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Long-term culture of HIV-1-infected cells with the transcription inhibitor K-37☆

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Abstract

We have previously reported that the fluoroquinoline derivative K-37 is a potent and selective inhibitor of HIV-1 replication in both acutely and chronically infected cells. K-37 blocks the HIV-1 transcription process through the inhibition of still unknown cellular factor(s). To gain further insight into the target of K-37 for HIV-1 replication, we have conducted long-term culture of acutely infected cells in the presence of K-37. When MOLT-4 and U937 cells were infected with HIV-1 and cultured in the absence of K-37, the p24 antigen levels in the culture supernatants reached a plateau within 12 days. In the presence of K-37 (0.25 and 0.5 μM), the elevation of p24 antigen levels was delayed but reached a similar plateau level on day 16 or later. At a concentration of 1 μM, K-37 markedly suppressed HIV-1 replication. However, viral breakthrough was observed after 1 month of the culture period in both MOLT-4 and U937 cells. We established MOLT-4 cell lines chronically infected with the breakthrough viruses (M_1 and U_1) or the corresponding wild-type strains (M_0 and U_0, respectively), and K-37 was examined for its inhibitory effects on HIV-1 replication in these cell lines. No substantial difference in anti-HIV-1 activity was observed between the two cell lines. However, acute infection experiments revealed that the infectivity of M_1 and U_1 was much lower than that of M_0 and U_0, respectively. Furthermore, both M_1 and U_1 had a G to T nucleotide mutation at position −215 in the second nuclear factor of activated T-cells-binding domain (−215 to −203) of the HIV-1 long terminal repeat.

Keywords: Fluoroquinoline; Transcription inhibitor; Tat; Breakthrough virus; Chronic infection

1. Introduction

Recent development of the highly active antiretroviral therapy with reverse transcriptase (RT) inhibitors and protease inhibitors in human immunodeficiency virus type 1 (HIV-1)-infected patients has achieved a more than 2 log 10 reduction of viral RNA levels in plasma for a considerable period of time (see Guidelines for the Use of

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Antiretroviral Agents in HIV-Infected Adults and Adolescents by Department of Health and Human Services, 2000). However, considering the fact that treatment failure is often associated with the emergence of drug resistance during long-term chemotherapy in patients, discovery of novel anti-HIV-1 agents with different mechanisms of action is still highly desirable.

Transcription of the viral genome (integrated proviral DNA) into its mRNA is an essential step in the HIV-1 replication cycle and is considered as a potential target for chemotherapeutic intervention (Butera, 2000; Daelemans et al., 1999; Karn, 1999). We have reported that the fluoroquinoline derivatives K-12 and K-37 are potent and selective inhibitors of HIV-1 replication not only in acutely infected cells but also in chronically infected cells (Baba et al., 1997, 1998; Okamoto et al., 1999). These compounds could reduce the viral mRNA synthesis in chronically infected cells without affecting the synthesis of a cellular mRNA. However, its precise target molecule is still unknown. To gain further insight into the mechanism of action, we have performed long-term culture of HIV-1-infected cells in the presence of K-37 and obtained breakthrough viruses. Interestingly, the long-term culture of HIV-1-infected cells with K-37 influenced the infectivity of the breakthrough viruses.

2. Materials and methods

2.1. Compounds

K-37 (7-(3,4-dehydro-4-phenyl-1-piperidinyl)-1,4-dihydro-6-fluoro-1-methyl-8-trifluoromethyl-4-oxoquinoline-3-carboxylic acid) (Fig. 1) and the HIV-1 protease inhibitor nelfinavir (NFV) were provided by Daiichi Pharmaceuticals (Tokyo, Japan) and Japan Tobacco (Takatsuki, Japan), respectively. These compounds were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mM to avoid any antiviral and cytotoxic effects of DMSO. The stock solution was stored at −20 °C until use.

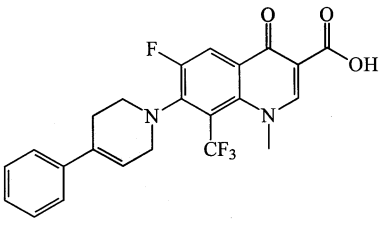

Fig. 1. Structure of K-37.

2.2. Cells and virus

The T-lymphoblastoid cell line MOLT-4 and the promonocytic cell line U937 were used for the long-term culture experiments. MT-4 cells (Miyoshi et al., 1982) and MOLT-4/III_B cells, MOLT-4 cells chronically infected with HIV-1 (Kikukawa et al., 1986), were used for the assays. The cells were grown and maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin G, and 100 μg/ml streptomycin (culture medium). HIV-1 (III_B strain) was used for the infection experiments. The virus was propagated and titrated in MT-4 cells and stored at −80 °C until use.

2.3. Long-term culture of infected cells

MOLT-4 and U937 cells (1×10^5 cells) were infected with HIV-1 at a multiplicity of infection (MOI) of 0.01. After a 2-h incubation for viral adsorption, the cells were washed thoroughly with culture medium to remove unadsorbed viral particles. The cells were seeded in a 24-well tray containing various concentrations of K-37. After a 4-day incubation at 37 °C, the cells were subcultured with fresh culture medium containing appropriate concentrations of the compound, then further cultured. At the time of subcultivation, the culture supernatants were collected and examined for their p24 antigen levels with a sandwich enzyme-linked immunosorbent assay kit (ZeptoMetrix, Buffalo, NY). During the cell culture, the number of viable cells was always monitored by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method (Pauwels et al., 1988).

2.4. Establishment of chronically infected cells with breakthrough viruses

The breakthrough viruses obtained in the long-term culture experiments were once propagated in MT-4 cells to remove the compound from the virus suspensions. MOLT-4 cells were infected with the breakthrough viruses and cultured in the absence of the compound. The cells were subcultured every 4–5 days with fresh culture medium for more than 1 month. To confirm the chronic infection, FACScan (Becton Dickinson, Mountain View, CA) analysis was performed to determine HIV-1 antigen expression and CD4 down-regulation on the cell surfaces with an HIV-1-specific polyclonal antibody and an anti-human CD4 monoclonal antibody (PharMingen, San Diego, CA), respectively. The anti-HIV-1 activity of K-37 was examined in the chronically infected cells based on the inhibition of p24 antigen production. The cells were incubated in the presence of various concentrations of the compound for 3 days. After incubation, the culture supernatants were collected and examined for the p24 antigen levels.

2.5. Virus replication assays

The culture supernatants of the chronically infected cells were titrated, and then MOLT-4 cells (1×10^5 cells) were infected with the supernatants at a MOI of 0.01. After a 2-h incubation for viral adsorption, the cells were washed thoroughly with culture medium to remove unadsorbed viral particles. Every 48 h, the culture supernatants of the infected MOLT-4 cells were collected and examined for their infectivity (virus titer) and p24 antigen levels.

2.6. Determination of nucleoside sequences of breakthrough viruses

Genomic DNA was extracted from chronically infected cells (5×10^5 cells) with a DNA Extraction Kit (WAKO, Tokyo, Japan) and amplified by polymerase chain reaction (PCR). The PCR consisted of 30 cycles with the primer pairs for either a part of HIV-1 gp120 including the third hypervariable (V3) domain or the total 5′-long terminal repeat (LTR) including the transactivating response element (TAR). The nucleotide sequences of the primer pairs were 5′-AATGTCAGCACAGTACAATGTACAC-3′ (+6521) and 5′-TCACAATTTCTGGGTCCCCTCCTGAG-3′ (+6917) for HIV-1 gp120 and 5′-GACAAGATATCCTTGATCTG-3′ (−426) and 5′-TTGGCGTACTCACCAGTC-3′ (+300) for the LTR. The numbers shown in parentheses indicate the nucleotide positions in the consensus sequence of HTLV-III_B strain (GeneBank accession number: X01762). HIV-1- negative control DNA was included in the PCR experiments to monitor cross-contamination. The amplified products were isolated by agarose gel electrophoresis and sequenced directly, using the dye-labeled primers above on a model 310 automated DNA sequencer (Applied Biosystems Inc., Foster City, CA).

3. Results

Prior to the long-term culture experiments, K-37 was examined for their inhibitory effects on HIV-1 replication in acutely and chronically infected cells to determine appropriate concentration of the compound for the long-term culture. The 50% effective concentrations (EC_{50}) of K-37 were 0.050 and 0.040 μM in acutely infected MOLT-4 cells and peripheral blood mononuclear cells (PBMC), respectively (data not shown). Furthermore, its EC_{50} was 0.070 and 0.041 μM in MOLT-4/III_B cells and tumor necrosis factor (TNF)-α-stimulated OM-10.1 cells, respectively (data not shown). The latter cells represent a myelomonocytic cell line latently infected with HIV-1 (Butera et al., 1991). K-37 did not show any cytotoxicity to these cells at concentrations up to 1 μM (data not shown). NFV was also active against HIV-1 replication in MOLT-4/III_B cells with an EC_{50} of 0.3 μM (data not shown).

Based on the antiviral activity and cytotoxicity results of K-37, the concentration of K-37 in the long-term culture experiments was fixed at 0.25, 0.5, and 1 μM. When MOLT-4 and U937 cells were infected with HIV-1 and cultured in the absence of K-37, the virus was growing rapidly,

and the p24 antigen levels in the culture supernatants reached a plateau (100–300 ng/ml) within 12 days (Fig. 2). On day 12 after virus infection, the HIV-1-induced cytopathicity was apparent in both MOLT-4 and U937 cells. Therefore, further subcultivation was halted, and the viruses in the culture supernatants of MOLT-4 and U937 cells were regarded as the wild-type strains M_0 and U_0, respectively. In the presence of 0.25 and 0.5 μM K-37, the elevation of p24 antigen levels was delayed but could reach the same plateau level on day 16 or later (Fig. 2). Interestingly, the HIV-1-induced cytopathicity was not so apparent as that observed in the absence of the compound, even when the p24 antigen levels reached the maximum (data not shown). These cells continued producing high levels of p24 antigen for at least 40 days without any morphological changes, suggesting the establishment of chronic infection.

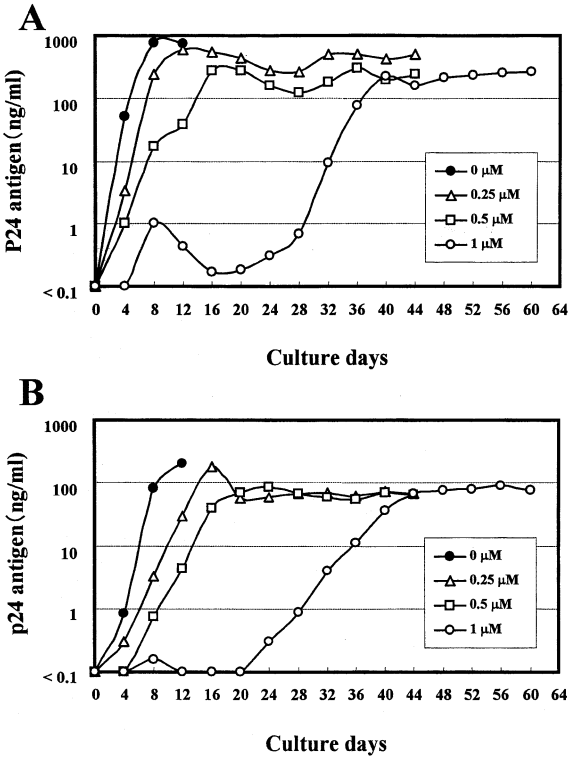

Fig. 2. Long-term culture of HIV-1-infected (A) MOLT-4 and (B) U_1 cells in the presence of various combinations of K-37. The cells were infected with HIV-1 and cultured in the presence of compound at the indicated concentrations. Every 4 days, the cells were subcultured, and the culture supernatants were examined for their p24 antigen levels.

At a concentration of 1 μM, K-37 markedly suppressed HIV-1 replication. The p24 antigen levels remained less than 1 ng/ml for 28 days (Fig. 2). However, viral breakthrough was observed after 1 month of the culture period. The cells also continued producing high levels of p24 antigen after 40 days. On day 60, further subcultivation was halted, and the viruses in the culture supernatants of MOLT-4 and U937 cells were regarded as the breakthrough viruses M_1 and U_1, respectively. To determine whether the breakthrough viruses had reduced susceptibility to K-37, we established MOLT-4 cells chronically infected with the M_0 and U_0 strains (MOLT-4/M_0 and MOLT-4/U_0, respectively) and the M_1 and U_1 strains (MOLT-4/M_1 and MOLT-4/U_1, respectively) and examined K-37 for its inhibitory effects on HIV-1 replication in these cells. As shown in Table 1, no substantial difference in anti-HIV-1 activity was identified among these cell lines and the reference line MOLT-4/III_B at a level of 50% inhibition, which has been maintained in our laboratory for a considerable period of time. MOLT-4/M_1 and MOLT-4/U_1 were approximately threefold less susceptible to K-37 than their counterparts (MOLT-4/M_0 and MOLT-4/U_0, respectively) at a level of 90% inhibition (Table 1). However, this difference was not statistically significant.

In the acute infection experiments with the wild-type strains (M_0 and U_0) and the breakthrough viruses (M_1 and U_1), the M_0 strain displayed the highest infectivity in MOLT-4 cells among the 4 strains. The infectivity of culture supernatant exceeded 2×10^5 50% cell culture infectious dose ($CCID_{50}$) per ml (Fig. 3A). In contrast, the infectivity of the M_1 strain was found to be much lower than that of the M_0 strain. Such a relationship was also observed with the U_0 and U_1 strains, although the infectivity of these strains was not comparable to that of the M_0 and M_1 strains. Interestingly, the p24 antigen levels of the M_1 and U_1 strains appeared to be similar or even slightly higher than those of the M_0 and U_0 strains, respectively (Fig. 3B).

Table 1
Inhibitory effects of K-37 on HIV-1 replication in MOLT-4 cells chronically infected with breakthrough viruses

Cell[a]	EC_{50}[b] (μM)	EC_{90}[c] (μM)	CC_{50}[d] (μM)	p24[e] (ng/ml)
MOLT-4/III_B	0.05	0.4	3.9	20
MOLT-4/M_0[f]	0.05±0.01[g]	0.3±0.1	1.9±0.5	30
MOLT-4/M_1	0.05±0.02	1.0±0.8	1.8±0.8	22
MOLT-4/U_0	0.05±0.01	0.4±0.2	1.9±1.0	50
MOLT-4/U_1	0.06±0.01	1.4±0.5	2.1±0.6	30

[a] Each cell line was incubated for 3 days in the presence of various concentration of K-37.
[b] Fifty percent effective concentration, based on the reduction of p24 antigen production in culture supernatants.
[c] Ninety percent effective concentration, based on the reduction of p24 antigen production in culture supernatants.
[d] Fifty percent cytotoxic concentration, based on the reduction of viable cell number.
[e] Mean p24 antigen level of the culture supernatants in the absence of K-37.
[f] MOLT-4/M_0 indicates MOLT-4 cells chronically infected with the M_0 strain. M_1 and U_1 are the breakthrough viruses, while M_0 and U_0 being the corresponding wild-type strains, respectively.
[g] All data represent means±standard deviations for three separate experiments, except for those of MOLT-4/III_B.

To elucidate whether the reduced infectivity of the breakthrough viruses (M_1 and U_1) is due to a mutation of the viral genome, we analyzed the nucleotide sequences of gp120 and the LTR of the breakthrough viruses and compared with those of the wild-type strains. No amino acid mutation of the sequenced gp120 region, including the V3 loop, was identified with the M_1 and U_1 strains (data not shown). On the other hand, both of the two breakthrough viruses had a G to T mutation at position −215 in the second nuclear factor of activated T-cells (NFAT)-binding site (−215 to −203) of the LTR (Fig. 4), whereas other regions of the LTR, including two AP1-binding sites, two nuclear factor (NF)-κB-binding sites, three SP1-binding sites, and the TATA box motif, and the transcription initiator/TAR region did not reveal differences in the breakthrough viruses as compared to the wild-type strains (data not shown).

4. Discussion

K-37 is a unique fluoroquinoline derivative, which selectively inhibits HIV-1 replication in both acutely and chronically infected cells. Its mechanism of action is clearly distinct from other known inhibitors of HIV-1, such as RT and protease inhibitors. We have previously demonstrated that K-12, a derivative of K-37, interrupts the late stage (post-integration of proviral DNA) of the HIV-1 replication cycle, presumably HIV-1 gene expression or transcription (Baba et al., 1997). Witvrouw et al. reported that K-12 did not affect the stages of viral adsorption, reverse transcription, or integration but did interfere with a stage prior to the processing by HIV-1 protease (Witvrouw et al., 1998). This study also showed that K-12 was inhibitory to some herpesviruses, suggesting that the compound targets a host cellular factor or factors involved in the transcription of viral genome. More recently, K-37 was found to suppress the replication of another human retrovirus, human T-lymphotoropic virus type 1 (HTLV-1), in chronically infected cells at the level of HTLV-1 LTR-driven Tax expression (Wang et al., 2002).

In this study, we have conducted long-term culture of HIV-1 infected cells with various concentrations of K-37 and observed viral breakthrough. The same experiment was carried out twice, and reproducible results were obtained (data not shown). Similar studies have been performed with HIV-1 RT and protease inhibitors to characterize drug-resistant mutants. In fact, some of the amino acid mutations responsible for drug-resistance were well illustrated by such studies (Schinazi et al., 2000). In our experiments, however, a mutant resistant to K-37 could not be obtained, because the breakthrough viruses (M_1 and U_1) were as susceptible to K-37 as the wild-type strains (M_0 and U_0) in chronically infected

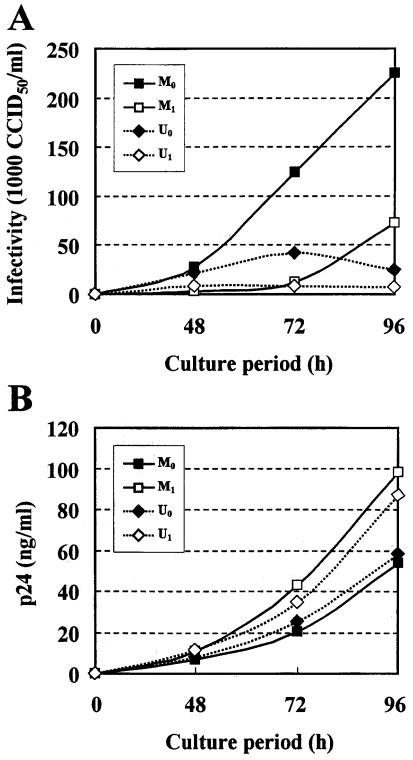

Fig. 3. (A) Infectivity and (B) p24 antigen levels of the culture supernatants of the cells infected with the wild-type strains and the breakthrough viruses. MOLT-4 cells were infected with the wild-type strains (M_0 and U_0) or the breakthrough viruses (M_1 or U_1) at a MOI of 0.01. After viral adsorption, the cells were washed thoroughly with culture medium to remove unadsorbed viral particles. Every 48 h, the culture supernatants were collected and examined for their infectivity and p24 antigen levels.

MOLT-4 cells (Table 1). Therefore, it is unlikely that the breakthrough was attributed to a mutation of virus-specific proteins. One possible explanation for this finding is that K-37 induces drug-transporter proteins, such as P-glycoprotein and multiple-drug-resistance-associated proteins, which have recently been suggested to play a role in the resistance to anti-HIV-1 drugs as well as anticancer drugs (Schuetz et al., 1999; Srinivas et al., 1998; Washington et al., 1998). Further studies are required to determine whether K-37 indeed induces the drug-transporter proteins.

Another interesting finding is the difference in viral infectivity between the wild-type strains and the corresponding breakthrough viruses. All of the strains (M_0, U_0, M_1, and U_1) were growing well in MOLT-4 cells, when determined by the p24 antigen levels in the culture supernatants (Fig. 3B). Furthermore, the p24 antigen levels of the breakthrough viruses (M_1 and U_1) were even higher than those of the wild-type strains (M_0 and U_0). In contrast, the infectivity of the breakthrough viruses proved significantly lower than that of their corresponding counterparts (Fig. 3A), suggesting that the cells infected with the breakthrough viruses produce a higher proportion of incomplete (or immature) viral particles than those infected with the wild-type strains. Similar phenomena are often observed between laboratory strains and clinical isolates of HIV-1.

It has been well documented that some parts of the envelope glycoprotein gp120 are important determinants for the infectivity and cell-tropism of HIV-1 (reviewed by Freed and Martin, 1995; Berger et al., 1999). Although the complete nucleotide sequence of gp120 was not determined in the present study, nucleotide substitutions that conferred amino acid mutations could not be identified in the sequenced region, including the V3 loop, of the breakthrough viruses (data not shown), indicating that K-37 did not induce mutations in gp120. Furthermore, K-37 did not generate any mutations of the nucleotide sequences of the HIV-1 LTR and TAR, except that both of the breakthrough viruses (M_1 and U_1) had a common mutation (G to T at position −215) in the second NFAT-binding site (Fig. 4). NFAT is a cellular factor ensuring a highly permissive state for HIV-1 replication in primary $CD4^+$ T cells (Kinoshita et al., 1998), presumably through the enhancement of HIV-1 gene expression (Cron et al., 2000). It is possible that the mutation in the NFAT-binding site impaired in part the infectivity of the breakthrough viruses. However, NFAT was also shown to act as a negative regulatory factor for HIV-1 replication (Lu et al., 1990).

At present, HIV-1 transcription inhibitors, including K-37, are not successful in the treatment of HIV-1-infected individuals. Preliminary experiments in animals revealed that this class of fluoroquinoline derivatives has considerable toxicity (unpublished data). Clinical trials of Ro 24-

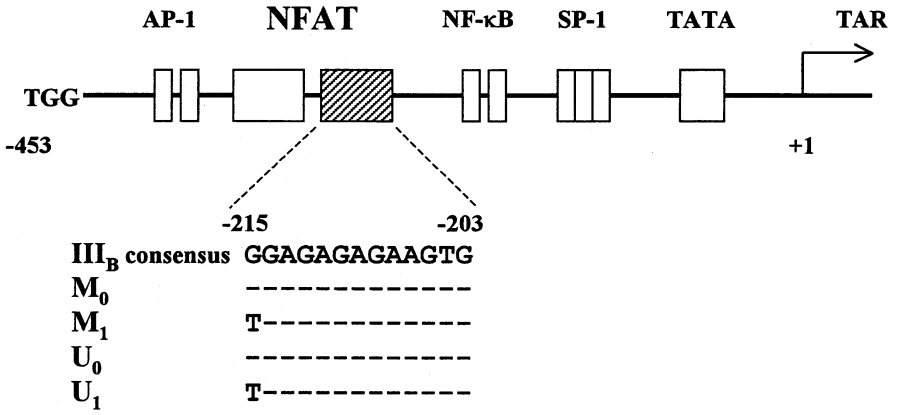

Fig. 4. Nucleotide sequences of the second NFAT-binding site of the breakthrough viruses. Boxes represent some cellular transcription factor-binding sites of the HIV-1 LTR. Dots indicate the nucleotide sequences identical to those of the NFAT-binding site of the consensus HTLV-III$_B$ strain (GeneBank accession number: X01762).

7429, the first Tat inhibitor described in the literature (Hsu et al., 1991), were halted due to lack of efficacy and some side effects in patients (Cupelli and Hsu, 1995). It is assumed that Ro 24-7429 targets a host cellular factor that binds to the transactivating response element (Braddock et al., 1994). HIV-1 remained sensitive after prolonged exposure to this compound (Hsu et al., 1993). Although K-37 is a more potent and selective inhibitor of HIV-1 than Ro 24-7429, its selectivity index (ratio of EC_{50} to CC_{50}) does not exceed 100 in acutely infected PBMC (data not shown).

In conclusion, the HIV-1 transcription inhibitor K-37 did not suppress viral breakthrough even at a subtoxic concentration after long-term culture of infected cells. The breakthrough viruses remained sensitive to the compound and showed reduced infectivity to the host cells. Thus, K-37 and its derivatives may have potential for the treatment of HIV-1 infection. However, further efforts have to be made to elucidate the target molecule and diminish toxicity in vivo.

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