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A Simple Screening System for Anti-HIV Drugs: Syncytium Formation Assay Using T-Cell Line Tropic and Macrophage Tropic HIV env Expressing Cell Lines—Establishment and Validation—

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The first step in cellular entry of HIV involves binding of the viral envelope glycoprotein complex (gp120/gp41) to specific receptor molecules on the target cells. The cell-cell fusion (syncytium formation) between env expressing cells and CD4+ cells mimics the viral infection of the host cells. To search for anti-HIV substances preventing this process, we constructed the recombinant cell lines, HeLa/CD4/Lac-Z and HeLa/T-env/Tat for T-cell tropic (HIV-1_{NL4-3}) system, and HOS/CD4/CCR5/Lac-Z and HeLa/M-env/Tat for macrophage tropic (HIV-1_{SF162}) system. When each pair of cells were co-incubated for 20 hours, the multinuclear giant cells (syncytia) were formed and β-galactosidase was expressed. These systems are less biohazardous because no infectious virus particles are used. Their validity in screening for anti-HIV substances which inhibit syncytium formation was confirmed using various known HIV entry inhibitors.

Progress of combination chemotherapy with HIV-1 reverse transcriptase and protease inhibitors has achieved long sustained suppression of viral replication in HIV-1-infected individuals[1]. However, considering a low compliance of long-term combination chemotherapy and emergence of the mutant viruses resistant to these drugs, it is still necessary to discover novel anti-HIV-1 agents with different mechanism of action[2].

The envelope glycoprotein gp120/gp41 of HIV-1 binds the cellular receptors and mediates the fusion between the viral and cellular membranes. This process is an attractive target for the drugs to prevent the HIV-1 infection of its target cells. We have tried screening for new antibiotics active against gp120-CD4 binding from soil microorganisms, and found isochromophilones I and II from *Penicillium multicolor*[3~5] and chloropeptin from *Streptomyces* sp.[6~8] by gp120-sCD4 binding assay.

The fusogenic properties of the HIV envelope can also lead to fusion between the env-expressing cell lines and other CD4+ cells[9]. When these cell lines are co-incubated, the large syncytia are formed. Recently, it is clarified that the cell-tropism of HIV-1 depends on species of chemokine receptors such as CXCR4 or CCR5, and that these receptors function as a co-receptor for the virus binding to

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the cell surface[10,11]. So, we developed two syncytium formation systems using the recombinant cell lines. The one is the T-cell line (T-) tropic system using HeLa/CD4/Lac-Z and HeLa/T-env/Tat. The other is the macrophage (M-) tropic system using HOS/CD4/CCR5/Lac-Z and HeLa/M-env/Tat. Then, we tried to validate the systems using the known HIV-1 entry inhibitors and to screen anti-HIV substances. The present paper deals with the establishment and validation of the screening systems.

Materials and Methods

Compounds

Benanomicin A[12] and cyanovirin-N[13] were generous gifts from Drs. T. TAKEUCHI (Microbial Chemistry Institute, Tokyo, Japan) and M. R. BOYD (National Cancer Institute, MD), respectively. T134[14] and T140[15] were generous gifts from Dr. H. NAKASHIMA (Kagoshima Univ., Kagoshima, Japan). TAK-779[16] was kindly provided by Takeda Chemical Industries Ltd. (Osaka, Japan). Blasticidin S and G-418 were purchased from Kaken Pharmaceutical Ltd. (Tokyo, Japan) and Gibco BRL, Life Technologies Inc. (Rochville, MD), respectively.

Plasmids

The plasmids pBluscript SK(−), pCH110 and pMAM2-BSD were purchased from Stratagene (La Jolla, CA), Amersham Pharmacia Biotech (Buckinghamshire, UK) and Kaken Pharmaceutical Ltd. (Tokyo, Japan), respectively. The plasmids pHIV-CAT and pCMV Tat were generous gifts from Dr. H. KATO[17] (Rockfeller Univ., NY). The synthetic *Cla* I-*Xho* I adapter set (5*-TCGAGCCATCGATAAG, 5*-TCGACTTATCGATGGC) was purchased from Nisshinbo (Tokyo, Japan). The plasmid pSF162 which contains a 3.2 kilobase (Kb) *Eco*R I-*Xho* I fragment encoding the *C*-terminal 35 amino-acid residues of *vpr*, *tat*, *rev*, *vpu* and *env* gene products and the *N*-terminal 36 or 38 amino-acid residues of *nef* gene product was a generous gift from Dr. T. SHIODA[18,19] (Osaka Univ. Research Institute for Microbial Diseases, Osaka, Japan). The plasmid pKF1 HIV_{NL4-3} *env* expression vector was derived from the pLNL6 retroviral vector and contains a CMV immediate early promoter (positions 425 to 1316 from pCEP4, Invitrogen), 69 bp of a murine endogenous retroviral sequence, HIV-1 genes *rev*, *vpu* and *env* (positions 5969 to 8809), and selectable marker *neo* gene[20].

The plasmid pNCRVE-SF162 *env* expression vector was constructed as follows. The *Eco*R I-*Xho* I fragment of pSF162 was subcloned into pBluscript SK(−) *Eco*R I-*Xho* I site (pBSF162), and *Cla* I-*Xho* I adaptor was inserted into the *Xho* I site of pBSF162 (pBSF162::*Cla* I). This plasmid was digested with *Nsi* I-*Cla* I and the 2.3 Kb fragment containing *env* gene was inserted into *Nsi* I-*Cla* I site of pKF1 to obtain pNCRVE-SF162. The *Tth*III I site of pCH110 was converted to the *Xho* I site with *Xho* I linker (Takara Shuzo, Kyoto, Japan) to obtain pCH110::*Xho* I. The *Xho* I-*Hind* III fragment of pHIV-CAT which contains the HIV LTR (position −167 to +80) was inserted into *Xho* I-*Hind* III site of the pCH110::*Xho* I to construct the β-galactosidase expression vector controlled by HIV LTR. This plasmid was designated as pHIV-lacZ.

Cells

Each cell lines were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and kanamycin (100 μg/ml). The transfection was carried out by calcium phosphate method[21]. The HOS/CD4/CCR5 and HOS/CD4/CXCR4 cells were obtained from NIH AIDS Research and Reference Reagent Program, National Institute of Health (USA).

HeLa cells were transfected with pNCRVE-SF162 and cultured in the medium containing 1 mg of G-418 per ml in a 100-mm diameter culture dish for 2 weeks. The colonies were cloned by limiting dilution and expanded in 25 cm^2 culture flasks (HeLa/M-env).

The plasmids pHIV-LacZ and pMAM2-BSD were co-transfected into HeLa/CD4[22] and HOS/CD4/CCR5 cells. The pCMV-Tat and pMAM2-BSD were co-transfected into HeLa/T-env (HeLa135)[20] and HeLa/M-env cells. The transfected cells were cultured in the medium containing 2 μg of blastcidin S per ml for 2 weeks. The colonies were cloned by limiting dilution and expanded in 25 cm^2 culture flasks to obtain HeLa/CD4/LacZ, HOS/CD4/CCR5/LacZ, HeLa/T-env/Tat and HeLa/M-env/Tat.

Western Blot Analysis

To detect env protein expressed in HeLa/M-env cells, 5×10^5 cells were cultured in a 60-mm diameter culture dish for 18 hours. The cells were washed with phosphate buffered saline (PBS), collected by a policeman and centrifuged at $800\times g$ for 5 minutes at 4°C. The cells were resuspended in lysis buffer (1% Nonidet P-40, 0.5% deoxycholic acid, 150 mM NaCl, 50 mM Tris-HCl, pH 8.0) and stood on a ice bath for 30 minutes. The cell lysate was centrifuged at $9,000\times g$ for 5 minutes at 4°C and the supernatant fluid was subjected to SDS-polyacrylamide gel electrophoresis. Proteins in the gel were electrophoretically transferred to PVDF membrane (Millipore, MA) using a

semi-dry blotting apparatus (Biocraft, Tokyo, Japan). The membrane was immersed with a 3% nonfat dry milk in PBS containing 0.5% Tween-20 (PBS-T) for 18 hours at 4°C. The membrane was washed with PBS-T three times and then incubated with a goat HIV-1 gp120 polyclonal antibody (Virostat, Portland, OR) diluted to 1/1000, followed by alkaline phosphatase conjugated rabbit anti-goat immunoglobulin G (Bethyl laboratories Inc., Montgomery, TX) diluted to 1/2000. The membrane was washed with PBS-T three times and rinsed with alkaline phosphatase buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris-HCl, pH 9.5). The membrane was developed with alkaline phosphate buffer containing 337.5 μg of nitroblutetrazorium (Boehringer-Mannheim, Mannheim, Germany) per ml and 175 μg of BCIP (Boehringer-Mannheim) per ml.

Reporter Gene Activation-mediated Syncytium Formation Assay

The combinations of HeLa/CD4/LacZ and HeLa/T-env/Tat, and HOS/CD4/CCR5/LacZ and HeLa/M-env/Tat cells were used for T-tropic and M-tropic syncytium formation assay, respectively. The cells (8×10^3 each) were co-incubated in 10% FBS DMEM containing serially diluted test compounds under 5% CO_2 for 20 hours at 37°C in a 96-well microplate. The test compound was dissolved in ethanol and diluted with DMEM. The final ethanol concentration in the culture mixture was less than 0.5%. After co-incubation the cells were lysed with 20 μl of 0.05% Tween-20 and the lysate was mixed with 100 μl of Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol) containing 80 μg of *o*-nitrophenyl-β-D-galactopyranoside for 80 minutes (HeLa/CD4/LacZ and HeLa/T-env/Tat system) or 50 minutes (HOS/CD4/CCR5/LacZ and HeLa/M-env/Tat system) at 37°C. The reaction was stopped with 25 μl of 2 M Na_2CO_3 and the absorbance at 405 nm was measured with a microplate photometer (Microwell system reader 510, Organon Tecnika N.V., Turnhout, Belgium).

Cytotoxicity Assay

The number of viable cells was determined by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) colorimetric assay[23]. Cells (1.6×10^4) were cultured with 10% FBS DMEM medium containing serially diluted test compounds under 5% CO_2 for 24 hours at 37°C in a 96-well plate and each well was supplemented with 10 μl of MTT dissolved with PBS (5 mg/ml). After incubation for 3 hours at 37°C, the cells were lysed with 100 μl of extraction buffer (*N,N'*-dimethylformamide containing 20% sodium dodecyl sulfate, pH 4.7) per well, and further incubated for 2 hours. The absorbance at 570 nm was measured by a microplate photometer (Microwell system reader 510).

Results and Discussion

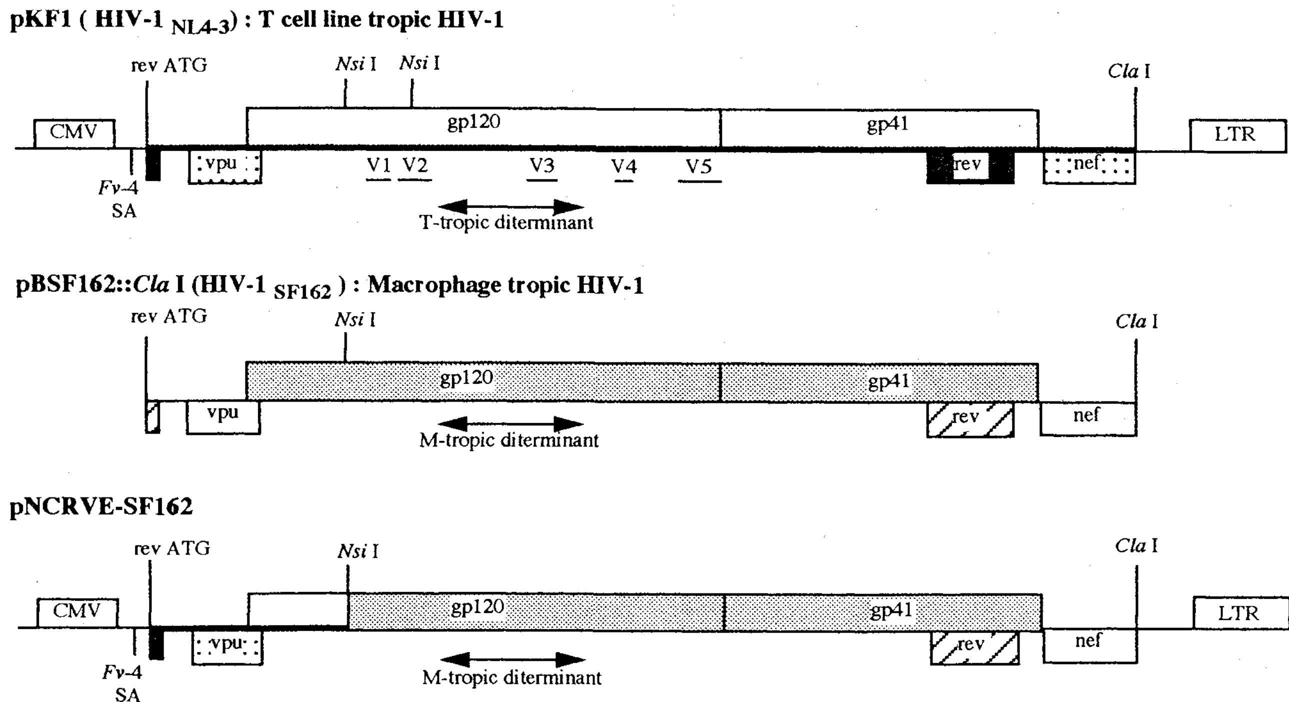
Construction of Cell Lines for Syncytium Formation

It is known that the envelope glycoprotein gp120/gp41 of HIV-1 binds the cellular receptors and mediates the fusion between the viral and cellular membranes, and that such fusion can also occur between the env-expressing cell lines and CD4+ cells. T- and M-tropic HIV-1 require the chemokine receptors CXCR4 and CCR5, respectively, as a co-receptor for entry into target cells in addition to CD4. The co-receptors CXCR4 and CCR5 for T-tropic and M-tropic viruses recognize the region of the viral envelope that determines infection of different cell types[24]. So, we tried to construct two env expression cell lines, which contain the envelope genes from T-tropic and M-tropic HIV-1, respectively. Furthermore, the *trans*-activation protein (tat) gene and Lac-Z gene controlled by HIV-1 LTR were introduced into env-expressing cells and CD4+ cells, respectively, to determine the formed syncytia colorimetrically.

The plasmid, pNCRVE-SF162 which expressed M-tropic $HIV\text{-}1_{SF162}$ envelope protein was constructed by the replacement of the *Nsi* I-*Cla* I fragment containing T-tropic determining *env* region of pKF1 to the corresponding restriction fragment (*Nsi* I-*Cla* I fragment) of pBSF162::*Cla* I containing M-tropic determining *env* region (Figure 1). To examine the tropism of env protein mediated by pNCRVE-SF162, HOS/CD4/CCR5 and HOS/CD4/CXCR4 cell lines were transfected with pNCRVE-SF162. As shown in Figure 2, only HOS/CD4/CCR5 cell line formed the multi-nuclear giant cells (syncytia), indicating that M-tropic *env* gene in pNCRVE-SF162 was expressed in HOS/CD4/CCR5 cells and presented env protein on the cell surface.

HeLa cells were transfected with pNCRVE-SF162 and then cultured in 10% FBS DMEM containing 1 mg of G418 per ml. The env expression level in the G418-resistant cells was determined by syncytium formation when the cells were incubated with HOS/CD4/CCR5 cells and by the western blotting analysis. Five clones, which showed a high syncytium formation ability, were analyzed by western blotting using goat anti-HIV_{IIIB} gp120 polyclonal antibody. The clone #48 and #57 among them showed the constitutive production of a large quantity of env protein (Figure 3).

Fig. 1. Construction of pNCRVE-SF162 containing M-tropic envelope gene.

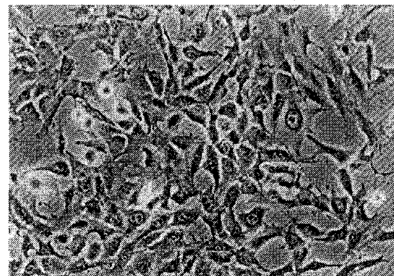


The pKF1 consists of a CMV promoter *Fv-4* derived sequences, HIV rev, vpu, env and the pLNL6 retroviral vector. Arrows indicate HIV-1 cell tropism determinant regions.

Fig. 2. Photomicrographs of HOS/CD4/CCR5 and HeLa/CD4/CXCR4 transfected with pNCRVE-SF162.

A. HOS/CD4/CCR5

B. HOS/CD4/CXCR4

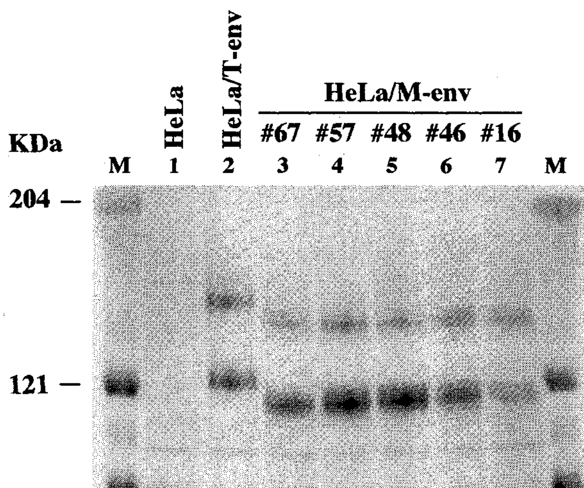


HOS/CD4/CCR5 (A) and HeLa/CD4/CXCR4 (B) cell lines were transfected with pNCRVE-SF162 and the transformants were stained with XC[25].

Clone #48 was used as HeLa/M-env in the following experiments.

Then, HeLa/T-env and HeLa/M-env cells were cotransfected with pCMV-Tat containing the HIV-1 *tat* gene and pMAM-BSD containing blasticidin S resistance gene, and blastcidin S resistant colonies were selected. To confirm expression of *tat* gene, the blasticidin S resistant cells were transfected with pHIV-LacZ and measured β-galactosidase activity to obtain HeLa/T-env/Tat and HeLa/M-env/Tat cells. HeLa/CD4 and HOS/CD4/CCR5 cells were cotransfected with pHIV-LacZ and pMAM-BSD in a similar manner, and blasticidin S resistant

Fig. 3. Western blot analysis of env protein expressed in HeLa/M-env cells.



The parent HeLa (lane 1), T-tropic env expressed HeLa/T-env (lane 2) and each of the established HeLa/M-env (lane 3~7).

Env protein was detected with primary Ab: HIV-1 IIIB gp120 goat polyclonal antibody and secondary Ab: Rabit anti-goat IgG-h+1 (alkaline phosphatase conjugated).

cells containing LTR-Lac Z gene were selected to obtain HeLa/CD4/LacZ and HOS/CD4/CCR5/LacZ. When HeLa/T-env/Tat and HeLa/CD4/LacZ, or HeLa/M-env/Tat and HOS/CD4/CCR5/LacZ were co-incubated, syncytia were formed at 8 hours after incubation and the number of syncytia was increased until 30 hours (Figure 4-A and B). On the other hand, no syncytia were formed in the combination of HeLa/T-env/Tat and HOS/CD4/CCR5/LacZ, or HeLa/M-env/Tat and HeLa/CD4/LacZ (Figure 4-C and D), indicating that the systems reflect the tropism of HIV envelope. Furthermore, β-galactosidase was expressed in the syncytia on the surface of the plate after 20 hours (Figure 4-E). The co-incubated cells could be used for the measurement of β-galactosidase activity as follows. When the co-incubated cells were lysed with Tween-20 and then incubated with ONPG at 37°C, the absorbances at 405 nm in the T- and M-tropic syncytium formation assays were increased linearly until 80 and 50 minutes, respectively (data not shown). These results indicate that the combinations of HeLa/T-env/Tat and HeLa/CD4/LacZ cells, and HeLa/M-env/Tat and HOS/CD4/CCR5/LacZ cells can be used for the T- and M-tropic syncytium formation assays, respectively, and that a β-galactosidase reporter gene is useful an indicator in both assay systems.

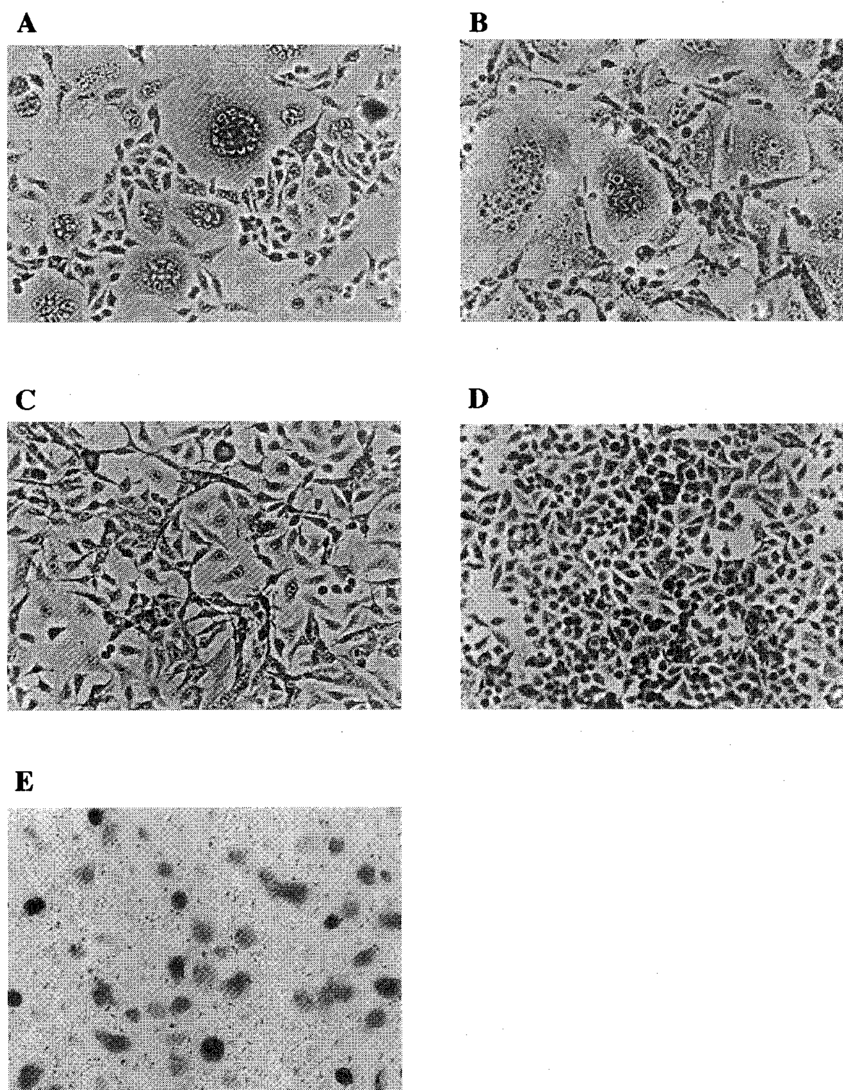
Validation of the Assay Systems Using Known HIV Entry Inhibitors

To confirm whether these assay systems reflect the tropism of HIV env protein, the inhibitory effects of the known HIV entry inhibitors were investigated using the systems. None of the test compounds employed were found to be cytotoxic at the concentrations used in the assay (data not shown). Figure 5 shows the syncytium formation in the presence of the inhibitors. Chloropeptin I[6], which was discovered as a gp120-CD4 binding inhibitor from *Streptomyces* sp. inhibited both T-tropic and M-tropic syncytium formation, with the 50% inhibitory concentration (IC_{50}) values of 2.0 and 2.4 μM, respectively. Cyanovirin-N[13], which was isolated from the cyanobacterium *Nostoc ellipsosporum* and found to interact with HIV env, also inhibited both systems with IC_{50} values of 40 and 50 nM, respectively. Benanomicin A[12], which is a mannose-binding antibiotic having antifungal and anti-HIV activities in the presence of calcium ions, also inhibited both syncytium formation with IC_{50} values of 10 and 12 μM, respectively. T134[14] and T140[15], which are known to bind CXCR4 receptor of the host cells and to exhibit a potent anti-HIV activity, inhibited T-tropic system with IC_{50} values of 0.5 and 0.3 nM, respectively, but not M-tropic system even at 1.0 μM. On the other hand, TAK-779[16], a small molecular non-peptide antagonist of CCR5 receptor, inhibited M-tropic system alone with IC_{50} values of 2 nM. These data indicate that the above systems are useful in measuring selectively the syncytium formation mediated by T- or M-tropic env protein and the chemokine receptor CXCR4 or CCR5, respectively. Thus, it was confirmed that the syncytium formation systems are useful in screening for anti-HIV substances having specific inhibitory activities against HIV entry to cells, and that the inhibitory activity against T- or M-tropism can be differentiated using the systems.

Screening of Syncytium Formation Inhibitors from Microorganisms

Using the above T-cell tropic system, we have screened syncytium formation inhibitors from microorganisms isolated in the Kitasato Institute and our laboratory. Over 8000 microogsnisms including actinomycetes and fungi were tested and we discovered a novel anti-HIV protein (actinohivin) inhibiting syncytium formation from the culture supernatant of the actinomycete strain K97-0003.

Fig. 4. Syncytium formation in the combination of each cell lines and X-gal staining of the syncytia formed.



(A) HeLa/T-env/Tat and HeLa/CD4/LacZ, (B) HeLa/M-env/Tat and HOS/CD4/CCR5/LacZ, (C) HeLa/T-env/Tat and HOS/CD4/CCR5/LacZ, (D) HeLa/M-env/Tat and HeLa/CD4/LacZ. Cells were co-incubated for 20 hours and stained with XC (×100). (E) *In situ* X-gal staining[26] of the syncytia formed in the combination (A) (×40).

The compound has been revealed to be a protein consisting of 114 amino acid residues and to inhibit T- and M-tropic syncytium formation with IC_{50} values of 60 and 700 nM, respectively, and the cytopathicity of HIV_{IIIB} in MT-4 cells with IC_{50} values of 230 nM[26]. Isolation, characterization, biological activities, and molecular cloning of actinohivin have been published in the separate papers[26,27].

Several assay systems for syncytium formation have been reported[9,28~30]. They depend on the use of infectious virus particles and/or the transfection with plasmids or bacteriophages. The assay system reported here does not depend on them but uses the recombinant cell lines constitutively expressing T- or M-tropic env protein or CD4 and co-receptor of HIV-1. Thus, our reporter gene activation-mediated syncytium formation assay provide a simple, less biohazardous and sensitive tool for screening of novel anti-HIV substances inhibiting HIV entry to cells.

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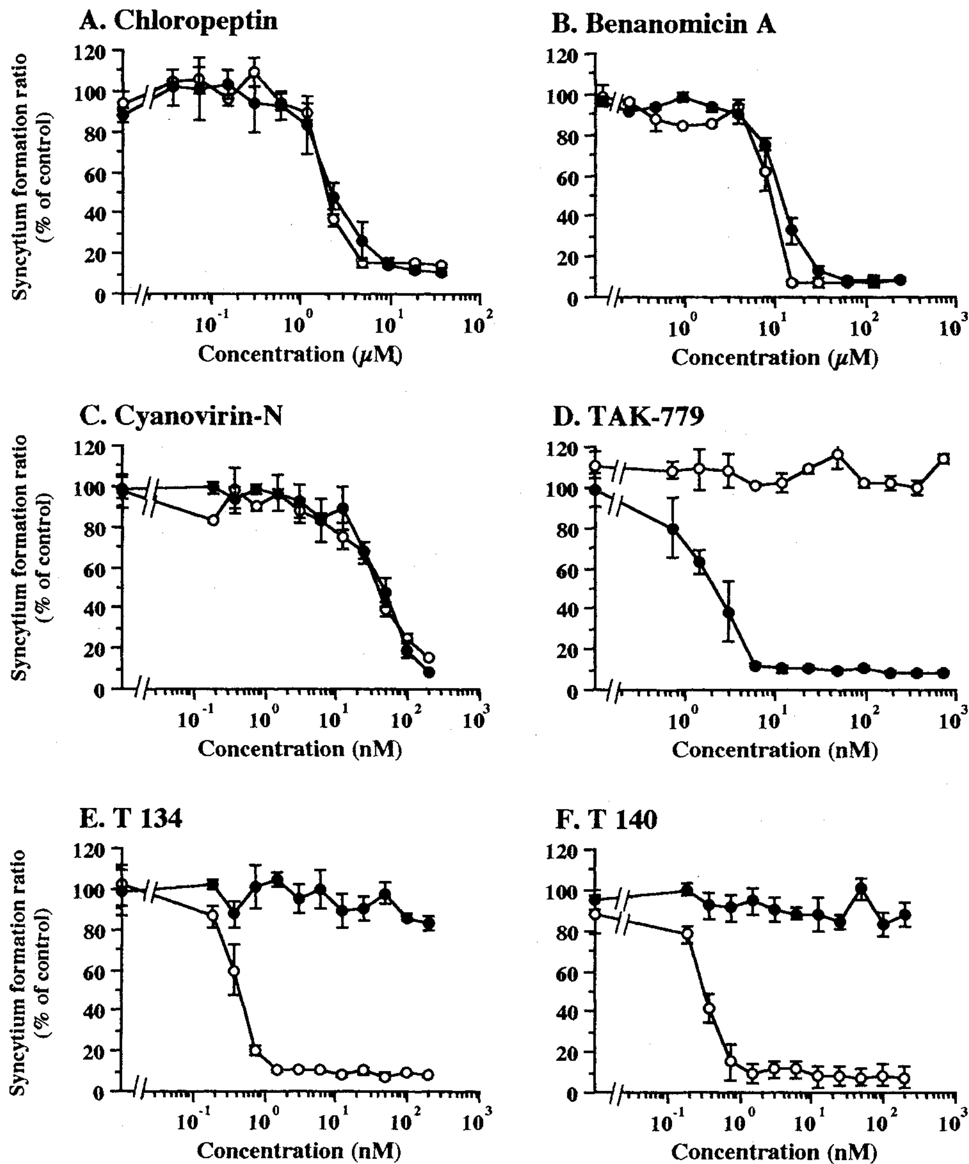
Fig. 5. Inhibitory effects of known HIV entry inhibitors on T- and M-tropic syncytium formation.



Syncytium formation rates (assay systems) in T-tropic (open circle) and M-tropic systems (closed circle) were determined. Points are averages (±standard derivations) of triplicate assays.

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