterocycle bearing a lone pair donor atom, such as a pyridine ring. It has been reported that the heteroaromatic nitrogen in the pyridine ring mimics the corresponding carboxyl oxygen in the diketo acid as a Lewis base equivalent.[11] The enolizable ketone at the α-position of diketo acids can be replaced with a phenolic hydroxyl group, indicating that the α-enol form of each diketo acid is its biologically active coplanar conformation.[11]

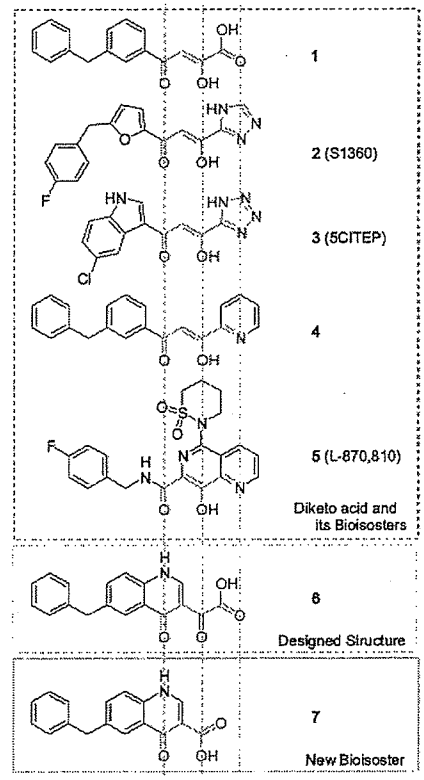


Figure 1. Structures of the diketo acid family and its new bioisoster.

All bioisosters of the diketo acid motif have the three functional groups that mimic a ketone, enolizable ketone, and carboxyl oxygen and can have a coplanar conformation (Figure 1). Therefore, we designed the structure of 4-quinolone-3-glyoxylic acid **6** as a new scaffold that maintained the coplanarity of diketo acid functional groups (Figure 1). Interestingly, not the 4-quinolone-3-glyoxlic acid **6** but its precursor 4-quinolone-3-carboxylic acid **7** showed integrase inhibitory activity. The 4-quinolone-3-carboxylic acid **7** only had two functional groups, a β-ketone and a carboxylic acid, which were coplanar. This result showed that the coplanar monoketo acid motif in 4-quinolone-3-carboxylic acid **7** could be an alternative to the diketo acid motif and provided novel insight into the structural requirements and the binding mode of this type of inhibitor. Quinolone **7** had an IC_{50} of 1.6 μM in the strand transfer assay, and structural modification of **7** led to a far more potent integrase inhibitor **12** with stronger antiviral activity (Table 1). Introduction of 2-fluoro and 3-chloro substituents into the distal benzene ring of **7** (**8**) led to a significant improvement of its inhibition of strand transfer (IC_{50} = 44 nM) and to the appearance of antiviral activity (EC_{50} = 0.81 μM). Compound **9**, bearing a hydroxyethyl group at the 1-position of the quinolone ring, was 1.8-fold more potent at inhibiting strand transfer (IC_{50} = 24 nM) and displayed about 11-fold stronger antiviral activity (EC_{50} = 76 nM) than **8**. Introduction of a methoxy group at the 7-position of the quinolone ring of **9** (**10**) led to a significant improvement of its inhibition of strand transfer (IC_{50} = 9.1 nM) and of antiviral activity (EC_{50} = 17.1 nM). Compound **11**, bearing an isopropyl group at the 1*S*-position of the hydroxyethyl moiety, was about 3-fold more potent at inhibiting strand transfer (IC_{50} = 8.2 nM) and about 10-fold stronger at inhibiting HIV

* To whom correspondence should be addressed. Phone: +81 72 681 9700. Fax: +81 72 681 9725. E-mail: hisashi.shinkai@ims.jti.co.jp.
† JT Inc.
‡ Kyoto University.

10.1021/jm0600139 CCC: $33.50
Published on Web 02/15/2006