

# Inhibition of the Tax-Dependent Human T-Lymphotropic Virus Type I Replication in Persistently Infected Cells by the Fluoroquinolone Derivative K-37

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## ABSTRACT

In the search for anti-human T-lymphotropic virus type-I (HTLV-I) compounds, we have evaluated several compounds for their inhibitory effects on HTLV-I replication in cell cultures. Among the test compounds, the fluoroquinolone derivative 7-(3,4-dehydro-4-phenyl-1-piperidinyl)-1,4-dihydro-6-fluoro-1-methyl-8-trifluoromethyl-4-oxoquinoline-3-carboxylic acid (K-37) was found to be a potent and selective inhibitor of HTLV-I replication in persistently infected cells, such as MT-2 and MT-4. When the cells were cultured in the presence of various concentrations of the compound, the 50% effective concentrations of K-37 for HTLV-I p19 antigen production were 0.44 and 0.24  $\mu$ M in MT-2 and MT-4 cells, respectively. K-37 did not affect the viability and proliferation of these cells at these concentrations, and its 50% cytotoxic concentrations to MT-2 and MT-4 cells were 5.7 and 1.1  $\mu$ M, respectively. The compound also showed selective inhibition of HTLV-I production in periph-

eral blood mononuclear cells obtained from patients with HTLV-I-associated myelopathy/tropical spastic paraparesis. Quantitative reverse transcription-polymerase chain reaction analysis revealed that K-37 selectively suppressed viral mRNA synthesis in MT-2 cells in a dose-dependent fashion. Furthermore, K-37 could inhibit the endogenous Tax-induced HTLV-I long terminal repeat (LTR)-driven reporter gene expression in MT-2 cells. Western blot analysis confirmed the reduced expression of Tax in MT-2 cells exposed to K-37. In contrast, when Tax was introduced into cells not infected with HTLV-I with a plasmid under the control of human cytomegalovirus promoter, the compound did not affect Tax-induced HTLV-I LTR-driven reporter gene expression. These results suggest that the inhibition occurred at the level of HTLV-I LTR-driven Tax expression.

Human T-lymphotropic virus type I (HTLV-I) is the first replication-competent human retrovirus (Takatsuki et al., 1977; Poiesz et al., 1980; Yoshida et al., 1982) and is clearly associated with some serious human diseases, such as adult T cell leukemia (ATL) and the neurological disorder HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (Gessain et al., 1985; Osame et al., 1986). Approximately 10 to 20 million people are thought to be infected with this virus worldwide. Although most infected persons remain asymptomatic and do not progress to disease, about 2 to 3% of the carriers will develop to ATL and another 2 to 3% to chronic inflammatory diseases of various organs and tissues,

including the central nervous system, eyes, lungs, or skeletal muscles.

The load of provirus is generally high in peripheral blood mononuclear cells (PBMCs) of patients with HAM/TSP, and the risk of this disease is positively correlated with the magnitude of the proviral load in PBMCs (Nagai et al., 1998). The mean proviral load is approximately 10 copies per 100 PBMCs in patients with HAM/TSP and 10-fold less in asymptomatic carriers. HTLV-I proviral load might be maintained either by lymphocyte proliferation, leading to the duplication of the HTLV-I genome at the time of cell division, or by classic retroviral replication. The relative contribution of

**ABBREVIATIONS:** HTLV-I, human T-lymphotropic virus type I; ATL, adult T cell leukemia; HAM/TSP, human T-lymphotropic virus type I-associated myelopathy/tropical spastic paraparesis; PBMC, peripheral blood mononuclear cell; RT, reverse transcriptase; HIV-1, human immunodeficiency virus type 1; AZT, zidovudine; LTR, long terminal repeat; K-37, 7-(3,4-dehydro-4-phenyl-1-piperidinyl)-1,4-dihydro-6-fluoro-1-methyl-8-trifluoromethyl-4-oxoquinoline-3-carboxylic acid; LVFX, levofloxacin; Ro 24-7429, 7-chloro-N-methyl-5-(1H-pyrrol-2-yl)-3H-1,4-benzodiazepin-2-amine; NF- $\kappa$ B, nuclear factor  $\kappa$ B; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RT-PCR, reverse transcription-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; bp, base pair(s); RSV, Rous sarcoma virus; mAb, monoclonal antibody; CC<sub>50</sub>, 50% cytotoxic concentration.

these two replication pathways to the total proviral load has not been determined. However, several lines of evidence suggest that the classic viral replication seems to more directly influence on the development of HAM/TSP. For instance, a high level of specific cytotoxic T-lymphocyte response was found in a majority of infected persons (Bieganowska et al., 1999; Jeffery et al., 1999). High antibody titles to the structural proteins of HTLV-I were found in patients with HAM/TSP (Kira et al., 1992). The HTLV-I tax/rex mRNA has been detected in particular in PBMCs of the patients with a high proviral load (Kinoshita et al., 1989; Gessain et al., 1991). The HIV-1 reverse transcriptase (RT) inhibitor lamivudine was shown to temporarily reduce the proviral load of five patients with HAM/TSP (Taylor et al., 1999), also suggesting that de novo HTLV-I infection plays a certain role in the increased proviral load.

If the high proviral load were attributed to the de novo acute infection of the target cells, inhibition of a crucial step in the viral replication cycle could reduce the proviral load, as demonstrated by human immunodeficiency virus type 1 (HIV-1) infection. HIV-1 RT inhibitor zidovudine (AZT) could suppress the production of HTLV-I Gag and reduce the proviral DNA, when HTLV-I-infected lymphocytes were cocultured with susceptible target cells. However, it is assumed that AZT had no antiviral activity in PBMCs already infected with HTLV-I (Matsushita et al., 1987; Macchi et al., 1997). In this study, we have focused on the transcriptional step of HTLV-I, because the inhibition of this step by a small-molecule compound may reduce the production of infectious virus particles and antigens in persistently (chronically) infected cells. To this end, several compounds, including anti-HIV-1 agents, have been examined for their inhibitory effects on HTLV-I antigen production in the persistently infected cells. We have found that the fluoroquinolone derivative K-37 is a potent and selective inhibitor of HTLV-I and that its mechanism of action is the inhibition of HTLV-I long terminal repeat (LTR)-driven Tax expression.

## Materials and Methods

**Compounds.** Fluoroquinolone derivatives K-37 (Baba et al., 1998) and levofloxacin (LVFX) (Fig. 1) were synthesized by Daiichi Pharmaceutical Co. (Tokyo, Japan); the HIV-1 Tat antagonist Ro24-7429 (Hsu et al., 1993) was kindly provided by Eisai Co. (Tsukuba, Japan). The nuclear factor  $\kappa$ B (NF- $\kappa$ B) inhibitor cepharranthine (Okamoto et al., 1998), the HIV-1 protease inhibitor nelfinavir, and the RT inhibitor lamivudine were provided by Kaken Shoyaku (Mitaka, Japan), Japan Tobacco Co. (Takatsuki, Japan), and Mitsubishi Chemical Corporation (Yokohama, Japan), respectively. All compounds were dissolved in dimethyl sulfoxide at 10 mM or higher concentrations to exclude any antiviral or cytotoxic effect of dimethyl sulfoxide and stored  $-20^{\circ}\text{C}$  until use.

**Cells.** MT-4 cells, MT-2 cells, and PBMCs were used in the antiviral assays. MT-4 and MT-2 cells are T cell lines persistently infected with HTLV-I (Miyoshi et al., 1981, 1982). The cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin G, and 100  $\mu\text{g}/\text{ml}$  streptomycin (culture medium). HTLV-I-infected PBMCs were donated under informed consent from patients with a clinical diagnosis of HAM/TSP. PBMCs were isolated from heparinized blood with Ficoll-Paque Plus (Pharmacia, Uppsala, Sweden) and washed three times with phosphate-buffered saline. PBMCs were cultured in RPMI1640 medium supplemented with 20% fetal bovine serum and antibiotics, and 100 U/ml interleukin-2 (Takeda Chemical Indus-

tries, Osaka, Japan). The non-HTLV-I-infected cell lines Jurkat, CEM, and MOLT-4 were also used in the experiments.

**Antiviral Assays.** The activity of the compounds against persistent HTLV-I infection was based on the inhibition of HTLV-I p19 antigen production in MT-2 and MT-4 cells. The cells ( $1 \times 10^5$  cells/ml) were cultured in the presence of various concentrations of test compounds. After a 3-day incubation at  $37^{\circ}\text{C}$ , the culture supernatants were collected and examined for their p19 antigen levels with a sandwich enzyme-linked immunosorbent assay kit (Cellular Products, buffalo, NY). The cytotoxicity of the compounds was determined in parallel with the antiviral activity by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (Pauwels et al., 1988). The anti-HTLV-I activity of the compounds was also evaluated in PBMCs isolated from patients with HAM/TSP. PBMCs ( $2 \times 10^5$  to  $1 \times 10^6$  cells/ml) were cultured in the presence of various concentrations of test compounds. After 6 days, the culture supernatants were collected and examined for their p19 antigen levels by enzyme-linked immunosorbent assay. The number of viable cells was determined by the MTT method.

**Quantitative Reverse Transcription-Polymerase Chain Reaction Analysis.** MT-2 cells ( $2 \times 10^5$  cells/ml) were incubated in the absence or presence of K-37 or cepharanthine for 2 days. Total RNA was extracted from the cells with the RNA extraction kit RNeasy B (Tel-Test, Friendswood, TX). The extracted RNA was subjected to quantitative RT-PCR analysis to determine HTLV-I mRNA, using GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA). For quantitative RT-PCR, the Taqman One-Step RT-PCR Master Mix Reagents Kit (Applied Biosystems) was used according to the manufacturer's instructions. The primer pair and the probe for HTLV-I incompletely spliced mRNA were 7141F (5'-CAAACCGTCAAGCACAGCTT-3', amino acid position 7140-7159), 7363R (5'-TCTCCAAACACGTAGACTGGGT-3', amino acid position 7341-7362), and 7308T (5'-TTCCAGGGTTTGGACAGAGTCTTCT-3', amino acid position 7307-7332) (Takenouchi et al., 1999). Non-specific inhibition of host cellular mRNA synthesis by K-37 was determined with the Taqman GAPDH Control Reagents Kit (Applied Biosystems).

**Plasmids.** Six plasmids (pCG-Tax, pCG-BL, WT-Luc, LTR-Luc, dN55-Luc, and RSV-Luc) were used in the experiments. pCG-Tax

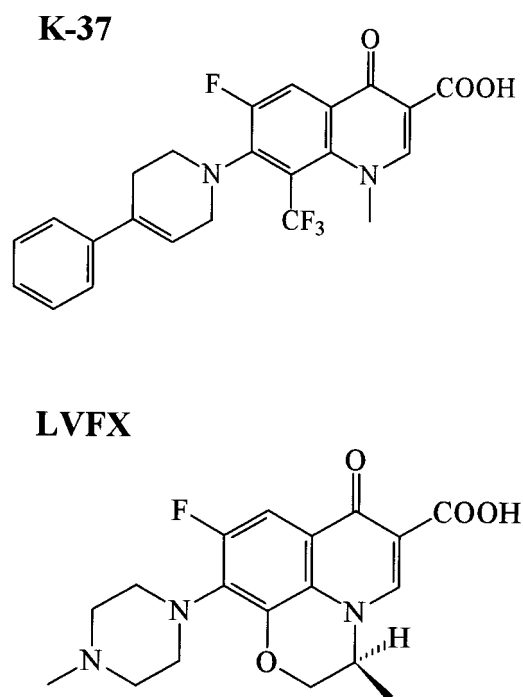
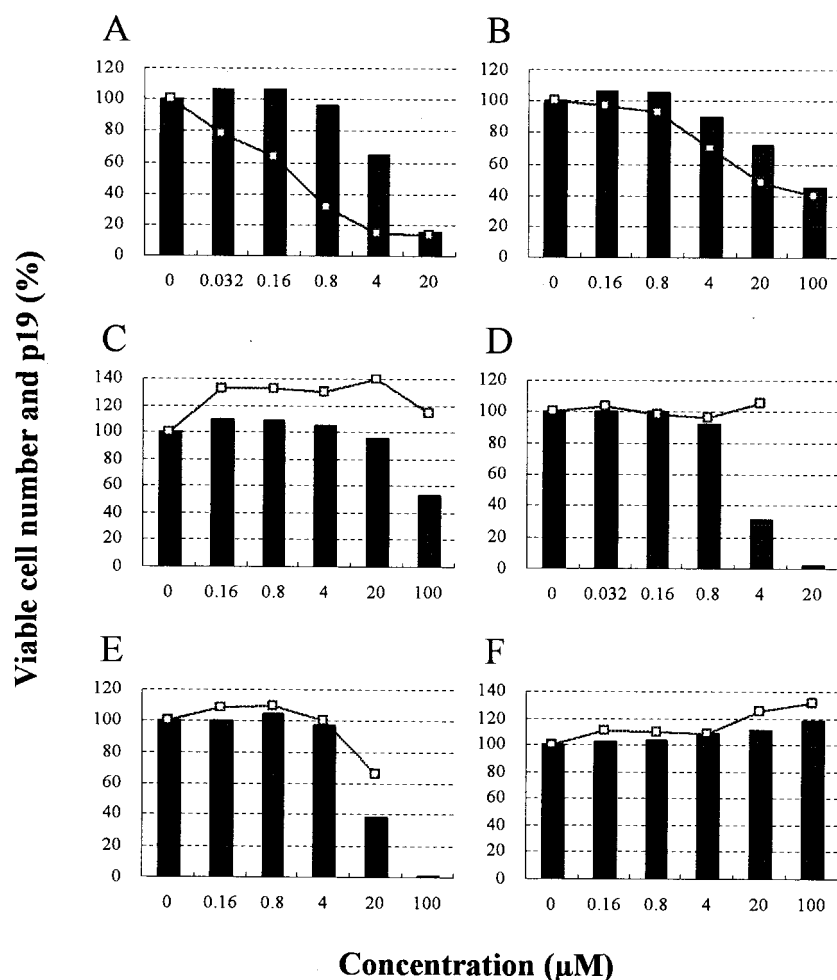


Fig. 1. Chemical structures of K-37 and LVFX.



**Fig. 2.** Inhibitory effects of selected compounds on HTLV-I replication in MT-2 cells. MT-2 cells were cultured in the presence of various concentrations of K-37 (A), Ro 24-7429 (B), LVFX (C), cepharanthine (D), nelfinavir (E), and lamivudine (F). After a 3-day incubation, the culture supernatants were collected and examined for their p19 antigen levels (lines). The viable cell number was determined by the MTT method (columns). All experiments were carried out in duplicate, and mean values are shown.

was constructed by inserting tax cDNA into the *Xba*I-*Bam*HI site of pCG-BL, and the expression of Tax is regulated by a human cytomegalovirus promoter (Fujisawa et al., 1991). All of the luciferase reporter plasmids were based on pGL2-Basic (Promega, Madison, WI). WT-Luc was constructed by ligation of the *Xba*I-*Xho*I fragment of WT/BL (Fujisawa et al., 1989) to *Nhe*I-*Xho*I-digested pGL2-Basic. It contained five tandem repeats of the 21-bp enhancer and HTLV-I promoter. dN55-Luc was an enhancer-deleted control reporter. The LTR sequences of HTLV-I and Rous sarcoma virus (RSV) were placed at the upstream of luciferase gene, and the generated plasmids were referred as LTR-Luc and RSV-Luc, respectively.

**Transfection and Luciferase Assays.** MT-2 cells ( $2 \times 10^6$  cells) were transfected with 2  $\mu\text{g}$  of a reporter plasmid, using DEAE-dextran. Jurkat, CEM, and MOLT-4 cells were cotransfected with 2  $\mu\text{g}$  of a reporter plasmid and 1  $\mu\text{g}$  of either pCG-Tax or pCG-BL (Fujisawa et al., 1989). After 2 h, the transfected cells were harvested and subcultured in the absence or presence of test compounds in a 96-well plate. After a 24-h incubation at 37°C, the cells were treated with 50  $\mu\text{l}$  of luciferase reaction buffer and 50  $\mu\text{l}$  of luciferin substrate (Luc-Screen; Applied Biosystems). Luciferase activity was measured sequentially by MicroLumat Plus LB96V microplate luminometer with an injection unit (Berthold Technologies, Bad Wildbad, Germany). The number of viable cells was determined by the MTT method.

**Western Blot Analysis.** Western blot analysis was performed as described previously (Tanaka et al., 1990). Briefly, MT-2 cells ( $2 \times 10^5$  cells/ml) were incubated in the absence or presence of K-37 or cepharanthine for 2 days. Cell lysates were obtained by treatment of cells with a low-salt extraction buffer (10 mM Tris-HCl, pH 8.0, containing 0.14 M NaCl, 3 mM  $\text{MgCl}_2$ , 1 mM dithiothreitol, 2 mM

phenylmethylsulfonyl fluoride, and 0.5% Nonidet P-40) on ice for 20 min, followed by centrifugation at 12,000  $g$  at 4°C for 10 min. Protein concentration was determined by a method described previously (Bradford, 1976). Total cell lysates (100  $\mu\text{g}$  of protein) were electrophoresed on a 10% polyacrylamide gel with SDS and transferred to a polyvinylidene difluoride membrane. The transferred proteins were reacted with the anti-HTLV-I p40 Tax monoclonal antibody (mAb) Lt-4 (Tanaka et al., 1990), followed by treatment with horseradish peroxidase-conjugated goat anti-mouse IgG (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). Antibody binding was visualized with an enhanced chemiluminescence Western blotting detection system (Amersham Biosciences). Coomassie Brilliant Blue R250 staining of the membrane was also performed to confirm the equal amount of protein loaded.

## Results

**Anti-HTLV-I Activity of Selected Compounds in Persistently Infected Cell Lines.** When several compounds were examined for their inhibitory effects on HTLV-I replication in MT-2 cells, only two compounds, K-37 and Ro 24-7429, were found to be selective inhibitors of HTLV-I replication. In particular, K-37 achieved approximately 70% inhibition of p19 antigen production in the culture supernatants at a concentration of 0.8  $\mu\text{M}$  (Fig. 2A). Whereas K-37 did not affect the proliferation and viability of MT-2 cells at this concentration, indicating the inhibition was selective to HTLV-I. Ro 24-7429 also showed some inhibition, yet its selectivity was lower than that of K-37 (Fig. 2B). In contrast,



LVFX, an antibacterial fluoroquinolone structurally related to K-37, was totally inactive against HTLV-I replication (Fig. 2C). We also tested cepharanthine, nelfinavir, and lamivudine, all of which have been shown anti-HIV-1 activity in vitro and/or in vivo. However, these compounds proved inactive against HTLV-I replication in MT-2 cells (Fig. 2, D-F).

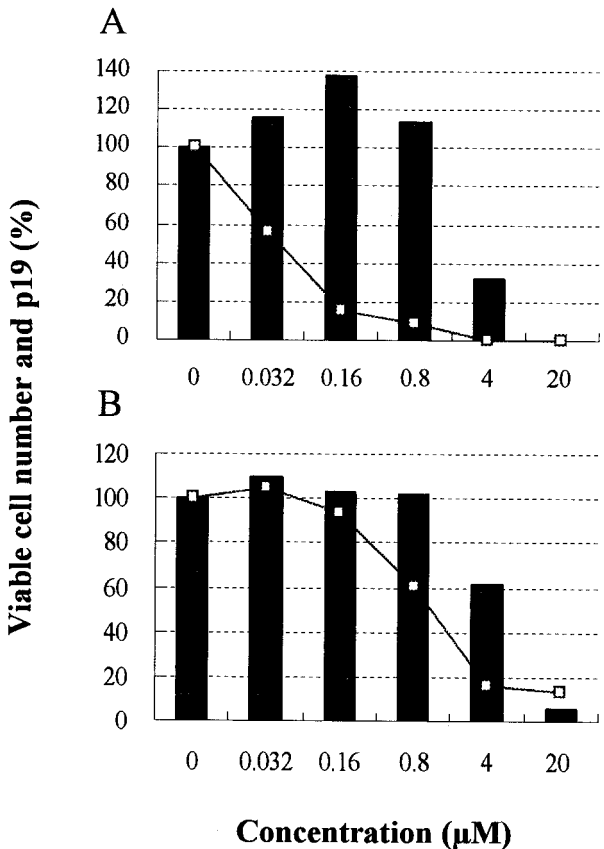
The anti-HTLV-I activity of K-37 was confirmed in MT-4 cells, another cell line persistently infected with HTLV-I. Table 1 summarizes the antiviral activity and cytotoxicity of the selected compounds in MT-2 and MT-4 cells. The  $EC_{50}$  values of K-37 were 0.44 and 0.24  $\mu$ M in MT-2 and MT-4 cells, respectively, whereas its 50% cytotoxic concentrations ( $CC_{50}$ ) were 5.7 and 1.1  $\mu$ M, respectively. Consequently, its selectivity indices, base on the ratio of  $EC_{50}$  to  $CC_{50}$ , were 13.0 and 4.6 in MT-2 and MT-4 cells, respectively. Although Ro 24-7429 had modest anti-HTLV-I activity in MT-2 cells, it did not display selective inhibition in MT-4 cells (Table 1). Under the standard culture conditions, MT-2 and MT-4 cells continuously produce a large amount of HTLV-I antigens without any stimuli. In fact, the levels of p19 antigen in the culture supernatants were 48.2 ng/ml in MT-2 cells and 2.7 ng/ml in MT-4 cells (data not shown).

**Anti-HTLV-I Activity of K-37 in PBMCs from HAM/TSP Patients.** It would be of particular interest to know whether K-37 could suppress the production of HTLV-I in PBMCs of infected persons. Therefore, K-37 was examined for its inhibitory effect on HTLV-I replication in PBMCs obtained from 2 patients with HAM/TSP. In general, in vitro cultivation of patient PBMCs induces the production of p19 in the culture supernatants after several days. In our experiments, approximately 60 pg/ml p19 antigen was detected for all samples after 6 days of cultivation (data not shown). Although the potency of K-37 differed from one patient to another, the compound was dose dependently reduced the levels of p19 antigen in all PBMC samples (Fig. 3A and 3B). The  $EC_{50}$ s were 0.040 and 1.1  $\mu$ M. In contrast, as demonstrated on MT-2 and MT-4 cells, the anti-HIV-1 agents nelfinavir and lamivudine were totally inactive in the patient PBMCs (data not shown).

**Inhibitory Effect of K-37 on HTLV-I Transcription.** Because K-37 had been shown to interfere with HIV-1 transcription (Baba et al., 1998), the compound was also expected to act as an HTLV-I transcription inhibitor. Therefore, quantitative RT-PCR analysis was conducted to determine

whether K-37 could prevent HTLV-1 mRNA synthesis in MT-2 cells. As shown in Fig. 4A, K-37 selectively suppressed HTLV-I mRNA synthesis in a dose-dependent fashion. On the other hand, it did not affect the GAPDH mRNA synthesis at a concentration of 0.8  $\mu$ M, indicating that K-37 selectively inhibited HTLV-I gene expression. As a control, the NF- $\kappa$ B inhibitor cepharanthine was also tested at the same time and found to have no inhibitory effect on HTLV-I mRNA synthesis (Fig. 4B).

**Inhibitory Effect of K-37 on Tax-Induced *trans*-Activation.** To determine whether K-37 affect Tax-induced *trans*-activation, transient luciferase assay in MT-2 cells were conducted. As shown in Fig. 5A, K-37 suppressed the HTLV-I LTR- or the 21-bp enhancer-driven reporter gene expression in a dose-dependent fashion, whereas no significant inhibition was observed for the RSV LTR-mediated gene expression or the basal transcription (dN55-Luc), suggesting that K-37 suppressed the endogenous Tax-induced *trans*-activation. Cepharanthine did not show any effect on such enhancer-driven reporter gene expression (data not shown). To further elucidate the effect of K-37 on the *trans*-activation, cotransfection experiments with the Tax-expression plasmid pCG-Tax and the HTLV-I LTR-Luc were carried out in Jurkat cells in the presence of various concentrations of the compound. Interestingly, K-37 did not display any inhibition of Tax-induced reporter gene expression even at a concentra-



**Fig. 3.** Inhibitory effects of K-37 on HTLV-I replication in PBMCs from patients with HAM/TSP. PBMCs were obtained from 2 different patients with HAM/TSP (A and B) and cultured in the presence of various concentrations of K-37. After a 6-day incubation, the culture supernatants were collected and examined for their p19 antigen levels (lines). The viable cell number was determined by the MTT method (columns). All experiments were carried out in duplicate, and mean values are shown.

**TABLE 1**  
Inhibitory effects of K-37 and other selected compounds on HTLV-I replication in MT-2 and MT-4 cells

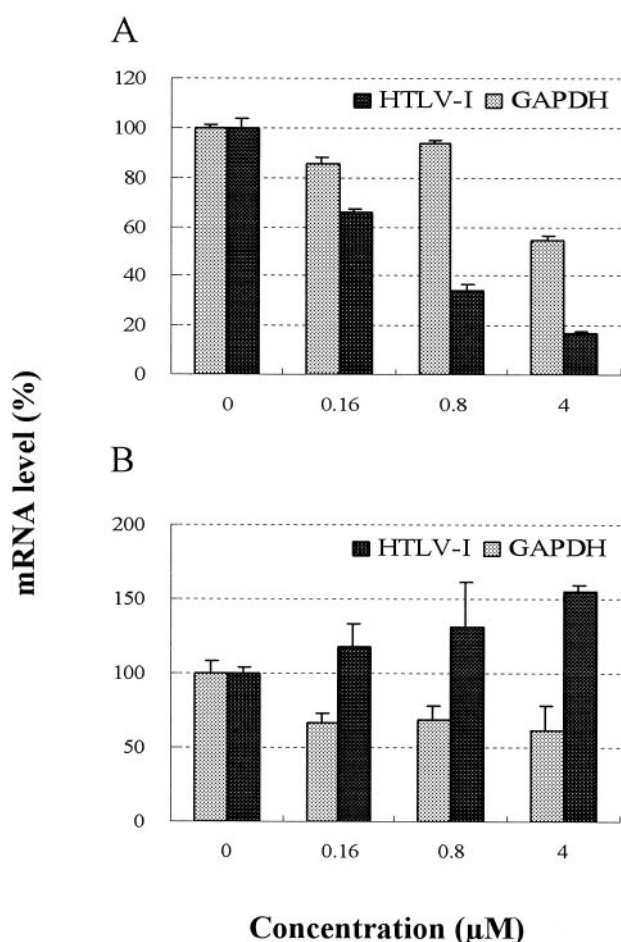
All data represent means  $\pm$  S.D. for three separate experiments.

Compound	Cell	$EC_{50}$	$CC_{50}$	SI
$\mu$ M				
K-37	MT-2	$0.44 \pm 0.13$	$5.7 \pm 1.0$	13.0
	MT-4	$0.24 \pm 0.14$	$1.1 \pm 0.2$	4.6
Ro 24-7429	MT-2	$17 \pm 1$	$72 \pm 3$	4.2
	MT-4	$> 24$	$24 \pm 4$	$< 1$
Cepharanthine	MT-2	$> 3.0$	$3.0 \pm 0.4$	$< 1$
	MT-4	$> 1.3$	$1.3 \pm 0.5$	$< 1$
Nelfinavir	MT-2	$> 16$	$16 \pm 2$	$< 1$
	MT-4	$> 7.1$	$7.1 \pm 1.9$	$< 1$
Lamivudine	MT-2	$> 100$	$> 100$	$> 1$
	MT-4	$> 100$	$> 100$	$> 1$

$EC_{50}$ , concentration required for 50% inhibition of p19 antigen production in culture supernatants;  $CC_{50}$ , concentration required for 50% inhibition of proliferation and viability of the cells; SI, selectivity index (ratio of  $CC_{50}$  to  $EC_{50}$ ).

tion of 4  $\mu$ M, when Tax was introduced into cells not infected with HTLV-I with pCG-Tax, a plasmid under the control of human cytomegalovirus promoter (Fig. 5B). The same results were also obtained by the cotransfection experiments in CEM and MOLT-4 cells (data not shown). These results indicate that the inhibitory effect of K-37 on the HTLV-I transcription was not caused by the inhibition of Tax-induced *trans*-activation itself but by the suppression of endogenous Tax expression in HTLV-I persistently infected cells.

**Effects on Tax Antigen Expression.** To confirm the suppression of endogenous Tax expression by K-37 in HTLV-I-infected cells, Western blot analysis with an anti-Tax mAb were conducted. A significant decrease of HTLV-I p40 Tax expression was identified in the presence of K-37 at 4  $\mu$ M in MT-2 cells (Fig. 6A). At this concentration, the Tax expression was decreased to 44% of the control (in the absence of K-37). Furthermore, the expression of p68 Env-pX fusion protein (Miwa et al., 1984) was also suppressed in a dose-dependent fashion. The suppression of endogenous Tax was selective, because an equal amount of protein loaded for Western blot analysis was confirmed by Coomassie Brilliant Blue staining (Fig. 6B). Again, cepharanthine had no effect on the endogenous Tax expression in MT-2 cells.

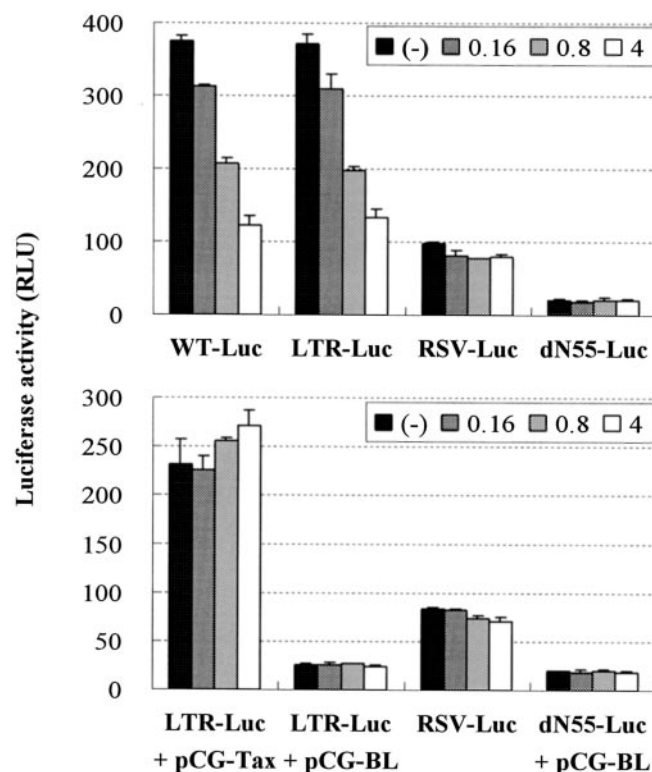


**Fig. 4.** Inhibitory effects of K-37 and cepharanthine on HTLV-I mRNA synthesis in MT-2 cells. The cells were incubated with K-37 (A) or cepharanthine (B) for 2 days. Total RNA was extracted from the cells, and quantitative RT-PCR for HTLV-I mRNA was performed. The cytotoxic effects of the test compounds on host cellular mRNA synthesis were determined by quantitative RT-PCR for GAPDH mRNA. Representative results for two independent experiments are shown.

## Discussion

Because a rapid and efficient assay system has not been established for in vitro infection of HTLV-I, effective chemotherapeutic agents against HTLV-I have not been studied extensively. We have examined several compounds, including anti-HIV-1 agents, for their inhibitory effects on HTLV-I replication in persistently infected cells. Using this system, we have found that the fluoroquinolone derivative K-37 is a potent and selective inhibitor of HTLV-I. K-37 was previously reported to be a potent and selective inhibitor of HIV-1 replication in both acutely and chronically infected cells (Baba et al., 1997, 1998; Okamoto et al., 1999). Although K-37 belongs to the family of fluoroquinolones, its properties are totally different from other antibacterial fluoroquinolones, such as LVFX. K-37 had little, if any, antibacterial activity (T. Ikeuchi, unpublished observations). In contrast, LVFX did not show any anti-HIV-1 activity (data not shown).

In this study, we have demonstrated that K-37 could inhibit HTLV-I replication in persistently infected cells through the suppression of Tax expression. HTLV-I Tax is a transcriptional activator of the viral genes and is essential for efficient viral replication (Sodroski et al., 1984; Chen et



**Fig. 5.** Inhibitory effects of K-37 on HTLV-I Tax-mediated *trans*-activation. Top, for Tax-mediated *trans*-activation in HTLV-I infected cells, MT-2 cells were transfected with 2  $\mu$ g of the reporter plasmids (WT-Luc, LTR-Luc, RSV-Luc, or dN55-Luc), incubated for 2-h, and subcultured in the presence of various concentrations of K-37. Bottom, for Tax-mediated *trans*-activation in uninfected cells, Jurkat cells were cotransfected with 2  $\mu$ g of the reporter plasmids (LTR-Luc, RSV-Luc, or dN55-Luc) with or without 1  $\mu$ g of pCG-Tax or pCG-BL, incubated for 2 h, and subcultured in the presence of various concentrations of K-37. After a 24-h incubation, the transfected cells were collected and examined for their luciferase activity. The activity was expressed as relative light unit (RLU). At the same time, the number of viable cells was determined by the MTT method. All experiments were carried out in triplicate, mean values, and standard deviations are shown. The results are representative of two independent experiments.

al., 1985; Felber et al., 1985; Fujisawa et al., 1985). The Tax protein activates the 21-bp enhancer of the HTLV-I LTR, resulting in enhanced transcription of the HTLV-I genome. It also activates the transcription of cellular genes for lymphokines, lymphokine receptors, protooncogenes, and some cell surface molecules. Therefore, Tax has been thought to play important role in the pathogenesis of ATL and HAM/TSP (Inoue et al., 1986; Yoshida and Seiki, 1987). As a consequence of the suppression of Tax expression, K-37 selectively inhibited the synthesis of HTLV-I unspliced and incompletely spliced mRNA but not GAPDH mRNA in MT-2 cells (Fig. 4A). More importantly, K-37 could also suppress HTLV-I gene expression in PBMCs from patients with HAM/TSP. A large proportion of HTLV-I-infected PBMCs (10–80%) isolated from patients with HAM/TSP become positive for Tax protein only after 6 h of cultivation in vitro (Hanon et al., 2000). Most of the infected cells in vivo, therefore, are capable of expressing Tax. At present, why the activity of K-37 in PBMCs varied from one donor to another is unclear (Fig. 3). Although their p19 antigen levels in the absence of the compound were similar (61 and 52 pg/ml), one patient whose PBMCs were highly sensitive to K-37 has progressive neurological symptoms, whereas the other patient is at a chronic (nonprogressive) stage of HAM/TSP (data not shown). In any cases, our findings may be of great value in developing a new strategy for the therapy of HAM/TSP.

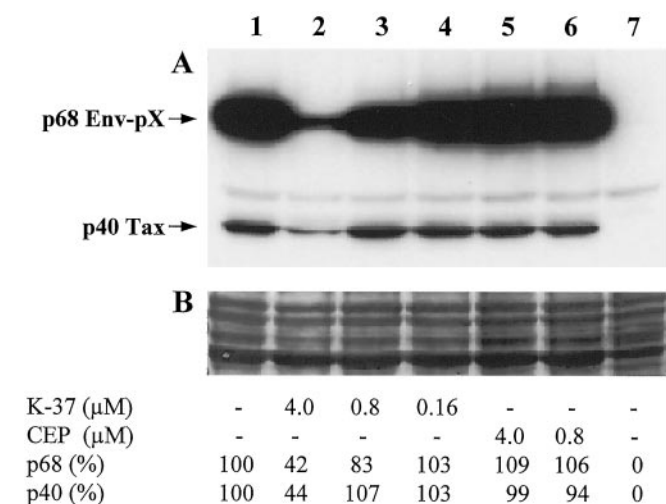
Although K-37 inhibited the endogenous Tax-mediated *trans*-activation for the reporter gene driven by the HTLV-I LTR or the 21bp enhancer in MT-2 cells, it did not affect the Tax-mediated *trans*-activation for the HTLV-I LTR-driven reporter gene in cells not infected with HTLV-I (Fig. 5, A and B). In the uninfected cells, Tax was introduced into the cells with a Tax expression plasmid driven by the human cyto-

megalovirus immediate-early promoter, which may be a reason for the unresponsiveness of cells not infected with HTLV-I by K-37. It was reported that K-37 could inhibit a RNA- dependent *trans*-activation, including HIV-1 Tat but could not inhibit DNA-dependent *trans*-activation (Okamoto et al., 2000). Interestingly, in addition to HIV-1 and HIV-2, the K-37 analog K-12 was inhibitory to the replication of murine retroviruses, which are devoid of accessory genes, such as tat and rev (Witvrouw et al., 1998), suggesting that K-12 and possibly K-37 target a cellular factor or factors necessary for the transcription of viral DNA. Furthermore, K-12 did not inhibit the tumor necrosis factor  $\alpha$ -induced activation of NF- $\kappa$ B, a potent activator of gene expression, in HIV-1-infected cells or the binding of NF- $\kappa$ B to its target DNA (Baba et al., 1997). Taken together, K-37 does not seem to directly block Tax molecule itself or Tax-LTR interaction but may interfere with cellular factors that play a key role in Tax expression or posttranscriptional steps. Further studies are required to elucidate the precise target molecule of K-37.

In conclusion, the fluoroquinolone derivative K-37 is the first compound to show potent and selective inhibition of HTLV-I replication through the suppression of endogenous Tax in persistently infected cells. Although K-37 was found to be rather toxic in vivo and not to be further developed for the treatment of HTLV-I infection (T. Ikeuchi, unpublished observations), such a therapeutic approach may have great potential for the discovery of novel agents active against HTLV-I.

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**Fig. 6.** Inhibitory effects of K-37 and cepharanthine on Tax expression in MT-2 cells. MT-2 cells were incubated in the presence of various concentrations of the test compounds for 2 days. Total cell lysate (100 μg of protein) was electrophoresed and transferred to a polyvinylidene difluoride membrane. A, the membrane was immunoblotted with the anti-HTLV-I p40 Tax mAb Lt-4, followed by treatment with horseradish peroxidase-conjugated goat anti-mouse IgG. The antigen expression was quantified with NIH image software (ver. 1.60; <http://rsb.info.nih.gov/nih-image/>) and expressed as percentage conversion. B, Coomassie Brilliant Blue staining of the membrane was also performed to confirm the equal amount of protein loaded for Western blot analysis. The samples analyzed were MT-2 cells in lanes 1 to 6 and Jurkat cells in lane 7. CEP, cepharanthine.



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