that if an inhibitor maintains strong hydrogen bond interactions with the wild-type protease, particularly with backbone atoms of multiple residues that are conserved (e.g., Asp29 and Gly27), then the loss of van der Waals contacts due to mutations may not result in a drastic loss of binding affinity. Thus, inhibitors without multiple strong hydrogen bond interactions with wild-type protease would be more susceptible to loss of binding due to loss of weaker van der Waals contacts than inhibitors with multiple hydrogen bond interactions. In this respect, we analyzed the hydrogen bond interactions of several PIs with wild-type protease (Table 5). It is noteworthy that only GRL-98065 and DRV have four hydrogen bond interactions with backbone atoms of Asp29 and Asp30 and of Asp30′. None of the other clinically approved PIs studied here have more than two hydrogen bond interactions with these residues. Thus, GRL-98065 is likely to preserve the hydrogen bond interactions and bind tightly with mutant protease.

The present data suggest that GRL-98065 has several advantages: (i) it exerts potent activity against a wide spectrum of drug-resistant HIV-1 variants, presumably due to its interactions with the main chains of the active-site amino acids Asp29 and Asp30; (ii) its unique contact with HIV-1 protease differs from that of other PIs; (iii) the viral acquisition of resistance is substantially delayed; and (iv) at least several PIs, including SQV and ATV, remain active in vitro against the virus selected in vitro with GRL-98065. It is of note that GRL-98065 possesses substantially favorable features as a potential therapeutic for AIDS, as described above; however, its oral bioavailability, pharmacokinetics/pharmacodynamics, biodistribution, etc., are yet to be determined in further rigorous preclinical and clinical testing.

ACKNOWLEDGMENTS

We thank the Center for Information Technology, National Institutes of Health, for providing computational resources.

This work was supported in part by the Intramural Research Program of the Center for Cancer Research, National Cancer Institute, National Institutes of Health grants GM62920 and GM53386, and in part by a Grant-in-aid for Scientific Research (Priority Areas) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (Monbu-Kagakusho), a Grant for Promotion of AIDS Research from the Ministry of Health, Welfare, and Labor of Japan (Kosei-Rohdosho; H15-AIDS-001), and a grant to the Cooperative Research Project on Clinical and Epidemiological Studies of Emerging and Re-emerging Infectious Diseases (Renkei Jigyo; no. 78, Kumamoto University) of Monbu-Kagakusho, the Georgia State University Molecular Basis of Disease Program, the Georgia Research Alliance, the Georgia Cancer Coalition, and National Institute of Health grants GM62920 and GM53386. The X-ray diffraction data were collected at beamline X-26C, National Synchrotron Light Source. Use of the National Synchrotron Light Source, Brookhaven National Laboratory, was supported by the U.S. Department of Energy, Office of Science, Office of Basic Energy Sciences, under contract no. DE-AC02-98CH10886.

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Available online at www.sciencedirect.com
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Bioorganic & Medicinal Chemistry 15 (2007) 7576–7580
Bioorganic & Medicinal Chemistry

Darunavir, a conceptually new HIV-1 protease inhibitor for the treatment of drug-resistant HIV

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Received 14 June 2007; revised 4 September 2007; accepted 7 September 2007
Available online 14 September 2007

Abstract—Our structure-based design strategies which specifically target the HIV-1 protease backbone, resulted in a number of exceedingly potent nonpeptidyl inhibitors. One of these inhibitors, darunavir (TMC114), contains a privileged, structure-based designed high-affinity P2 ligand, 3(*R*),3a(*S*),6a(*R*)-bis-tetrahydrofuranylurethane (bis-THF). Darunavir has recently been approved for the treatment of HIV/AIDS patients harboring multidrug-resistant HIV-1 variants that do not respond to previously existing HAART regimens.

The AIDS (acquired immunodeficiency syndrome) epidemic has become one of the most pressing medical concerns of our time.[1] The World Health Organization (WHO), as of 2006, estimated that over 40 million people are infected with HIV (human immunodeficiency virus), the causative agent of AIDS.[2] During replication in the HIV life-cycle, *gag* and *gag-pol* gene products are produced as precursor polyproteins which are subsequently processed by a virally encoded protease to provide structural proteins (p17, p24, p9 and p7) and essential viral enzymes, including protease (PR), reverse transcriptase (RT) and integrase (IN).[3] All three retroviral enzymes have been identified as potential drug targets. Specifically, the critical function of HIV protease has made it an important target for the treatment of HIV/AIDS. The approval of the first protease inhibitor (PI), saquinavir and its introduction into highly active antiretroviral therapy (HAART), with reverse transcriptase inhibitors, led to significantly enhanced HIV management and improved the quality of life of HIV/AIDS patients.[4]

Since the advent of saquinavir, a number of PIs have been introduced in the regimens of HAART. Thus improved HAART regimens have shown reduced viral load, increased CD4+ T-cell counts[5] and drastically lowered AIDS-related deaths in the US and industrialized nations.[6] While HAART proved to be a large step forward, there are still serious drawbacks with the first generation anti-protease therapeutics. These include: (1) severe side effects and drug toxicities, (2) higher therapeutic doses due to 'peptide-like' character, (3) costly synthesis which leads to high treatment cost, and perhaps the most alarming, (4) the rapid emergence of drug resistance. Indeed, the emergence of multidrug-resistant HIV strains has greatly compromised current HAART regimens. It has been reported that treatment failure has ultimately occurred in at least 40–50% of patients, who initially achieved favorable viral suppression with HAART to undetectable levels.[7] Furthermore, persistent viral replication (plasma HIV RNA > 500 copies/mL) has been reported under HAART in 10–40% of antiviral therapy-naive individuals as a result of transmission of drug-resistant HIV-1 variants.[8] The management and effective treatment options for HIV/AIDS clearly depend upon the development of PIs and other novel anti-HIV therapeutics, which can effectively combat drug-resistant HIV strains, possess better pharmacokinetic properties, have no or less toxicities, and come at a reduced cost of synthesis.

Keywords: Protease inhibitors; Darunavir; Design and synthesis.
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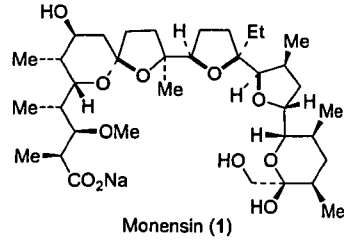
doi:10.1016/j.bmc.2007.09.010

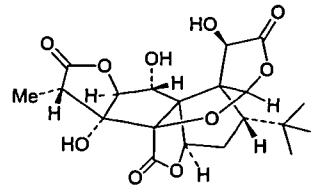
1. Design of nonpeptide ligands to eliminate peptidic character

Our initial investigation primarily focused on reducing peptidic features, molecular weight, and structural complexity of protease inhibitors. In this context, we have designed a number of nonpeptidic high-affinity ligands for the HIV protease substrate binding site based upon various available three-dimensional structures of the protein-ligand complex.[9] Particularly, we planned to design conformationally constrained nonpeptidic molecules of a cyclic or heterocyclic nature to maximize the active site interactions. One of the important elements in our design strategy is the incorporation of a stereochemically defined and conformationally constrained cyclic ether template that could replace peptide bonds and mimic their biological mode of action by retaining critical interactions in the active site.[9,10] The idea of designing cyclic ether-based ligands emerged from our observation that numerous bioactive natural products are comprised of these cyclic ether motifs. Of particular interest, ionophore antibiotic, monensin (**1**, Fig. 1)[11] and platelet activating factor antagonist, ginkgolide B (**2**),[12] which feature these cyclic ether subunits, do not suffer from oral bioavailability problems inherent to peptide and peptidomimetic-based inhibitor drugs.

Indeed, our structure-based design strategy led to the development of a number of cyclic ether-derived nonpeptide P2-ligands for the HIV protease substrate binding site. We have documented an intriguing potency enhancing effect of 3(*S*)-tetrahydrofuranyl urethane in inhibitors containing a hydroxyethylene isostere or a hydroxyethylsulfonamide isostere.[13] Incorporation of 3(*S*)-tetrahydrofuran into a (*R*)-(hydroxyethyl)sulfonamide isostere afforded a highly potent inhibitor which later became amprenavir.[14] It is noteworthy to mention that the tetrahydrofuranyl subunit is inherent in both monensin and various ginkgolides (Fig. 1).



Monensin (**1**)



Ginkgolide B (**2**)

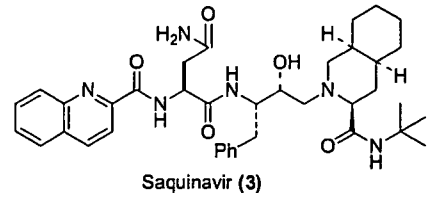
Figure 1. Structures of monensin and ginkgolide B.

2. Design of bis-THF: an inspiration from bioactive natural products

Analysis of a number of protein-ligand X-ray structures of 3(*S*)-tetrahydrofuranyl urethane-bearing inhibitors revealed weak hydrogen bonding between the tetrahydrofuranyl oxygen and the main chain aspartic acids (Asp-29 and Asp-30) as well as van der Waals interactions in the S2-site. Based upon this structure, our subsequent objective became to design a ligand that would maximize the hydrophobic and hydrogen bonding interactions with the residues in the S2-site. Our critical analysis of the saquinavir (**3**)-bound protease X-ray crystal structure led us to design and develop a stereochemically defined bicyclic tetrahydrofuran (bis-THF) ligand that appeared to effectively hydrogen bond with both Asp-29 and Asp-30 NHs. The bicyclic ring of the bis-THF is also poised to offset the loss of P3 hydrophobic binding of the quinoline ring in saquinavir. Inhibitor **4** (enzyme IC_{50} of 1.8 nM and antiviral CIC_{95} of 46 nM, Fig. 2) has shown improved aqueous solubility, reduced peptidic features and molecular weight compared to saquinavir.[9] Subsequent detailed studies established that the stereochemistry, the position of the oxygen atoms, ring sizes and substituents are all essential for potency.[9] The X-ray crystal structure of **4**-bound protease revealed that the bis-THF ring oxygens are involved in effective hydrogen bonding interactions with both the backbone NH's of Asp-29 and Asp-30 present in the S2 subsite. In essence, the bis-THF ligand, a subunit of ginkgolides (bicyclic acetal) remarkably mimics the binding of the P2 asparagine carboxamide and the P3 quinaldic amide carbonyl of saquinavir.

3. Design and development of 'darunavir' to combat drug resistance

Our analysis of protein-ligand complexes of wild-type and mutant proteases and an overlay of the corresponding protein backbones showed only minimal distortion of the backbone conformation, particularly in the active site of the protease.[15] This is also apparent in other reported high resolution X-ray structures of related inhibitor/ligand complexes.[16] This observation led us



Saquinavir (**3**)

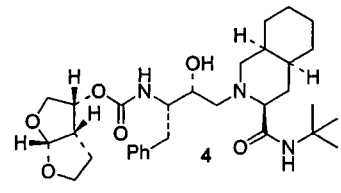


Figure 2. Structures of saquinavir and a bis-THF inhibitor.

to speculate that an inhibitor making extensive hydrogen bonding interactions with the protein backbone of the wild-type enzyme will also maintain potency against mutant strains. Our inhibitor design strategy to combat drug-resistance then focused on optimizing of the ligand-binding site interactions so as to make maximum interactions in the active site, including hydrophobic, electrostatic and most critical, hydrogen bonding with the backbone atoms located in the S2 to S2′-subsites of protease. As mentioned previously, inhibitor **4**-bound X-ray structure of HIV protease revealed that while the P2 bis-THF ligand makes extensive interactions including backbone hydrogen bonding in the S2-subsite, similar hydrogen bonding in the S2′-site are mostly absent.

With the objective of designing an inhibitor that can make robust hydrogen bonding throughout the S2-S2′-subsites, we investigated the effect of a P2 bis-THF ligand with a number of different isosteres.[9] Incorporation of the P2 bis-THF in (*R*)-(hydroxyethyl) sulfonamide isosteres led to a number of potent PIs, with impressive drug resistance profiles. For instance, inhibitor **5** (UIC-PI or UIC94003 and later TMC-126), incorporating a P2 bis-THF and a *p*-methoxysulfonamide as the P2′ ligand exhibited remarkably potent enzyme inhibitory potency (K_i value 14 pM, Fig. 3) and antiviral activity (ID_{50} value of 1.2 nM) in CEM cell lines.[17] It was also profiled against numerous mutant HIV proteases. The K_i was less than 100 pM in every case and the K_{imut}/K_{iwt} was no greater than five. This indicates a low level of resistance even for enzymes with multiple mutations which have been shown to be resistant to clinically active inhibitors. TMC-126 has shown a remarkable drug resistance profile and has maintained high potency in the presence of human serum albumin.[17]

Inhibitor **6** (UIC 94017, later known as TMC-114) with a bis-THF as the P2 ligand and *p*-aminosulfonamide as the P2′ ligand has also exhibited very impressive inhibitory properties. It displayed an enzyme inhibitory potency (K_i) of 16 pM and an antiviral ID_{50} of 4.7 nM in CEM cell lines. It showed an antiviral ID_{90} of 10 nM, and a $TD_{50} > 100$ μM in cell culture assays.[18] Inhibitor-bound X-ray structure analysis revealed that both P2 and P2′-ligands of inhibitor **6** are involved in extensive hydrogen bonding with the protein backbone. This may be responsible for its potency and wide-spectrum activity against multi-PI-resistant HIV-1 variants. It was tested against a panel of 20 HIV variants resistant to current PI's, but there was no greater than a 5-fold increase in ID_{50} values, indicating it remained active against the resistant strains. In addition, the P2′-amine group provided more favorable pharmacokinetic properties compared to the P2′-methoxy group in inhibitor **5**. Subsequently, it was selected for clinical development and renamed darunavir.[19]

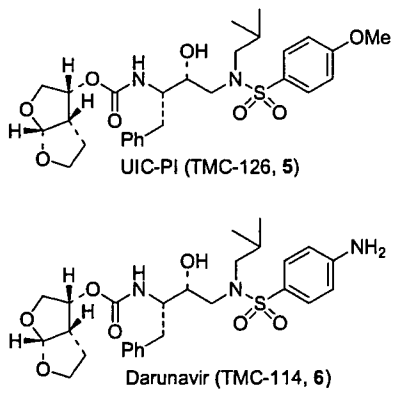


Figure 3. Structure of darunavir and inhibitor **5**.

Clinical development of darunavir was conducted by Tibotec-Virco, Belgium.[18b] POWER 1 and POWER 2 clinical trials of ritonavir-boosted darunavir (DRV/r) were carried out with treatment-experienced patients who were no longer benefiting from available PIs. Over a period of six months, both studies showed that combination therapy using DRV/r led to a reduction in viral load below 50 copies/mL in 45% of participants compared with only 12% of participants given another available PI. CD4+ cell counts in the DRV/r group rose by an average of 92 cells/mm^3 over the six month period compared with an average increase of 17 CD4+ cells/mm^3 for participants receiving another PI during this time.[20] POWER 3, a non-randomized, open-label trial was conducted to assess the long-term efficacy and safety of DRV/r 600/100 mg BID in treatment-experienced patients. The primary efficacy endpoint was the proportion of patients with $\geqslant 1$ log10 reduction in HIV RNA by week 24. Reduction of HIV RNA with an efficacy endpoint of $\geqslant 1$ log10 was observed in 65% of patients. Reductions in HIV RNA levels to < 400 copies/mL and < 50 copies/mL were observed in 57% and 40% of patients, respectively. DRV received accelerated approval by the FDA on June 23, 2006.[20] Recent studies have shown that darunavir, when used in combination with the fusion inhibitor FUZEON, can substantially increase the chances of reaching undetectable viral load.[21]

4. Recent PIs based on the bis-THF ligand

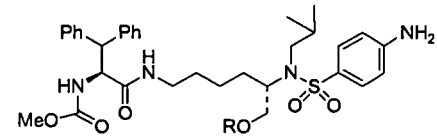
The success of Darunavir and the evidence indicating the importance of the bis-THF P2 ligand has led to an expansion of the 'backbone binding concept', and produced several novel and active PIs. Ritonavir has recently been modified with the addition of the P2-bis-THF ligand, and initial SAR results revealed a new potent inhibitor.[22] Introduction of a fused benzodioxolane and other related functionalities as P2′ sulfonamides have shown significant potency enhancement and drug-resistance properties.[23] GlaxoSmithKline researchers have investigated various structural modifications at the P1 and P1′ positions of inhibitors containing a bis-THF group as the P2-ligand and a benzodioxolane sulfonamide as the P_2'-ligand.[24] One of the inhibitors has shown IC_{50} values in the single digit nanamolar range as well as K_i's in the femtomolar range.[25] This inhibitor was later renamed brecanavir and had undergone extensive clinical studies. However

brecanavir development was later terminated reportedly due to its difficulty to formulate. Tibotec researchers have discovered a new series of fused benzoxazole and benzothiazole ligands to fit the S2′ domain. These new inhibitors have shown broad-spectrum antiviral activity against PI resistant mutants, as well as excellent pharmacokinetic properties.[26]

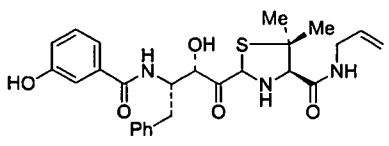
5. Beyond the bis-THF ligand and darunavir: recent developments in protease inhibitors

Recently, Procyon pharmaceuticals has reported a new class of protease inhibitors based on L-lysine. Uniquely, two novel sulfonamide based PIs (PL-100, **7** and PPL-100, **8**, Fig. 4) have displayed good protease inhibitory activity.[27] They have shown enzyme inhibitory activity less than 20 nM, but their preliminary cross-resistance results are very impressive. Against 14 viral stains from highly PI-experienced patients, PL-100 showed a 4.5 average fold-increase in IC_{50} values.[28] The phosphate ester prodrug, PPL-100 (**8**), showed a superior pharmacokinetic profile.[29] AG-001859, **9** is another recently identified compound which exhibited potency against resistant strains of HIV.[30] This new compound is an allophenylnorstatin-based PI, and has shown K_i's for wild-type and mutant proteases as low as 0.1 nM. When AG-001859 was tested against 44 PI-resistant HIV-1 isolates, it displayed excellent potency with a median EC_{50} of 34 nM (range 5.3–420 nM).[30] AG-001859 was selected for further testing and has started a phase 1 clinical trial.

We recently developed a new P2 ligand, based upon the 'backbone binding' design concepts. Inhibitor **10** contains a stereochemically defined bicyclic hexahydrocyclopentanofuran as a P2 ligand and a hydroxymethylphenylsulfonamide group as the P2′ ligand (Fig. 4) It has shown potent antiviral activity (K_i = 4.5 pM, IC_{50} = 1.8 nM) and effective drug-resistance properties against a panel of multi-PI-resistant HIV-1 isolates with IC_{50} values ranging from 4–52 nM.[31]



7 R = H (PL-100)
8 R = $OPO(OH)_2$ (PPL-100)



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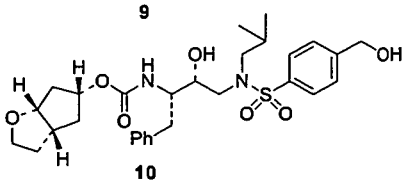

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Figure 4. Structures of recent protease inhibitors.

6. Future directions of anti-protease treatment

The future management of HIV/AIDS should rely upon the development of therapies that are less toxic and more effective in combating drug-resistance. Since protease inhibitors are very important components of current HAART regimens, design and development of new PIs with improved pharmacological properties and better drug-resistance profiles are of great importance. In this context, our design strategies target the active site protein-backbone as there is minimal change in the backbone conformations of the wild-type and mutant proteases. Of particular note, we have developed a new generation of PIs bearing a structure-based designed bis-THF ligand that effectively fills in the hydrophobic pocket and maximizes hydrogen bonding interactions with the backbone atoms of the S2-site. A number of bis-THF-derived inhibitors are exceedingly potent and have maintained very impressive potency against multidrug-resistant HIV-1 variants. One of these inhibitors, darunavir, has been recently approved by the FDA as the first treatment of drug-resistant HIV. Our detailed structural analysis of darunavir-bound wild-type and mutant proteases have documented extensive hydrogen bonding interactions with the active site backbone atoms. This design concept targeting the backbone may serve as an important guide to combat drug resistance. Further development of novel PIs with designed functionalities is currently the focus of our ongoing investigation.

Acknowledgments

Financial support by the National Institutes of Health (GM 53386; A.K.G.), the Japan Health Sciences Foundation (International Research Grant SA14801; H.M. and A.K.G.), and the Intramural Research Program of Center for Cancer Research, National Cancer Institute, NIH (HM) is gratefully acknowledged. We also thank Dr. Bruno Chapsal for helpful discussion.

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Antiviral Chemistry & Chemotherapy 18:201–211

Potent and selective inhibition of Tat-dependent HIV-1 replication in chronically infected cells by a novel naphthalene derivative JTK-101

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In search for effective human immunodeficiency virus type 1 (HIV-1) transcription inhibitors, we have evaluated more than 100,000 compounds for their inhibitory effects on HIV-1 long terminal repeat (LTR)-driven reporter gene expression, and identified a novel naphthalene derivative, JTK-101. This compound could suppress tumour necrosis factor (TNF)-α-induced HIV-1 production in latently infected OM-10.1 cells at nanomolar concentrations. JTK-101 could also potently inhibit constitutive HIV-1 production in MOTL-4/III$_B$. However, the antiviral activity of JTK-101 was found to be much weaker in acutely infected cells and the chronically infected cells U937/III$_B$ cells than in OM-10.1 and MOLT-4/III$_B$ cells. JTK-101 selectively suppressed TNF-α-induced HIV-1 mRNA synthesis in OM-10.1 cells in a dose-dependent fashion. JTK-101 modestly inhibited TNF-α-induced HIV-1 LTR-driven reporter gene expression, but potently inhibited Tat-induced gene expression. Immunoblot analysis revealed that low-level expression of the Tat cofactors CDK9 and cyclin T1 might contribute to the diminished antiviral activity in U937/III$_B$ cells. Furthermore, JTK-101 could not inhibit HIV-1 replication in chronically infected monocytes/macrophages, in which CDK9 and cyclin T1 were undetectable. These results suggest that JTK-101 exerts its anti-HIV-1 activity through the inhibition of known or unknown Tat cofactors, presumably CDK9/cyclin T1.

Keywords: CDK9/cyclin T1, HIV-1, naphthalene derivative, NF-κB, Tat

Introduction

Significant progress in the treatment of human immunodeficiency virus type 1 (HIV-1) infection has been achieved by the advent of highly active antiretroviral therapy (HAART), which targets different steps in the viral replication cycle with multiple inhibitors (Yeni *et al.*, 2004). At present, one entry inhibitor, eight nucleoside or nucleotide reverse transcriptase inhibitors (NRTIs), three non-NRTIs (NNRTIs) and eight protease inhibitors (PIs) are available for the treatment of HIV-1 infection. HAART with these inhibitors has significantly decreased plasma viraemia to undetectable levels and has considerably improved the survival of infected individuals (Pomerantz & Horn, 2003). However, considering the drug resistance and side effects of long-term HAART, discovery of novel HIV-1 agents with different mechanisms of action is still highly desirable. In addition, the reservoir cells containing latent HIV-1 are capable of producing infectious particles after cellular activation, which leads to a rebound of the viral load after interruption of HAART (Pierson *et al.*, 2000). Therefore, HAART cannot be terminated unless such reservoir cells have been eradicated or viral recovery from the cells can be completely suppressed. In this regard, inhibitors that selectively prevent HIV-1 gene expression can potentially inhibit the recovery of latent virus from resting memory T cells as well as infected monocytes/macrophages (M/Ms), which are also considered to be a long-surviving chronically infected cell population in HIV-1-infected patients.

Molecular analyses of HIV-1 replication have revealed a concerted complexity that regulates the viral life cycle. Among the various steps of the HIV-1 life cycle, transcription from the integrated proviral DNA is considered to be a crucial step for viral replication, as amplification of the viral genetic information is attainable only through transcription (Cullen, 1991; Jones & Peterlin, 1994; Okamoto, 1995). The viral-encoded transactivator protein Tat stimulates transcriptional elongation through its interaction with the transactivation response (TAR) RNA structure. Tat also interacts with cellular cofactors, such as positive transcription elongation factor b (P-TEFb), a complex composed of cyclin T1 and cyclin-dependent

kinase 9 (CDK9; Peng *et al.*, 1998; Price, 2000; Wei *et al.*, 1998). CDK9 hyperphosphorylates the carboxy-terminal domain of RNA polymerase II, and induces efficient promoter clearance and transcriptional elongation. In addition to the viral protein Tat, several cellular factors are known to regulate HIV-1 gene expression (Peterlin & Trono, 2003). Among these factors, nuclear factor κB (NF-κB) is the most potent activator of HIV-1 gene expression (Nobel & Baltimore, 1987). In general, NF-κB exists in an inactive form in the cytoplasm, where it is bound to the inhibitory molecule IκBα. Stimulation of the cells with several cytokines, such as tumour necrosis factor-α (TNF-α), leads to the immediate degradation of IκBα and activates NF-κB, resulting in immediate translocation of NF-κB from the cytoplasm to the nucleus (Roulston *et al.*, 1995). HIV-1 gene expression is initiated and enhanced by the activation of NF-κB and subsequent binding to the specific DNA motifs in the enhancer region of the HIV-1 long terminal repeat (LTR). However, complex and unknown machinery may also be involved in the regulation of HIV-1 gene expression.

Several compounds have been reported to suppress HIV-1 gene expression and replication through the inhibition of Tat or NF-κB. In our previous studies, the fluoroquinoline derivative K-37 proved to be a potent and selective HIV-1 transcription inhibitor in both acutely and chronically infected cells at nanomolar concentrations (Baba *et al.*, 1998). K-37 was an inhibitor of not only Tat but also other RNA-dependent transactivators. Although its target molecule remains to be elucidated, the aminoquinolone WM5, which is structurally related to K-37, was found to interact with the bulge region of the TAR (Parolin *et al.*, 2003; Richter *et al.*, 2004).

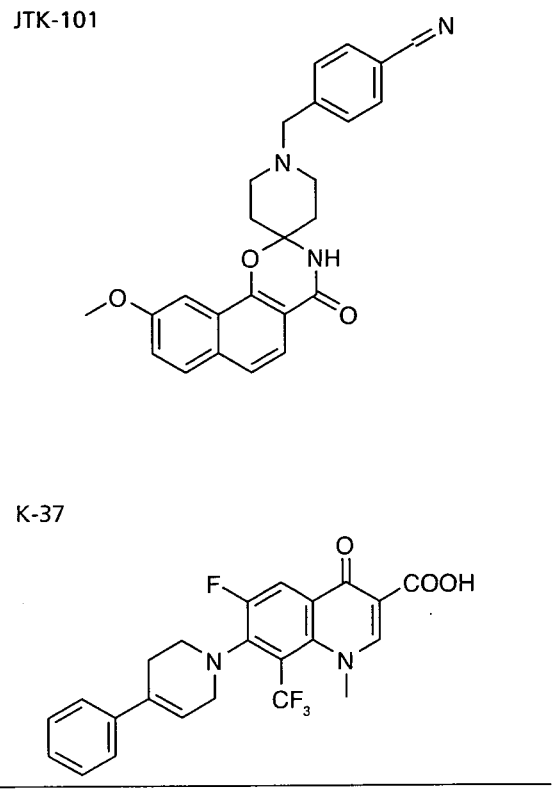
In our recent extensive search programme for novel HIV-1 transcription inhibitors, more than 100,000 compounds have been examined for their inhibitory effects on HIV-1 LTR-driven reporter gene expression in cell cultures. Among the test compounds, several compounds showed selective inhibition of HIV-1 replication in chronically infected cells; the novel naphthalene derivative JTK-101 (Figure 1) was selected as the representative of the active compounds because it exhibited the highest selectivity. JTK-101 is a more potent and selective transcription inhibitor of HIV-1 than K-37 in latently and chronically infected cells. Studies of its mechanism of action suggest that JTK-101 is an inhibitor of Tat cofactors, presumably CDK9/cyclin T1.

Materials and methods

Compounds

JTK-101 (Figure 1) was synthesized by Japan Tobacco Co. (Takatsuki, Japan) and the fluoroquinoline derivative K-37 (Figure 1) was provided by Daiichi Pharmaceutical Co.

Figure 1. Chemical structures of JTK-101 and K-37



(Tokyo, Japan). Lamivudine (3TC), zidovudine (AZT), and the histone deacetylase inhibitor trichostatin A (TSA) were purchased from Sigma (St. Louis, MO, USA). All compounds were dissolved in DMSO at 10 mM or higher concentrations to exclude any antiviral or cytotoxic effect of DMSO and stored at -20°C until use.

Cells and virus

Peripheral blood mononuclear cells (PBMCs), CEM, MOLT-4, OM-10.1 cells, MOLT-4/III_B, and U937/III_B cells were used in antiviral assays. OM-10.1 cells are a clone of HL-60 cells latently infected with HIV-1. MOLT-4/III_B and U937/III_B cells are MOLT-4 and U937 cells chronically infected with HIV-1 (III_B strain), respectively. PBMCs were obtained from healthy donors and stimulated with phytohaemagglutinin (PHA; Sigma). W-3 and KM-3 cells were clones of CEM cells that stably integrate an HIV-1 LTR-driven secreted alkaline phosphatase (SEAP) gene. The integrated HIV-1 LTR contains two intact NF-κB-binding sites in W-3 cells, whereas both of the sites are mutated in KM-3 cells. M/Ms were isolated from healthy donors and cultivated according to the procedure described previously (Perno *et al.*, 1988). Two strains of HIV-1 (III_B and Ba-L) were used in antiviral assays. III_B and Ba-L are CXCR4- and CCR5-using strains,

respectively. One CCR5-using HIV-1 isolate (CTV), and one CCR5- and CXCR4-using HIV-1 isolate (HE) were also used in antiviral assays.

Antiviral assays

The activities of the compounds against chronic HIV-1 infection were based on the inhibition of HIV-1 p24 antigen production. OM-10.1 cells (1×10^5 cells/ml) were incubated in the absence or presence of the compounds for 2 h and stimulated with 1 ng/ml TNF-α (Boehringer-Mannheim, Mannheim, Germany), whereas MOLT-4/III_B and U937/III_B cells (1×10^5 cells/ml) were cultured in the absence or presence of the test compounds without any stimulation. After 3 days of incubation at 37°C, the culture supernatants were collected and their p24 antigen levels were determined with a sandwich enzyme-linked immunosorbent assay kit (Cellular Products, Buffalo, NY, USA). The cytotoxicity of the test compounds for the chronically infected cells were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (Pauwels *et al.*, 1988).

The assay procedure for measuring the anti-HIV-1 activity of the compounds in chronically infected M/Ms was also based on the quantitative detection of p24 antigen in the culture supernatants. The isolated M/Ms (5×10^4 cells/ml) were cultured in RPMI 1640 medium supplemented with 10% heat-activated fetal calf serum, 10% heat-inactivated human AB serum, penicillin G (100 U/ml), and streptomycin (100 μg/ml). At day 7, differentiated M/Ms were infected with HIV-$1_{Ba\text{-}L}$ (10 ng of p24 per 5×10^4 cells). After 24 h incubation, the cells were washed and cultured for a further 9 days. After extensive washing, chronically infected M/Ms were cultured in the absence or presence of the test compounds for 4 days without medium change. The culture supernatants were collected and examined for their p24 antigen levels. The cytotoxicity of the test compounds for M/Ms was also determined by the MTT method.

The compounds' activities against acute HIV-1 infection were based on the inhibition of virus-induced cytopathicity in CEM cells and p24 antigen production in PBMCs, as described previously (Baba *et al.*, 1998). CEM cells (1×10^5 cells/ml) were infected with the virus at a multiplicity of infection of 0.01 and cultured in the presence of various concentrations of the compounds. After 4 days of incubation at 37°C, the CEM cells were subcultured at a ratio of 1:5 with fresh culture medium containing appropriate concentrations of the test compounds and further cultured. For the assays in PBMCs, the cells (1×10^5 cells/ml) were infected with HIV-1 at a multiplicity of infection of 0.1. After virus adsorption for 2 h, the cells were extensively washed to remove unadsorbed virus particles and cultured in the presence of various concentrations of the test compounds. After 6 days incubation at 37°C, the culture supernatants were collected and examined for their p24 antigen levels. The cytotoxicity of the test compounds were also determined by the MTT method.

Quantitative RT-PCR analysis

OM-10.1 cells (2.5×10^5 cells/ml) were incubated in the absence or presence of the JTK-101 for 2 h, stimulated with 1 ng/ml TNF-α, and further incubated for 24 h. Total RNA was extracted from the cells with an RNA extraction kit (Promega, Madison, WI, USA). The extracted RNA was subjected to quantitative RT-PCR analysis to determine HIV-1 mRNA, using GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). For quantitative RT-PCR, Taqman One-Step RT-PCR Master Mix Reagents Kit (Applied Biosystems) was used according to the manufacturer's instructions. Using the sequence of the HIV-1 molecular clone HXB2, a primer pair and a probe were designed downstream of the transcription initiation site for HIV-1 mRNA. The primer pairs and the probes were 581F (5′-TGGTAACTAGAGTCCCTCAGACC-3′, nucleotide position 582–605), 683R (5′-AGCTCCTCTG-GTTTCCCTTTC-3′, nucleotide position 662–682) and 620T (5′-TGGAAAATCTCTCTAGCAGTGGCGCC-GAAC-3′, nucleotide position 619–647). Non-specific inhibition of host cellular mRNA synthesis by JTK-101 was determined with Taqman GAPDH Control Reagents kit (Applied Biosystems).

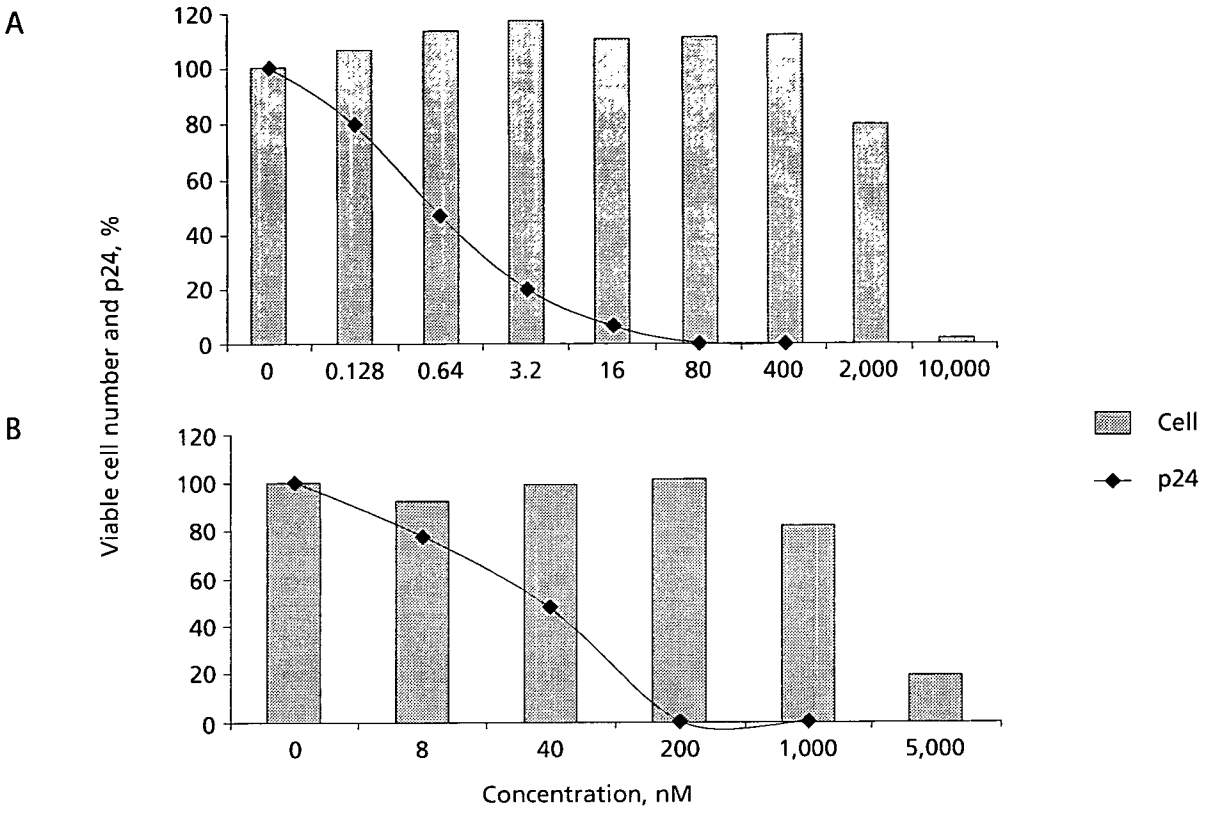
Reporter gene assays

W-3 and KM-3 cells were either treated with 10 ng/ml TNF-α or transfected with 1 μg of plasmid expressing HIV-1 Tat, which contains the second exon under the control of the simian virus 40 promoter (modification of pSV2tat72), by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The cells were cultured in the presence of various concentrations of the test compounds. After 2 days of incubation at 37°C, the SEAP activities in the culture supernatants were determined by chemiluminescence. The SEAP activities were measured using the GreatEscape SEAP detection kit (CLONTECH, Palo Alto, CA, USA), according to the manufacturer's instructions. The chemiluminescence intensity was measured with a LB96P luminometer (Berthold, Wildbad, Germany). At the same time, the number of viable cells was determined by the MTT method.

Immunoblot analysis

Immunoblot analysis was performed as described previously (Wang *et al.*, 2002). Briefly, cells extracts were prepared by incubating cells in lysis buffer (50 mM Tris [pH 8.0], 120 mM NaCl and 0.5% NP-40) containing protease inhibitor cocktail and phenylmethylsulphonyl fluoride (Sigma, St. Louis, MO, USA). Protein concentra-

Figure 2. Inhibitory effects of JTK-101 and K-37 on HIV-1 replication in TNF-α-stimulated OM-10.1 cells



(A) Inhibitory effects of JTK-101. **(B)** Inhibitory effects of K-37. OM-10.1 cells were incubated in the absence or presence of the test compounds for 2 h, stimulated with TNF-α (1 ng/ml), and further incubated. After 3 days of incubation, the p24 antigen levels of culture supernatants (lines) were measured by ELISA. At the same time, the number of viable cell was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (columns). The experiments were repeated three times and representative results are shown.

tions were determined by Bio-Rad protein assay, and equal amount of total protein was loaded onto 10% sodium dodecyl sulphate-polyacrylamide gels. The immunoblotting procedure using enhanced chemiluminescence for detection was also described previously. CDK9 was detected using a rabbit polyclonal antibody (C-20 sc-484; Santa Cruz Biotechnology, Santa Cruz, CA, USA), Cyclin T1 was detected using a goat polyclonal antibody (T-18 sc-8127; Santa Cruz Biotechnology), and actin was detected with goat polyclonal antibody (C-11 sc-1615; Santa Cruz Biotechnology). Complexes were then detected using an anti-rabbit horseradish peroxidase-conjugated secondary goat antibody and an anti-goat horseradish peroxidase-conjugated secondary rabbit antibody (MP Biomedicals, Aurora, OH, USA), and visualized by enhanced chemiluminescence western blotting detection system (Amersham Biosciences, Buckinghamshire, UK). For chronically infected M/Ms, the isolated M/Ms were kept uninfected or infected with HIV-$1_{Ba\text{-}L}$ (10 ng of p24 per 5×10^4 cells). After chronic infection of M/Ms with HIV-$1_{Ba\text{-}L}$, the M/M cells and culture supernatants were collected on day 17 for cellular extract preparation and p24 antigen detection, respectively. HIV-1 p24 levels in four donors ranged between 30 and 200 ng/ml (data not shown). As a control, PBMCs obtained from the same donors and stimulated with PHA for 3 days were also used for western blot analysis for CDK9 and cyclin T1.

Results

Antiviral activity in chronically infected cell lines

We first evaluated JTK-101 for its inhibitory effects on HIV-1 replication in chronically infected cells. OM-10.1 cells produce little or no HIV-1 under basal conditions, but do produce a significant level of virus after stimulation with TNF-α or phorbol 12-myristate 13-acetate (PMA; Butera *et al.*, 1991). In fact, the level of HIV-1 p24 antigen in culture supernatant was less than 1 ng/ml in unstimulated OM-10.1 cells, yet it reached 100 ng/ml or more after stimulation with 1 ng/ml TNF-α (data not shown). As shown in Figure 2A, JTK-101 suppressed p24 antigen production in

Table 1. Inhibitory effects of JTK-101 and other selected compounds on HIV-1 replication in chronically infected cells*

Compound	Cells	EC_{50}†, μM	CC_{50}‡, μM	SI§
JTK101	OM-10.1	0.0014 ±0.0005	3.8 ±0.2	2,714
	MOLT-4/III_B	0.0057 ±0.0025	1.3 ±0.4	228
K-37	OM-10.1	0.033 ±0.012	2.1 ±0.3	63
	MOLT-4/III_B	0.074 ±0.033	>5.0	>68
3TC	OM-10.1	>20	>20	-
	MOLT-4/III_B	>20	>20	-

*All data represent means ±SD for three separate experiments. †Concentration required for 50% inhibition of p24 antigen production in culture supernatants. ‡Concentration required for 50% inhibition of cell proliferation and viability. §Selectivity index (ratio of CC_{50} to EC_{50}).

TNF-α-stimulated OM-10.1 cells in a dose-dependent fashion. The compound completely prevented p24 antigen production at a concentration of 0.08 μM. However, it did not reduce cell viability and proliferation at a concentration of 2 μM. The EC_{50} and CC_{50} of JTK-101 were 0.0014 and 3.8 μM, respectively. K-37 (Figure 1), another potent HIV-1 transcription inhibitor, could also suppress the production of p24 antigen in a dose-dependent fashion (Figure 2B). The EC_{50} and CC_{50} of K-37 was 0.033 and 2.1 μM, respectively (Table 1). Thus, their selectivity indexes, based on the ratio of their CC_{50}s to EC_{50}s, were 2,714 and 63 for JTK-101 and K-37, respectively, indicating that JTK-101 is a much more potent and selective inhibitor of HIV-1 replication in chronically infected cells than K-37.

The inhibitory effects of JTK-101 on HIV-1 replication were also evaluated in MOLT-4/III_B and U937/III_B cells, both of which continuously produce a large amount of virus without any stimuli (data not shown). As shown in Figure 3A, JTK-101 efficiently suppressed HIV-1 production in MOLT-4/III_B cells even at very low concentrations, yet higher concentrations are required to completely block viral production in MOLT-4/III_B cells than in OM-10.1 cells. Again, K-37 was less active than JTK-101. The EC_{50}s of JTK-101 and K-37 in MOLT-4/III_B cells were 0.0057 and 0.074 μM, respectively (Table 1). Interestingly, little, if any, suppression of HIV-1 production by JTK-101 was observed in U937/III_B cells even at high concentrations (Figure 3B). In contrast, K-37 had similar inhibitory effect on HIV-1 production in MOLT-4/III_B and U937/III_B cells (Figure 3C and 3D). The NRTI 3TC was totally inactive in these chronically infected cells, such as OM-10.1 and MOLT-4/III_B (Table 1).

Antiviral activity in acutely infected cells

In the next experiment, JTK-101 was examined for its inhibition of HIV-1 (III_B strain) replication in acutely infected CEM cells and PBMCs. Although JTK-101 could suppress p24 antigen production in culture supernatants at non-toxic concentrations, the compound was found to be less inhibitory to HIV-1 replication in acutely infected cells than in chronically infected cells (Tables 1 and 2). The EC_{50} and CC_{50} of JTK-101 for HIV-1_{IIIB} in CEM cells were 0.031 and 1.0 μM, respectively, and its EC_{50} and CC_{50} in PBMCs were 0.39 and 1.2 μM, respectively (Table 2). Furthermore, JTK-101 also showed similar antiviral activity against the dual-tropic isolate HE and the R5 clinical isolate CTV in acutely infected MOLT-4 cells and PBMCs, respectively (data not shown). Unlike JTK-101, K-37 appeared to be equally inhibitory to HIV-1 replication in acutely and chronically infected cells (Tables 1 and 2). AZT was a highly potent inhibitor of HIV-1 replication in acutely infected CEM cells.

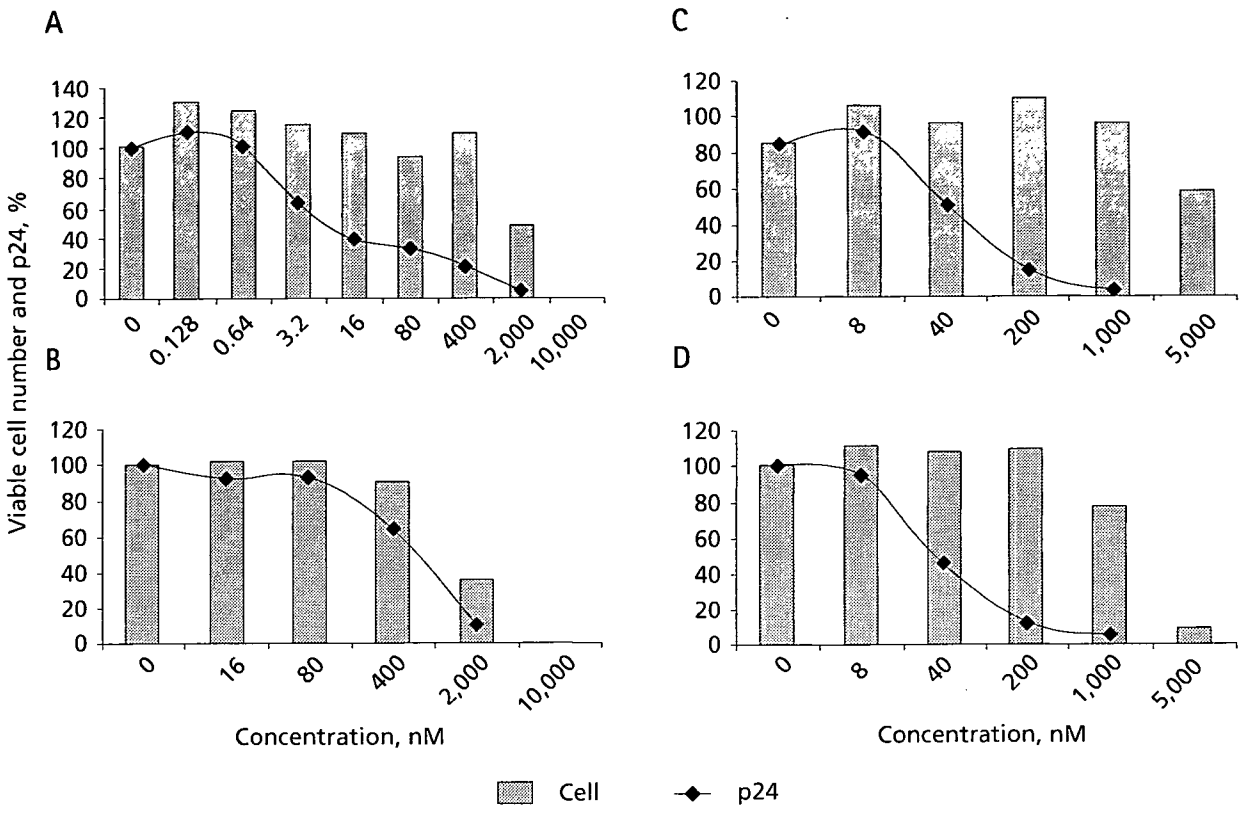
Inhibitory effect on HIV-1 transcription

As JTK-101 was selected through screening in an HIV-1 LTR-driven reporter gene expression system and showed potent anti-HIV-1 activity in chronically infected cells, the compound was assumed to be an HIV-1 transcription inhibitor. Therefore, quantitative RT-PCR analysis was conducted to determine whether JTK-101 could prevent HIV-1 mRNA synthesis in TNF-α-stimulated OM-10.1 cells. As shown in Figure 4, JTK-101 selectively suppressed TNF-α-induced HIV-1 mRNA synthesis in a dose-dependent fashion. Even at a concentration of 1 nM, the compound could prevent HIV-1 mRNA synthesis by 60% in the cells. By contrast, it did not affect glyceraldehydes-3-phosphate dehydrogenase (GAPDH) mRNA synthesis at concentrations up to 100 nM, indicating that JTK-101 selectively inhibits HIV-1 growth at the transcriptional level.

Inhibitory effects on TNF-α- and Tat-induced transactivation

To elucidate whether JTK-101 primarily inhibits Tat or the cellular transcriptional factor NF-κB, transfection experiments with a Tat expression plasmid into W-3 and KM-3 cells were conducted. Transfection with the Tat expression plasmid induced an increase of SEAP production in both W-3 and KM-3 cells. In contrast, treatment with TNF-α

Figure 3. Inhibitory effects of JTK-101 and K-37 on HIV-1 replication in MOLT-4/III_B and U937/III_B cells



(A) Inhibitory effects of JTK-101 in MOLT-4/III_B cells. **(B)** Inhibitory effects of JTK-101 in U937/III_B cells. **(C)** Inhibitory effects of K-37 in MOLT-4/III_B cells. **(D)** Inhibitory effects of K-37 in U937/III_B cells. MOLT-4/III_B and U937/III_B cells were cultured in the absence or presence of the test compounds without any stimuli. After 3 days of incubation, the p24 antigen levels of culture supernatants (lines) were measured by antigen ELISA. At the same time, the number of viable cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (columns). The experiments were repeated three times and representative results are shown.

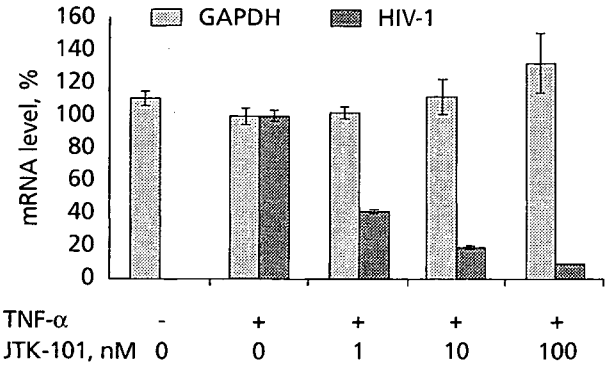
Table 2. Inhibitory effects of JTK-101 and other selected compounds on HIV-1 replication in acutely infected cells*

Compound	Cells	EC_{50}†, μM	CC_{50}‡, μM	SI§
JTK101	CEM	0.031 ±0.007	1.0 ±0.5	32
	PBMC	0.39 ±0.25	1.2 ±0.9	3.1
K-37	CEM	0.11 ±0.07	1.8 ±0.6	16
	PBMC	0.095 ±0.074	3.2 ±0.5	34
AZT	CEM	0.0026 ±0.0005	>1	>385

*All data represent means ±SD for three separate experiments. †Concentration required for 50% inhibition of p24 antigen production in culture supernatants. ‡Concentration required for 50% inhibition of cell proliferation and viability. §Selectivity index (ratio of CC_{50} to EC_{50}).

(10 ng/ml) induced an increase of SEAP production in W-3 cells, but not KM-3 cells, because two NF-κB binding sites of the HIV-1 LTR were mutated in KM-3 cells (Baba *et al.*, 1999). In both W-3 and KM-3 cells, JTK-101 could reduce the Tat-induced SEAP production in a dose-dependent fashion (Figure 5A). Interestingly, JTK-101 reduced the Tat-induced SEAP production more efficiently in KM-3 cells than in W-3 cells – its IC_{50} values in W-3 and KM-3 cells were 110 and 4.5 nM, respectively. K-37 inhibited Tat-induced SEAP production less than JTK-101. However, there was no substantial difference between K-37's activity in W-3 and KM-3 cells. The IC_{50} of K-37 in W-3 and KM-3 cells were 318 and 236 nM, respectively (Figure 5B). Furthermore, JTK-101 could reduce the TNF-α-induced SEAP production in W-3 cells with an IC_{50} of 229 nM, whereas K-37 had no effect on the

Figure 4. Inhibitory effects of JTK-101 on HIV-1 mRNA synthesis in OM-10.1 cells



The cells were incubated with the compound for 2 h, stimulated (+) with tumour necrosis factor (TNF)-α (1 ng/ml), and further incubated. After 24 h incubation, total RNA was extracted from the cells, and quantitative RT-PCR for HIV-1 mRNA was performed. The cytotoxic effects of the test compounds on host cellular mRNA synthesis were determined by quantitative RT-PCR for glyceraldehydes-3-phosphate dehydrogenase (GAPDH) mRNA. Representative results for two independent experiments are shown.

TNF-α-induced SEAP production (Figure 5C and 5D). In both cell systems, the compounds did not affect basal SEAP production (data not shown). These results suggest that JTK-101 is capable of inhibiting both Tat- and NF-κB-triggered gene expressions. However, the compound seems to predominantly interfere with a Tat-associated mechanism rather than a NF-κB-associated one. Both JTK-101 and K-37 were found to efficiently inhibit PMA- and TSA-induced HIV-1 production in OM-10.1 cells (data not shown).

CDK9/cyclin T1 level and JTK-101 activity

As the expression of CDK9 and cyclin T1 affect the transactivation by Tat, the protein levels of CDK9 and cyclin T1 were evaluated in several chronically infected cells. As shown in Figure 6, CDK9 and cyclin T1 were highly expressed in the T-lymphoblastoid cell lines CEM and MOLT-4. However, only low levels of the molecules were detected in the promonocytic cell line U937. Chronic infection of MOLT-4 and U937 cells with HIV-1 did not significantly alter the expression of CDK9 and cyclin T1. Furthermore, like MOLT-4/III_B cells, OM-10.1 cells displayed high level expression of CDK9 and cyclin T1. These results suggest that the poor activity of JTK-101 against HIV-1 production in U937/III_B is partly attributed to the low level expression of CDK9 and cyclin T1.

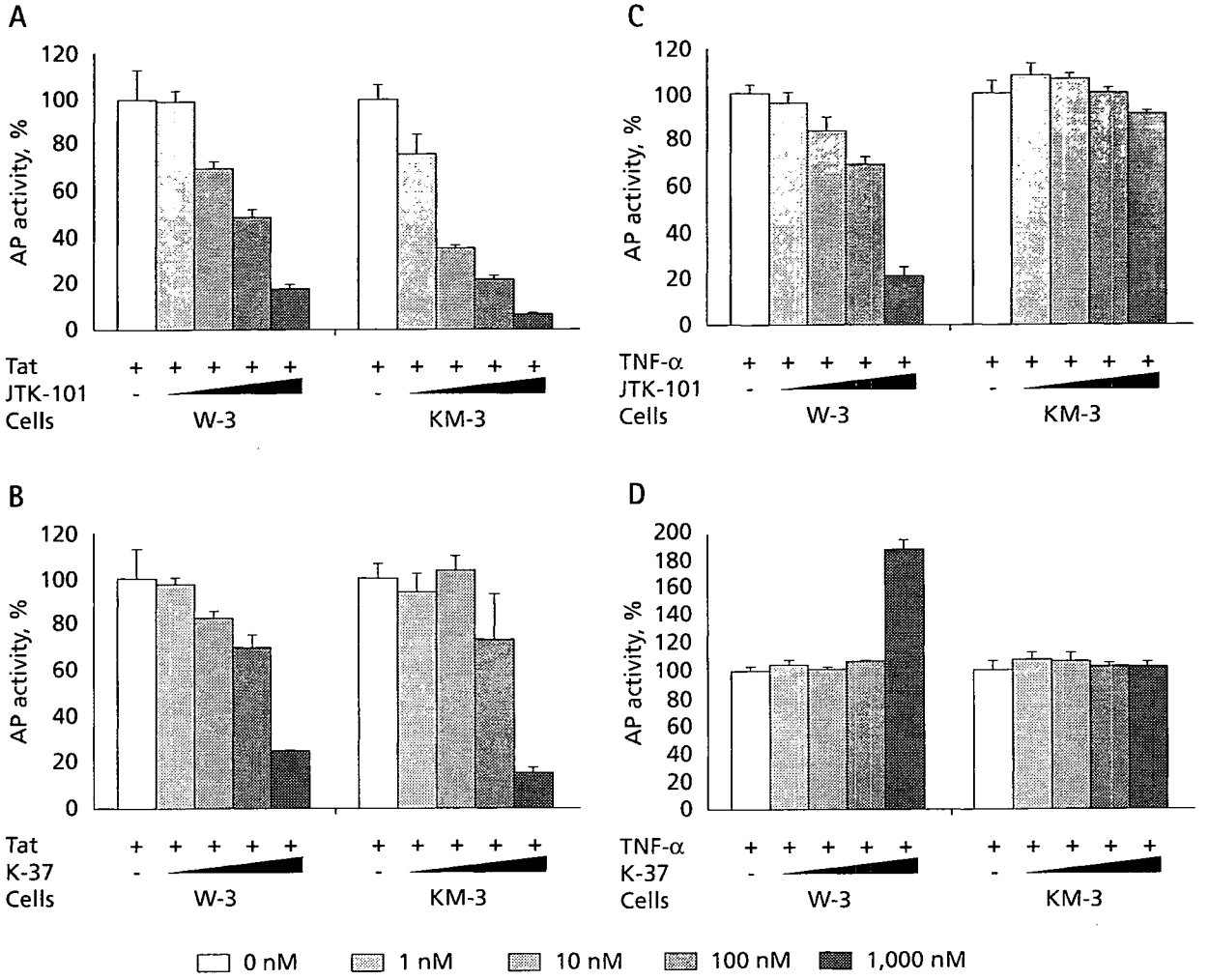
It was reported that the downregulation of cyclin T1 expression at a late stage of M/Ms differentiation contributed to low or absent Tat transactivation function at this stage (Liou *et al.*, 2002). Therefore, the level of CDK9 and cyclin T1 expression and the anti-HIV-1 activity of JTK-101 were examined in differentiated and chronically infected M/Ms. As expected, the expression of cyclin T1 was an undetectable level in both uninfected and chronically infected M/Ms after 17 days of cultivation (Figure 7A). CDK9 was also undetectable, although activated PBMCs obtained from the same donor displayed a high level of cyclin T1 and CDK9 expression. Furthermore, JTK-101 did not inhibit HIV-1 production in chronically infected M/Ms (Figure 7B), whereas K-37 did inhibit HIV-1 production in a dose-dependent fashion (data not shown). These results suggest that the interaction of JTK-101 with either cyclin T1 or CDK9, or both, is needed to exert its anti-HIV-1 activity.

Discussion

The presence of reservoir cells that contain latent viruses results in the prodcution of infectious particles upon cellular activation, which leads to a rebound of the viral load after interruption of HAART (Pierson *et al.*, 2000). The persistence of these virus reservoirs, despite prolonged HAART treatments, represents a major obstacle to the eradication of HIV-1 in infected patients (Finzi *et al.*, 1997; Wong *et al.*, 1997). Therefore, therapeutic targets for HIV-1 replication at the level of transcriptional activation hold great potential for further attempts at clearing viral latency. In this study, we have identified JTK-101, a novel naphthalene derivative, as a potent and selective transcription inhibitor of HIV-1 in latently and chronically infected cells. JTK-101 did not prevent proviral DNA synthesis (data not shown), suggesting that viral entry, uncoating, reverse transcription and integration are not the target of this compound. Furthermore, the inhibition of HIV-1 transcription by JTK-101 is potent and selective. Quantitative RT-PCR analysis revealed that JTK-101 almost completely inhibited HIV-1 mRNA synthesis without altering the level of GAPDH mRNA in TNF-α-treated OM-10.1 cells at a concentration of 100 nM (Figure 4).

K-37, an anti-HIV-1 fluoroquinoline derivative, also displayed selective inhibition of HIV-1 replication in acutely and chronically infected cells (Tables 1 and 2). However, its antiviral activity was weaker than that of JTK-101. K-37 was capable of inhibiting RNA-dependent transactivation mediated by Tat, but did not inhibit DNA-dependent transactivation mediated by NF-κB (Baba *et al.*, 1998; Okamoto *et al.*, 2000). Furthermore, K-37 did not inhibit but stimulated the NF-κB-mediated transactivation at the highest concentration tested (1,000 nM), yet the mechanism was still unknown (Figure 5D). Unlike K-37, JTK-101 could inhibit not only Tat but also NF-κB-mediated transactivation of the HIV-1 LTR (Figure 5). Although JTK-101 was highly inhibitory to Tat-mediated transactivation, a much higher concentration

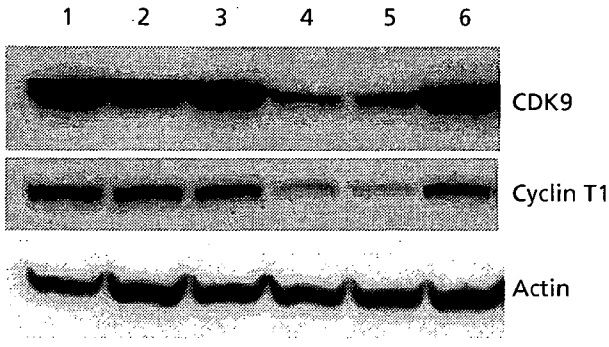
Figure 5. Inhibitory effects of JTK-101 and K-37 on HIV-1 Tat-induced or TNF-α-induced transactivation in W-3 and KM-3 cells



For HIV-1 Tat-induced transactivation **(A and B)**, W-3 and KM-3 cells were transfected with the Tat expression plasmid (1 μg). For tumour necrosis factor (TNF)-α-induced transactivation **(C and D)**, W-3 and KM-3 cells were treated with or without TNF-α (10 ng/ml). The cells were cultured in the presence of various concentrations of the compounds. After 2 days of incubation, the culture supernatants were collected and examined for their secreted alkaline phosphatase (SEAP) levels. At the same time, the number of viable cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) methods. Transfection with the Tat expression plasmid induced 8.8- and 6.3-fold increase of SEAP production in W-3 and KM-3 cells, respectively. While TNF-α stimulation induced 3.5- and 0.9-fold increases of SEAP production in W-3 and KM-3 cells, respectively. Effects of JTK-101 **(A and C)** and K-37 **(B and D)** on TNF-α- or HIV-1 Tat-induced transactivation were expressed as percent inhibition of SEAP activity. All experiments were carried out in duplicate and expressed as means (ranges). Representative results for two independent experiments are shown.

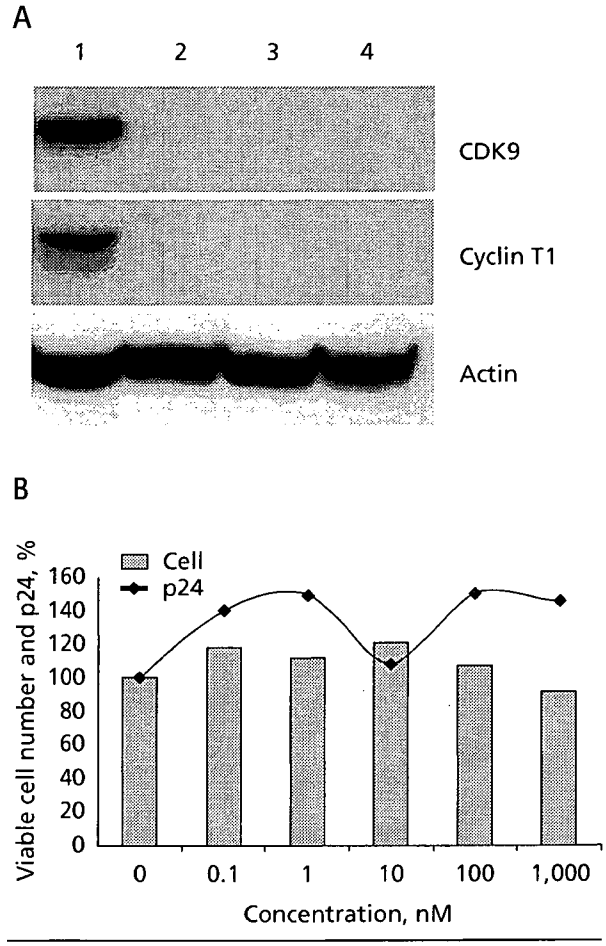
was required to inhibit NF-κB-mediated transactivation. Thus, it is assumed that JTK-101 exerts its potent anti-HIV-1 activity primarily through the inhibition of Tat function rather than NF-κB. Furthermore, JTK-101 was still active against HIV-1 replication in OM-10.1 cells, even when added to culture medium 24 h after stimulation with TNF-α (data not shown). The compound showed a similar inhibitory effect on TSA-induced HIV-1 production in OM-10.1 cells (data not shown), further suggesting that NF-κB was not a major target. Although the cellular transcription factor NF-κB plays an important role in triggering HIV-1 gene expression, the activation of NF-κB leads to rapid production of Tat, which may be necessary to maintain continuous HIV-1 gene expression in latently infected cells. Therefore, as well as NF-κB inhibitors, a Tat inhibitor could be effective in restricting the recovery of latent virus from resting T cells *in vivo*. Stevens *et al.* (2007) recently reported that N-aminoimidazole derivatives interfered with viral replication at a post-integrational level by inhibiting HIV-1 mRNA transcription. However,

Figure 6. Western blot analysis for CDK9 and cyclin T1 expression in various cell lines



Whole cell-lysates were fractionated by 10% sodium dodecyl sulphate-polyacrylamide gels, and western blot analysis was performed with anti-CDK9, anti-cyclin T1 or anti-actin polyclonal antibodies. The analysed samples were CEM cells (lane 1), MOLT-4 cells (lane 2), MOLT-4/III$_B$ cells (lane 3), U937 cells (lane 4), U937/III$_B$ cells (lane 5) and OM-10.1 cells (lane 6).

Figure 7. CDK9 and cyclin T1 expression in chronically infected M/Ms and anti-HIV-1 activity of JTK-101



(A) The isolated monocytes/macrophages (M/Ms) were kept uninfected (lane 2) or infected with HIV-1$_{Ba-L}$ on day 2 (lane 3) or day 7 (lane 4). After 24 h incubation, the cells were washed and further incubated. On day 17, the cells were harvested for cell extracts preparation. Western blot analysis was performed, as described in Figure 6. The culture supernatants were also collected on day 17 for p24 antigen detection. Their p24 levels of infected M/Ms on days 2 and 7 were 221.5 and 27.3 ng/ml, respectively. As a control, peripheral blood mononuclear cells (PBMCs) from same donors stimulated with phytohaemagglutinin (PHA) for 3 days were also examined (lane 1). **(B)** Inhibitory effects of JTK-101 on HIV-1 replication in chronically infected M/Ms. The isolated M/Ms were cultured for 7 days and infected with HIV-1$_{Ba-L}$. After 24 h of incubation, the cells were washed and cultured for further 9 days. After extensive washing, chronically infected M/Ms were cultured in the presence of various concentrations of JTK-101. After 4 days of incubation, the p24 antigen levels of culture supernatants (lines) were measured by ELISA. At the same time, the number of viable cell was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (columns). The experiments were repeated four times with PBMCs obtained from different donors; representative results are shown.

unlike JTK-101, these compounds enhanced NF-κB binding at the HIV-1 promoter. Furthermore, they suppressed viral transcription via potent inhibitory effects on the recruitment of Tat to the HIV-1 promoter and on the transcriptional processivity of RNA polymerase II during the viral transcription process (Stevens *et al.*, 2007).

In spite of robust inhibition of HIV-1 replication in the chronically infected cells OM-10.1 and MOLT-4/III$_B$, JTK-101 did not produce a significant inhibitory effect in U937/III$_B$ cells and primary M/Ms. It showed less inhibitory to HIV-1 replication in acutely infected cells, especially in acutely infected PBMCs, than in chronically infected cells This difference in anti-HIV-1 activity between acute and chronic stages of infection could be attributed to a distinct role of Tat in the infection stage or to the possibility that the compound interacts with known or unknown cellular factors involved in Tat-mediated transactivation.

Several lines of evidence have suggested that Tat function is largely dependent upon the interaction with the cellular transcription factor TAK/P-TEFb, a complex containing cyclin T1 and CDK9 (Herrmann & Rice, 1995; Mancebo *et al.*, 1997). In fact, several CDK inhibitors were found to potently suppress Tat functions and inhibited HIV-1 replication in cell cultures (Heredia *et al.*, 2005; Wang *et al.*, 2001). Our preliminary experiments demonstrated that JTK-101 could inhibit CDK9 with an IC$_{50}$ of 0.3 μM, whereas it did not show any inhibition of CDK7 and casein kinase II (data not shown). Although direct interaction between JTK-101 and CDK9 has not been demonstrated, its activity was totally dependent on the expression of CDK9 and cyclin T1 in infected cells. The expression of TAK/P-TEFb, especially cyclin T1, was regulated during differentiation from monocytes to macrophages. PMA, vitamin D$_3$ and other agents cause the human myelomonocytic cell line HL-60 and the promonocytic cell line U937 to differentiate into terminal cells exhibiting macrophage characteristics, accompanied by a dramatic increase in their cyclin T1 levels (Herrmann *et al.*,

1998). Furthermore, the cyclin T1 level in freshly isolated monocytes increases during the first 2–3 days in the differentiation process then starts decreasing to a very low level after 10 days (Liou *et al.*, 2002). As cyclin T1 is essential for the Tat function, it is possible that cyclin T1 regulates HIV-1 replication in certain types of cells. In fact, it has been reported that the level of cyclin T1 expression regulates Tat transactivation of the HIV-1 LTR in promonocytic cells and primary M/Ms (Herrmann *et al.*, 1998; Liou *et al.*, 2002). The transactivation activity of Tat was always weak or absent when cyclin T1 expression was very low. In such cells as U937 and primary M/Ms, JTK-101 may not be able to intervene between Tat and cyclin T1/CDK9 and exert its anti-HIV-1 activity.

Another interesting finding is that the inhibitory effect of JTK-101 on Tat-mediated transactivation was still affected by the function of NF-κB. JTK-101 was more efficient in suppressing Tat-mediated transactivation when the functional NF-κB binding sites were removed from the HIV-1 LTR (Figure 5). A previous study showed that Tat-mediated transactivation of the HIV-1 LTR was strictly dependent on the HIV-1 enhancer, especially NF-κB, in human blood $CD4^+$ T-lymphocytes (Alcami *et al.*, 1995). NF-κB-independent Tat transactivation could occur in transformed lymphoblastoid T-cell lines, but not in normal T lymphocytes (Alcami *et al.*, 1995). Thus, the absolute dependence of Tat function on κB responsive elements in T lymphocytes might bring about the much reduced anti-HIV-1 activity of JTK-101 observed in acutely infected PBMCs as compared with acutely infected CEM cells. Furthermore, Tat upregulates cytokine gene expression via a TAR-independent pathway and induces the production of proinflammatory cytokines, such as TNF-α and interleukin 1β, which activate NF-κB signal transduction pathways (Biswas *et al.*, 1995).

In conclusion, the novel naphthalene derivative JTK-101 is a potent and selective inhibitor of HIV-1 replication in cell cultures. Although its precise target molecule remains to be elucidated, the compound suppresses HIV-1 gene expression through the inhibition of known or unknown Tat cofactors, presumably CDK9/cyclin T1.

Acknowledgements

This work was supported in part by a research grant from the Ministry of Health Labor and Welfare, Japan.

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Received 5 April 2007, accepted 15 May 2007

JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 2008, p. 792–795 Vol. 46, No. 2
0095-1137/08/$08.00+0 doi:10.1128/JCM.01470-07

Dual-Reporter Phenotypic Assay for Human Immunodeficiency Viruses[▽]

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Received 22 July 2007/Returned for modification 27 September 2007/Accepted 5 December 2007

We have established a novel human immunodeficiency virus (HIV) tandem-reporter assay using HIV receptor-transduced NP-2 cells with long terminal repeat-controlled β-galactosidase, inserted internal ribosome entry site, and secretary alkaline phosphatase genes. This assay allows users to detect replication of clinical isolates, indicating its useful application as an HIV phenotypic assay.

Assays for detecting the emergence of resistant variants, as well as evaluating clinical efficacy, provide useful information regarding chemotherapy for human immunodeficiency virus (HIV) infection. To date, two types of assay systems have been developed and approved, namely, genotypic and phenotypic assays (13, 31). Genotypic assays detect genetic mutations that are associated with drug resistance and lead to rapid and sensitive detection of the emergence of resistant variants (9), although they only provide estimated resistance profiles (16, 30). Lists of significant resistance-associated mutations in reverse transcriptase (RT), protease, and envelope genes are maintained by some organizations and Universities, such as the International AIDS Society-USA (http://iasusa.org/resistance mutations); Los Alamos National Laboratory, Los Alamos, NM (http://resdb.lanl.gov/Resist_DB); and the Stanford University, Stanford, CA (http://hivdb.stanford.edu/index.html). PCR-based genotypic assays are heavily dependent on the primers used. Therefore, some biases must unfortunately be presumed, since most primer-matched sequences are preferentially amplified, resulting in some discordance with phenotypic assays (21, 33) that are time-consuming and require tedious procedures because isolation of replication-competent viruses is required. To date, phenotypic assays for clinical isolates have been mainly performed in experiments with a p24 production assay in phytohemagglutinin-stimulated peripheral blood mononuclear cells (27, 32, 34).

For more rapid and simple phenotypic assays, recombinant viruses containing the region responsible for resistance have been utilized instead of isolated viruses (14, 18, 35). However, since protease resistance mutations are introduced simultaneously with gag mutations (4, 25), cloning of the entire gag and protease coding region is occasionally required. Recently, mutations for 3′-azido-3′-deoxythymidine (AZT) resistance have been identified in the connection and/or RNase H subdomain (5, 7, 26), which no commercially available genotypic and phenotypic assays include for the analysis. Moreover, the mechanism of resistance to a fusion inhibitor, enfuvirtide, is a complex issue, since mutations in not only the gp41 coding region (6, 24) but also the V3 region (29) and the CD4-binding site (2) of gp120 influence the susceptibility, indicating that patient-derived viruses are ideally required for evaluation of drug susceptibility.

Recently, Hachiya et al. established a simple and rapid phenotypic assay using MAGIC5 cells (CCR5-transduced MAGI cells) (11). This system efficiently isolates clinical HIV variants and has proven to be useful for evaluating drug susceptibility (12). However, the expression of transduced receptors on MAGIC5 cells declines during prolonged culture, as described for MAGI cells (17). Therefore, in order to obtain HIV isolates efficiently and perform the assay, relatively fresh cells are required. More recently, we established a tetrazolium-based colorimetric assay for monitoring replications of not only CXCR4 (X4)-tropic but also CCR5 (R5)-tropic HIVs and drug susceptibilities (17). We reported that NCK45 cells stably express HIV receptors on their cell surface and provide reproducible results (17). Since this assay depends on the cytopathic effect induced by HIV, it appears to be insufficient for assessing infections with no or a few cytopathic variants. Furthermore, it requires 7 days of culture to obtain the drug susceptibility. In the present study, we have established a novel single long terminal repeat (LTR)-driven tandem two-reporter system using the internal ribosome entry site (IRES) (15), which enables the evaluation of drug susceptibility within 2 days for various HIVs, including clinical isolates.

To construct an LTR-driven reporter vector, an amplified LTR region (the −138 to +89 region of the transcriptional start site of HIV-1 molecular clone pNL4-3) was inserted into pβgal-Basic (Clontech Laboratories, Inc., Palo Alto, CA) between the NheI and HindIII sites (pLTR-βgal). The 5′ region (HindIII to EcoRV) of the β-galactosidase gene was replaced with the responsible β-galactosidase fragment with nuclear localization signal sequence (MPKKKRK) amplified from genomic DNA of MAGI cells (20). Fragments of IRES and secretory alkaline phosphatase (SEAP) were amplified from pIRES2-EGFP and pSEAP2-Basic (Clontech Laboratories, Inc.), respectively. A puromycin-resistance gene (Puro^r) under the control of the phosphoglycerate kinase promoter as a selection marker was inserted at the SalI site of the vector (pLTR-β-Gal/SEAP-Puro^r), as shown in Fig. 1A. All fragments were verified by sequencing.

The pLTR-β-Gal/SEAP-Puro^r plasmid was transfected into NCK45 cells (CD4, CXCR4, and CCR5-transduced NP-2 cells

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▽ Published ahead of print on 19 December 2007.

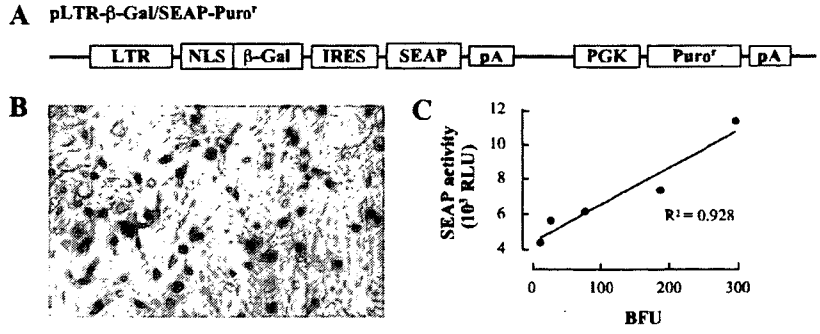

FIG. 1. Establishment of a cell line with β-galactosidase (β-Gal) and SEAP genes driven by an LTR. (A) Schematic diagram of the vector used in the present study, which simultaneously expresses genes for β-Gal and SEAP under the control of the HIV-1 LTR promoter (pLTR-β-Gal/SEAP-Puro^r). The enhancer region (positions −138 to +89) of the LTR, the nuclear localization signal (NLS) derived from the T-antigen of simian virus 40, the IRES, the phosphoglycerate kinase promoter (PGK), and the polyadenylation signal (pA) are also shown. (B) Microscopic image of X-Gal-stained NCK45-β-Gal/SEAP cells at 48 h after virus inoculation. (C) Correlation between β-Gal and SEAP activities in culture supernatants. NCK45-β-Gal/SEAP cells were infected with HIV at various infectious doses and incubated for 48 h. Culture supernatants were examined for their SEAP activities and expressed as relative right units (RLU). BFU, blue-cell-forming units.

derived from a glioma) (17, 36) to detect intracellular Tat expression through the LTR-driven tandem reporter genes. At 48 h after transfection, the cells were cultured in Dulbecco modified Eagle medium (Sigma, St. Louis, MO) supplemented with 5% heat-inactivated fetal calf serum, 0.5 mg of G418 disulfate (Nacalai Tesque, Kyoto, Japan)/ml, 0.2 mg of hygromycin B (Calbiochem, La Jolla, CA)/ml, and 10 μg of puromycin (Sigma)/ml and designated NCK45-β-Gal/SEAP cells. The expression levels of receptors on NCK45 cells confirmed by a flow cytometer (17) were 97, 83, and 99%, while those on H9 cells as a control were 65, 73, and 0.3% for CD4, CXCR4, and CCR5, respectively.

To evaluate anti-HIV agents, NCK45-β-Gal/SEAP cells (5×10^4 cells/ml) in Dulbecco modified Eagle medium-based culture medium supplemented with 5% fetal calf serum, penicillin, and streptomycin were seeded onto 96-well plates. On the following day, the cells were inoculated with sample viruses at 60 blue-cell-forming units (BFU)/well, incubated for 48 h, and then cultured in the presence of various concentrations of drugs. After 48 h of culture, the cells were fixed with 1% formaldehyde and 0.2% glutaraldehyde for 3 min, washed three times with phosphate-buffered saline, and incubated with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) for 2 h (Fig. 1B). To evaluate the SEAP activities, the culture supernatants were harvested and analyzed by using a Great EscAPe SEAP chemiluminescent detection kit (BD Biosciences Clontech, Palo Alto, CA) according to the manufacturer's protocol. Samples were measured by using a Wallac 1450 MicroBeta Jet Luminometer (Perkin-Elmer, Wellesley, MA). For comparison, MAGI-CCR5 cells were analyzed as previously described (17).

The activities of various anti-HIV agents toward NCK45-β-Gal/SEAP cells were compared to those in the MAGI assay. The BFU and SEAP activities were well correlated with the viral input (Fig. 1C; correlation coefficient $R^2 = 0.928$). We tested various anti-HIV agents (Table 1): DS5000, an adsorption inhibitor; AZT and 2′,3′-dideoxycytidine (ddC), RT inhibitors; T-140 (37), a CXCR4 antagonist; and TAK-779, a CCR5 antagonist (1). The antiviral activities of the compounds determined by each reporter in NCK45-β-Gal/SEAP cells were comparable to those obtained using MAGI cells, although some were statistically significant (Table 1). Intracellular nucleoside/nucleotide metabolisms, especially thymidine kinase (10), and expression levels of receptors (19) may alter the 50% effective concentrations of the AZT and CCR5 antagonists, respectively.

NCK45-β-Gal/SEAP cells also supported the replication of various clinical isolates, as well as laboratory strains. The clin-

TABLE 1. Comparison of anti-HIV activities in MAGI and NCK45-β-Gal/SEAP cells

Compound	Target	Mean EC_{50} (μM)[a] ± SD						CC_{50} (μM)[b] (NCK45-β-Gal/SEAP)
		HIV-1_{IIIB}			HIV-1_{Ba-L}			
		MAGI	NCK45-β-Gal/SEAP		MAGI	NCK45-β-Gal/SEAP		
			β-Gal	SEAP		β-Gal	SEAP	
DS5000	gp120	0.14 ± 0.025	0.076 ± 0.026	0.07 ± 0.0034	0.36 ± 0.052	0.49 ± 0.069	0.42 ± 0.15	>100
AZT	RT	0.031 ± 0.013*	0.0043 ± 0.0022	0.0035 ± 0.0007	0.05 ± 0.029	0.0035 ± 0.0007	0.0094 ± 0.0063	>100
ddC	RT	0.4 ± 0.16	0.53 ± 0.12	0.42 ± 0.14	0.48 ± 0.17	0.72 ± 0.067	0.67 ± 0.2	>100
T-140	CXCR4	0.006 ± 0.0006	0.006 ± 0.0002	0.0025 ± 0.0008	>100	>100	>100	>100
TAK-779	CCR5	>100	>100	>100	0.003 ± 0.0019*	0.035 ± 0.0088	0.027 ± 0.0098	>100

[a] EC_{50}, 50% effective concentration. Data represent mean values of at least three independent experiments. HIV-1_{IIIB} and HIV-1_{Ba-L} utilize CXCR4 (X4) and CCR5 (R5) as coreceptors, respectively. *, The EC_{50} values obtained from MAGI and NCK cells (both β-galactosidase and SEAP) were statistically significant (Student t test, $P < 0.01$).

[b] CC_{50}, 50% cytotoxic concentration. The CC_{50} was determined by the MTT method after 2 days exposure of compounds as described previously (11).