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Inhibition of Human Immunodeficiency Virus Type 1 Replication and Cytokine Production by Fluoroquinoline Derivatives

MASANORI BABA, MIKA OKAMOTO, MASAKI KAWAMURA, MASAHIKO MAKINO, TOMOE HIGASHIDA, TOHRU TAKASHI, YOUICHI KIMURA, TOHRU IKEUCHI, TOSHIFUMI TETSUKA, and TAKASHI OKAMOTO

Division of Human Retroviruses, Center for Chronic Viral Diseases, Faculty of Medicine, Kagoshima University, Kagoshima 890, Japan (M.B., M.O., M.K., M.M.), New Product Research Laboratories, Daiichi Pharmaceutical Co., Ltd., Tokyo 134, Japan (T.H., T.Takashi, Y.K., T.I.) and Department of Molecular Genetics, Nagoya City University Medical School, Nagoya 467, Japan (T.Tetsuka, T.O.)

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ABSTRACT

We have recently identified 8-difluoromethoxy-1-ethyl-6-fluoro-1,4-dihydro-7-[4-(2-methoxyphenyl)-1-piperazinyl]-4-oxoquinoline-3-carboxylic acid (K-12) as a potent and selective inhibitor of human immunodeficiency virus type 1 (HIV-1) transcription. In the search for more effective derivatives and their mode of action, we have found 7-(3,4-dehydro-4-phenyl-1-piperidinyl)-1,4-dihydro-6-fluoro-1-methyl-8-trifluoromethyl-4-oxoquinoline-3-carboxylic acid (K-37) and 8-difluoromethoxy-1,4-dihydro-6-fluoro-7-(3,4-dehydro-4-phenyl-1-piperidinyl)-1-[4,(1,2,4-triazol-1-yl)methylphenyl]-4-oxoquinoline-3-carboxylic acid (K-38) to be more potent inhibitors of HIV-1 replication than K-12. The EC_{50} values of K-37 and K-38 for $HIV\text{-}1_{IIIB}$ were 27 and 3.8 nM in peripheral blood mononuclear cells, respectively. These values were approximately 3- and 24-fold lower than the EC_{50} of K-12. K-38 was also a more potent inhibitor of HIV-1 replication in chronically infected cells, such as tumor necrosis factor α-stimulated OM-10.1 cells. K-37 and K-38 proved to be more cytotoxic than K-12 for a variety of cell lines as well as peripheral blood mononuclear cells. These compounds were more inhibitory of Tat-induced HIV-1 long terminal repeat-driven gene expression than K-12, which suggests that their mechanism of action is attributable in part to the inhibition of Tat function. Interestingly, K-37 and K-38 could suppress the production of tumor necrosis factor α and interleukin 6 in phytohemagglutinin-stimulated peripheral blood mononuclear cells and the expression of intercellular adhesion molecule 1 in tumor necrosis factor α-stimulated human umbilical vein endothelial cells at their nontoxic concentrations. In contrast, another K-12 derivative, 1,4-dihydro-8-dimethylaminomethyl-6-fluoro-7-[4-(2-methoxyphenyl)-1-piperadinyl]-1-methyl-4-oxoquinoline-3-carboxylic acid (K-42), had anti-HIV-1 activity and cytotoxicity profiles similar to those of K-12, but K-42 scarcely inhibited the cytokine production and intercellular adhesion molecule 1 expression.

Transcription of the viral genome (integrated proviral DNA) into its mRNA is an essential step in the replicative cycle of HIV-1 and is considered to be a potential target for chemotherapeutic intervention to restrict HIV-1 replication (Cullen, 1991; Jones and Peterlin, 1994). In addition to the viral *trans*-activator protein Tat, several lines of evidence suggest that cellular factors play an important role in regulating HIV-1 gene expression (Roulston *et al.*, 1995; Baba 1997). Among these factors, NF-κB is the most potent activator of HIV-1 gene expression (Nabel and Baltimore, 1987; Griffin *et al.*, 1989). 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 TNF-α, leads to the immediate degradation of IκBα and activates NF-κB, resulting in the translocation of NF-κB from the cytoplasm to the nucleus (Roulston *et al.*, 1995; Thanos and Maniatis, 1995). NF-κB binds to the specific DNA motifs in the HIV-1 LTR and stimulates viral transcription. However, a complex and unknown machinery may also be involved in the regulation of HIV-1 gene expression.

In addition to the cytokine stimulation, HIV-1 gene expression can be induced by oxidative stress, suggesting that the activation of NF-κB is redox-regulated (Staal *et al.*, 1990; Schreck *et al.*, 1991). Thus, various antioxidants have been

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ABBREVIATIONS: HIV-1, human immunodeficiency virus type 1; NF, nuclear factor; TNF-α, tumor necrosis factor α; LTR, long terminal repeat; NAC, *N*-acetyl-L-cysteine; K-12, 8-difluoromethoxy-1-ethyl-6-fluoro-1,4-dihydro-7-[4-(2-methoxyphenyl)-1-piperazinyl]-4-oxoquinoline-3-carboxylic acid; IL, interleukin; ICAM, intercellular adhesion molecule; K-37, 7-(3,4-dehydro-4-phenyl-1-piperidinyl)-1,4-dihydro-6-fluoro-1-methyl-8-trifluoromethyl-4-oxoquinoline-3-carboxylic acid; K-38, 8-difluoromethoxy-1,4-dihydro-6-fluoro-7-(3,4-dehydro-4-phenyl-1-piperidinyl)-1-[4,(1,2,4-triazol-1-yl)methylphenyl]-4-oxoquinoline-3-carboxylic acid; K-42, 1,4-dihydro-8-dimethylaminomethyl-6-fluoro-7-[4-(2-methoxyphenyl)-1-piperazinyl]-1-methyl-4-oxoquinoline-3-carboxylic acid; BTC, 5-methoxy-3-(1-methylethoxy)-benzo[*b*]thiophene-2-carboxamide; PBMC, peripheral blood mononuclear cell; PHA, phytohemagglutinin; HUVEC, human umbilical vein endothelial cell; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; CAT, chloramphenicol acetyltransferase; SI, selectivity index; HTLV-I, human T-lymphotropic virus type I

examined for their inhibitory effects on HIV-1 replication. Among the antioxidants, NAC has been most extensively studied and found to inhibit HIV-1 replication (Roederer *et al.*, 1992). Although a number of papers describing the anti-HIV-1 activity of NAC *in vitro* have been published, the clinical efficacy of NAC has not been proven yet, probably because of its modest anti-HIV-1 activity and low selectivity. In general, the inhibitors that interact with host cellular factors have low selectivity (little difference between their effective concentration and cytotoxic threshold), which may cause considerable toxicity to the host cells. Therefore, the use of such compounds for the treatment of HIV-1 infection would be limited, unless they could have a certain degree of selectivity to HIV-1 replication.

The anti-HIV-1 activities of fluoroquinoline derivatives were first described in an European patent, but their mechanism of action was not reported (Kimura and Kogushi, 1993). We have recently found a series of fluoroquinoline derivatives to be potent and selective inhibitors of HIV-1 replication not only in acutely infected cells but also in chronically infected cells (Baba *et al.*, 1997). We demonstrated that K-12, a representative compound of the series, significantly reduced the synthesis of HIV-1 mRNA in chronically infected cell lines without altering the synthesis of a host mRNA, indicating that K-12 is a selective inhibitor of HIV-1 transcription (Baba *et al.*, 1997). However, the compound did not significantly inhibit Tat activity or NF-κB activation. In this study, we have synthesized several K-12 derivatives, examined their biological activities in a variety of cell systems, and found that some derivatives are more potent inhibitors of HIV-1 replication and Tat-induced HIV-1 LTR-driven gene expression than K-12. Interestingly, they have proved inhibitory of TNF-α and IL-6 production and ICAM-1 expression.

Materials and Methods

Compounds. Four fluoroquinoline derivatives (Fig. 1), K-12, K-37, K-38, and K-42, were synthesized by Daiichi Pharmaceuticals, Tokyo, Japan. BTC, a benzothiophene derivative (Boschelli *et al.*, 1994), was also synthesized in the same laboratory. Lamivudine was kindly provided by Mitsubishi Chemical, Yokohama, Japan. All compounds were dissolved in dimethyl sulfoxide at concentrations of 20 mM or higher to exclude any antiviral or cytotoxic effect of dimethyl sulfoxide.

Cells and virus. MT-4 cells (Miyoshi *et al.*, 1982), MOLT-4 cells (Kikukawa *et al.*, 1986), U937 cells, OM-10.1 cells (Butera *et al.*, 1991), and PBMCs were used in the antiviral and cytotoxicity assays. The cell lines were grown and maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin. PBMCs were obtained from healthy donors, stimulated with 2 μg/ml PHA, and cultured with RPMI-1640 medium containing 20% fetal bovine serum, antibiotics, and 20 units/ml IL-2 (Boehringer-Mannheim, Mannheim, Germany). HUVECs were purchased from Kurabo (Osaka, Japan). HIV-1_{IIIB} (T cell-tropic strain) and HIV-$1_{Ba\text{-}L}$ (macrophage-tropic strain) were used in the antiviral assays. HIV-1_{IIIB} and HIV-$1_{Ba\text{-}L}$ were propagated in MT-4 cells and PBMCs, respectively. Titers (HIV-1_{IIIB}) and p24 antigen level (HIV-$1_{Ba\text{-}L}$) of viral stocks were determined, and the stocks were stored at −80° until use.

Cytotoxicity assays. The cytotoxicities of the compounds for the cell lines (MT-4, MOLT-4, U937, OM-10.1, and PBMCs) were based on the cell viability. Except for OM-10.1 cells, mock-infected cells were used in the assays. The cells (1×10^5 cells/ml) were cultured in the presence of various concentrations of the test compounds. After a 3-day incubation (for OM-10.1), a 4-day incubation (for MT-4 and U937), or a 7-day incubation (for MOLT-4 and PBMCs) at 37°, the number of viable cells was determined by the MTT method (Pauwels *et al.*, 1988). The cytotoxicities of the compounds for PBMCs were also evaluated by the inhibition of mitogen-induced cell proliferation. Briefly, PBMCs (2.5×10^5 cells/ml) were stimulated with 2 μg/ml PHA and cultured in the presence of various concentrations of the test compounds. At 16 hr before the end of culture period (6 days), 1 μCi of [^{3}H]thymidine was added into the culture medium and incubated at 37° for 16 hr. Then the cells were harvested, and their acid-insoluble materials were analyzed for radioactivity.

Antiviral assays. The activities of the compounds against acute HIV-1 infection were based on the inhibition of virus-induced cytopathicity in MT-4 and MOLT-4 cells and p24 antigen production in PBMCs, as previously described (Baba *et al.*, 1991). MT-4 and MOLT-4 cells (1×10^5 cells/ml) were infected with HIV-1_{IIIB} at a multiplicity of infection of 0.02 and 0.1, respectively, and were cultured in the presence of various concentrations of the test compounds. After a 4-day incubation at 37°, MOLT-4 cells were subcul-

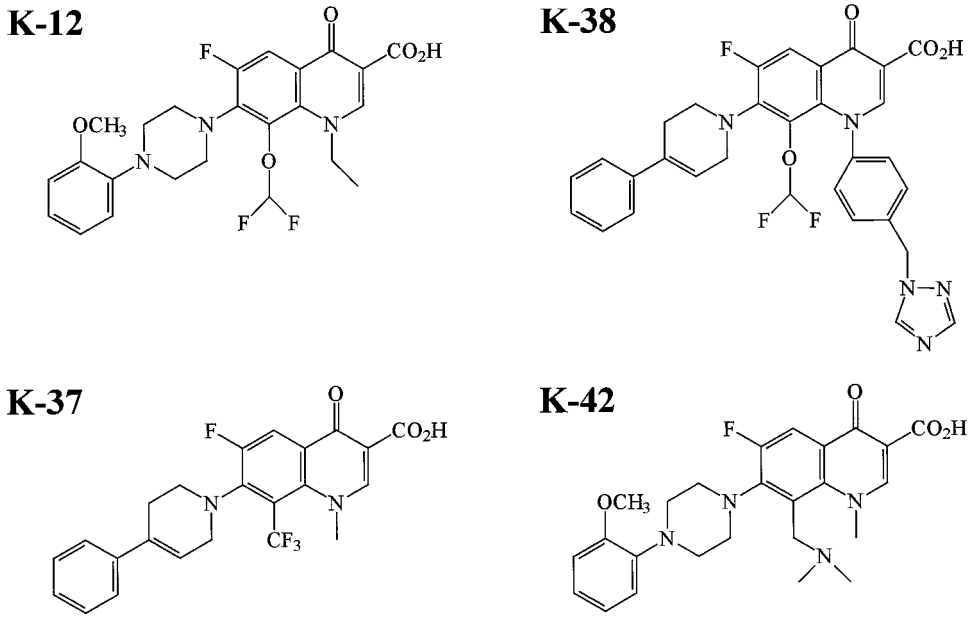

Fig. 1. Structural formulae of fluoroquinoline derivatives.

tured at a ratio of 1:5 with fresh culture medium, containing appropriate concentrations of the test compounds, and further incubated. The number of viable MT-4 and MOLT-4 cells was measured by the MTT method on days 4 and 7 after virus infection, respectively. For the PBMC assays, the cells (1×10^5 cells/ml) were infected with HIV-1_{IIIB} (a multiplicity of infection of 0.1) or HIV-$1_{Ba\text{-}L}$ (1.0 ng of p24). After virus adsorption for 2 hr, the cells were extensively washed to remove unadsorbed virus particles and cultured in the presence of various concentrations of the test compounds. After a 7-day incubation at 37°, the culture supernatants were collected and determined for their p24 antigen levels with a sandwich ELISA kit (Cellular Products, Buffalo, N.Y.). The activities of the compounds against chronic infection were based on the inhibition of p24 antigen production in OM-10.1 cells. OM-10.1 cells (1×10^5 cells/ml) were incubated in the absence or presence of the compounds for 2 hr, stimulated with 1 ng/ml TNF-α (Genzyme, Cambridge, MA), and further incubated. After a 3-day incubation at 37°, the culture supernatants were collected and examined for their p24 antigen levels.

Transfection assays. HeLa cells (2×10^6 cells) were cotransfected with 5 μg of a plasmid expressing CAT under the control of the HIV-1 LTR (pUC-BENN-CAT) (Gendelman *et al.*, 1986) and 0.5 μg of a plasmid expressing HIV-1 Tat under the control of the simian virus 40 promoter (pSV2*tat*72) by a liposome-mediated transfection method. After transfection, the cells were incubated in the absence or presence of compounds for 2 days. Total cell extracts (200 μg) were incubated with ^{14}C-labeled chloramphenicol and acetyl coenzyme A, and their acetylated forms were determined by thin-layer chromatography. CAT activity was quantified by a model BAS1000Mac image analyzer (Fuji Film, Tokyo, Japan).

Cytokine production assays. PBMCs (1×10^6 cells/ml) were stimulated with 2 μg/ml PHA and cultured in the presence of various concentrations of the test compounds. After a 24-hr incubation at 37°, the culture supernatants were collected and examined for their TNF-α and IL-6 concentrations with cytokine-detection ELISA kits (R&D Systems, Minneapolis, MN). At the same time, the viable cell number was determined by the MTT method to exclude the cytotoxic effects of the compounds on cytokine production.

ICAM-1 expression assay. Confluent HUVECs in a microtiter plate were stimulated with 100 ng/ml TNF-α. After a 4-hr incubation at 37°, the cells were extensively washed with Hanks' balanced salt solution containing 0.1% bovine serum albumin and incubated with an anti-ICAM-1 monoclonal antibody (clone 84H10, Cosmobio, Tokyo, Japan) for 45 min at room temperature. The cells were washed and further incubated with a horseradish peroxidase-conjugated goat antimouse-IgG (Cappel, West Chester, PA) for 90 min at room temperature. The cells were washed again and treated with 2,2′-azinobis(3-ethylbenzthiazoline sulfonate) as a substrate. The expression of ICAM-1 was determined colorimetrically with a microplate reader (Bio-Rad, Hercules, CA).

Results

Cytotoxicity of fluoroquinolines. Before the evaluation for anti-HIV-1 activities, the fluoroquinoline derivatives were examined for their inhibitory effects on the viability and proliferation of a variety of cell lines and PHA-stimulated PBMCs. Among the test compounds, K-38 was found to be the most cytotoxic for all cell lines (Table 1). The IC_{50} values for the viability of MT-4, MOLT-4, and U937 cells were 0.12, 0.22, and 0.22 μM, respectively. These values were approximately 44- to 66-fold smaller than those of K-12 (Table 1). K-38 was also the most inhibitory of the viability and PHA-induced proliferation of PBMCs. On the other hand, K-42 displayed a cytotoxic profile similar to that of K-12. For instance, the IC_{50} values of K-12 and K-42 were 8.1 and 9.7 μM in MOLT-4 cells, respectively (Table 1). K-37 proved more cytotoxic than K-12 and K-42; however, it was less inhibitory of the viability and proliferation of the host cells than K-38. Furthermore, MT-4 cells appeared to be more sensitive to all compounds than MOLT-4 and U937 cells, as determined under the same assay conditions (Table 1).

Antiviral activity in acute infection. When the fluoroquinoline derivatives were evaluated for their inhibitory effects on HIV-1 replication in PBMCs, all compounds suppressed the production of p24 antigen in culture supernatants at their nontoxic concentrations (Fig. 2). Among the compounds, K-38 was the most active, and it achieved 90% and 80% inhibition of HIV-1_{IIIB} and HIV-$1_{Ba\text{-}L}$ replication, respectively, at a concentration of 32 nM. K-37 was less active than K-38 but more active than K-12 and K-42. Similar to their cytotoxic profiles, K-12 and K-42 were equally inhibitory of HIV-1 replication in PBMCs. The EC_{50} values of K-12, K-37, K-38, and K-42 for HIV-1_{IIIB} were 85, 27, 3.8, and 130 nM, respectively. Thus, their SIs, defined as the ratio of IC_{50} to EC_{50} values, were more than 100, indicating that all compounds are potent and selective inhibitors of HIV-1 replication in acutely infected PBMCs. Although the compounds also displayed potent and selective inhibition of HIV-1 replication in MOLT-4 cells, they were found to be modest inhibitors in MT-4 cells (Table 2). All compounds proved to be approximately 5- to 10-fold less active in MT-4 cells than in MOLT-4 cells (Table 2). Because all compounds displayed higher cytotoxicity for MT-4 cells than for MOLT-4 cells (Table 1), their SIs could not exceed 10 in MT-4 cells (Table 2). In our preliminary studies on structure-activity relationships, decarboxylation of K-12 resulted in a complete loss of its anti-HIV-1 activity (data not shown). Furthermore, both 7-(4-arylpiperazinyl) and 7-(4-arylpiperidinyl) derivatives exhibited selective inhibition of HIV-1 replication, yet 7-(4-cycloalkylpiperazinyl) or 7-[4-(2-pyrimidinyl)piperidinyl] derivatives did not show any anti-HIV-1 activities (data not shown).

TABLE 1
Inhibitory effects of fluoroquinoline derivatives on cell proliferation
All data represent mean values ± standard deviation for at least three separate experiments.

Compound	IC_{50}[a]				
	MT-4	MOLT-4	U937	PBMC	
			μM		
K-12	5.4 ± 0.8	8.1 ± 1.5	9.6 ± 0.2	16.3 ± 4.0	(14.7 ± 0.2)[b]
K-37	0.53 ± 0.05	1.6 ± 0.2	1.5 ± 0.3	5.0 ± 0.7	(3.3 ± 0.4)
K-38	0.12 ± 0.05	0.22 ± 0.09	0.22 ± 0.04	1.5 ± 0.2	(0.52 ± 0.19)
K-42	3.5 ± 0.6	9.7 ± 1.2	9.5 ± 0.5	>20	(18.3 ± 9.3)

[a] Based on the reduction of viable cell number in mock-infected cells.
[b] IC_{50} values for PBMCs were also determined by the inhibition of [^{3}H]thymidine incorporation into the cells stimulated with PHA.

Antiviral activity in chronic infection. We have previously demonstrated that K-12 is a highly potent and selective inhibitor of HIV-1 replication in chronically infected cells (Baba *et al.*, 1997). Therefore, the fluoroquinoline derivatives were also examined for their inhibitory effects on HIV-1 production in TNF-α-stimulated OM-10.1 cells. OM-10.1 cells produce little or no HIV-1 under basal conditions but do produce significant levels of virus after stimulation with various substances, such as TNF-α and phorbol 12-myristate 13-acetate (Butera *et al.*, 1991; Baba, 1997). In fact, the level of p24 antigen in culture supernatants was less than 1 ng/ml in unstimulated OM-10.1 cells, yet it increased to more than 200 ng/ml after stimulation with 1 ng/ml TNF-α (data not shown). Table 3 shows the EC_{50} and IC_{50} values of the compounds in TNF-α-stimulated OM-10.1 cells. All fluoroquinoline derivatives, including K-12, displayed selective inhibition of HIV-1 replication in this assay system. However, the reverse transcriptase inhibitor lamivudine was totally inactive. The most active compound was K-38 followed by K-37. Unlike the anti-HIV-1 activity in acute infection, K-42 was 4-fold less active than K-12 (Table 3). Although the activities of K-37 and K-38 were higher than those of K-42, they were much more cytotoxic for OM-10.1 cells than K-42, resulting in smaller SIs (Table 3).

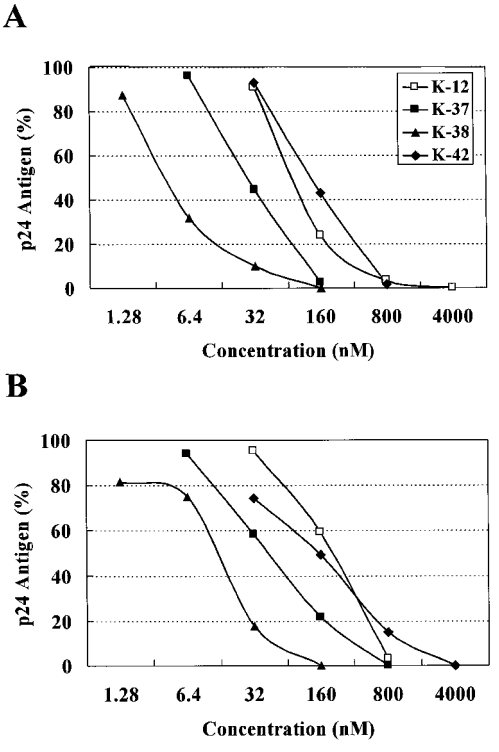

Fig. 2. Inhibitory effects of fluoroquinoline derivatives on HIV-1 replication in PBMCs. PHA-stimulated PBMCs were infected with A, HIV-1_{IIIB}, and B, HIV-$1_{Ba\text{-}L}$, and cultured in the presence of various concentrations of the test compounds. After a 7-day incubation, the p24 antigen levels of the culture supernatants were determined. The amounts of p24 antigen are expressed as the percent of control (no compound). All experiments were carried out in duplicate, and mean values are shown.

TABLE 2
Inhibitory effects of fluoroquinoline derivatives on HIV-1 replication in MT-4 and MOLT-4 cells
All data represent mean values ± standard deviation for at least three separate experiments.

Compound	Cell	EC_{50}[a]	IC_{50}[b]	SI[c]
		μM		
K-12	MT-4	0.64 ± 0.06	5.4 ± 0.8	8.4
	MOLT-4	0.11 ± 0.07	8.1 ± 1.5	74
K-37	MT-4	0.20 ± 0.06	0.53 ± 0.05	2.7
	MOLT-4	0.020 ± 0.010	1.6 ± 0.2	80
K-38	MT-4	0.043 ± 0.015	0.12 ± 0.05	2.8
	MOLT-4	0.0090 ± 0.0030	0.22 ± 0.09	24
K-42	MT-4	0.93 ± 0.41	3.5 ± 0.6	3.8
	MOLT-4	0.18 ± 0.06	9.7 ± 1.2	54
3TC	MT-4	0.63 ± 0.32	>20	>32

[a] Based on the inhibition of virus-induced cytopathicity.
[b] Based on the reduction of viable cell number in mock-infected cells.
[c] Ratio of IC_{50} to EC_{50}.

Effects on Tat-induced transactivation. Although our previous study demonstrated that K-12 did not significantly inhibit Tat-induced *trans*-activation (Baba *et al.*, 1997), another group has recently found that K-12 is able to inhibit Tat-induced expression of stably integrated HIV-1 LTR-driven alkaline phosphatase gene in CEM cells (Takeuchi and Yoshimatsu, personal communication). To elucidate whether the fluoroquinoline derivatives indeed affect the Tat-induced *trans*-activation, cotransfection experiments with a Tat-expressing plasmid and an HIV-1 LTR-driven CAT-expressing plasmid were carried out in HeLa cells in the presence of various concentrations of the compounds. An approximately 70-fold increase in CAT activity was observed in the absence of compounds, compared with that of the cells transfected with CAT-expressing plasmid alone (Fig. 3). Except for K-42, the Tat-induced CAT expression was suppressed by the fluoroquinoline derivatives in a dose-dependent fashion. In particular, K-38 proved to be a highly potent inhibitor of CAT expression. It achieved 62% and 93% inhibition at a concentration of 0.16 and 0.8 μM, respectively (Fig. 3). Furthermore, K-37 and K-12 were 5- and 25-fold less inhibitory, respectively, of CAT expression than K-38, which seems in accord with their anti-HIV-1 activities.

Effects on cytokine production and ICAM-1 expression. To further investigate biological activities of the fluoroquinoline derivatives, we conducted experiments to determine whether the compounds inhibited the production of TNF-α and IL-6 in PHA-stimulated PBMCs. When PBMCs were stimulated with 2 μg/ml PHA and incubated in the presence of various concentrations of the test compounds, K-12 and K-42 did not suppress the proliferation of stimulated PBMCs at concentrations up to 4 μM during a 24-hr incubation period (Fig. 4A). However, K-37 reduced the viable cell number to 70% of the control culture at a concentration of 4 μM, and K-38 reduced it to 43% and 58% at concen-

TABLE 3
Inhibitory effects of fluoroquinoline derivatives on HIV-1 replication in TNF-α-stimulated OM-10.1 cells
All data represent mean values ± standard deviation for at least three separate experiments.

Compound	EC_{50}[a]	IC_{50}[b]	SI[c]
		μM	
K-12	0.050 ± 0.020	9.3 ± 2.5	186
K-37	0.040 ± 0.001	1.53 ± 0.12	38
K-38	0.017 ± 0.005	0.30 ± 0.10	18
K-42	0.20 ± 0.03	18.3 ± 9.3	92
3TC	>20	>20	

[a] Based on the reduction of p24 antigen in culture supernatants.
[b] Based on the reduction of viable cell number.
[c] Ratio of IC_{50} to EC_{50}.

trations of 0.8 and 4 μM, respectively. In contrast, the production of TNF-α and IL-6 was almost completely inhibited by the presence of K-37 and K-38 at 0.8 and 4 μM, respectively. (Fig. 4B). K-12 moderately suppressed the production of TNF-α in a dose-dependent fashion. Interestingly, little, if any, inhibition was observed for K-42 even at the highest concentration examined (4 μM). Similar results were obtained for the production of IL-6, where K-38 was found to be the most potent inhibitor of PHA-induced IL-6 production in PBMCs. Again, K-42 was the weakest inhibitor of IL-6 production (Fig. 4C).

In the next experiment, we examined whether the fluoroquinoline derivatives could inhibit the TNF-α-induced expression of ICAM-1 in HUVECs. As shown in Fig. 4, K-38 proved highly inhibitory of ICAM-1 at a concentration of 0.05 μg/ml (0.084 μM). Although K-38 was slightly cytotoxic to the host cells at 0.5 μg/ml, it did not affect the viability of HUVECs at 0.05 μg/ml (data not shown). K-37 was less active than K-38; however, it appeared to be as active as the ICAM-1 inhibitor BTC (Boschelli *et al.*, 1994). K-12 was a modest inhibitor of ICAM-1, and K-42 scarcely suppressed the expression of this molecule (Fig. 5). Thus, the fluoroquinoline derivatives may also have various biological activities, yet such biological activities are not always associated with their anti-HIV-1 activities, as observed with K-42.

Discussion

We have recently found a series of fluoroquinoline derivatives to be potent and selective inhibitors of HIV-1 replication in both acute and chronic infections (Baba *et al.*, 1997). Northern blot analysis revealed that K-12, the most potent congener of the series, selectively prevented the accumulation of HIV-1 mRNA in chronically infected cells in a dose-dependent fashion, which indicates that the compound belongs to a family of HIV-1 transcription inhibitors. Although the exact target molecule of K-12 remains obscure, K-12 did not inhibit the TNF-α-induced translocation of NF-κB to the nucleus or the intranuclear level of Sp1 (Baba *et al.*, 1997). Moreover, the *in vitro* binding of NF-κB or Sp1 to its target DNA was not affected by the presence of K-12. A cotransfection experiment with a Tat expression plasmid and an HIV-1 LTR-driven CAT-expressing plasmid demonstrated that K-12 did not significantly reduce the Tat-induced CAT expression. In addition, K-12 proved inhibitory of the replication of the murine retrovirus LP-BM5, which is devoid of accessory genes such as *tat* and *rev* (Morse *et al.*, 1992). Thus, the fluoroquinoline derivatives appear to interact with a cellular factor or factors that play a key role in HIV-1 transcription. However, this does not exclude the possibility that K-12 is not inhibitory of the Tat-mediated activation of HIV-1 transcription, because the Tat-mediated HIV-1 activation may involve complex interactions with known and unknown cellular transcriptional factors (Veschambre *et al.*, 1995; Zhou and Sharp, 1995). In fact, the present study clearly demonstrated that the anti-HIV-1 activity of fluoroquinoline derivatives closely correlated with the inhibition of Tat-in-

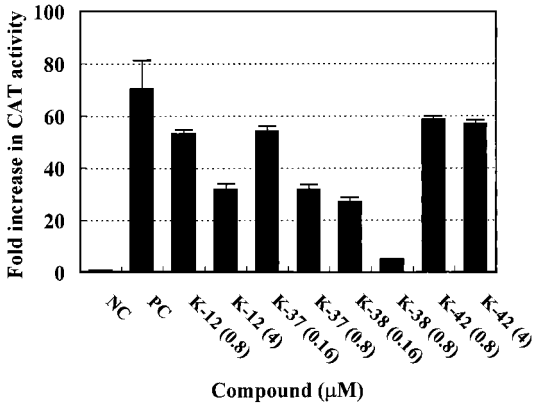

Fig. 3. Inhibitory effects of fluoroquinoline derivatives on Tat-induced *trans*-activation. HeLa cells were cotransfected with an HIV-1 LTR-driven CAT-expressing plasmid (pUC-BENN-CAT) and a Tat-expressing plasmid (pSV2*tat*72). The cells were incubated in the absence or presence of compounds for 2 days. Total cell extracts were incubated with ^{14}C-labeled chloramphenicol and acetyl coenzyme A, and their acetylated forms were determined by thin-layer chromatography. CAT activity was quantified by a model BAS1000Mac image analyzer and expressed as fold increase, compared with that in the cells transfected with the CAT-expressing plasmid alone (*NC*). PC indicates the cells cotransfected with both plasmids but incubated in the absence of compounds. All data represent mean values ± range for two separate experiments.

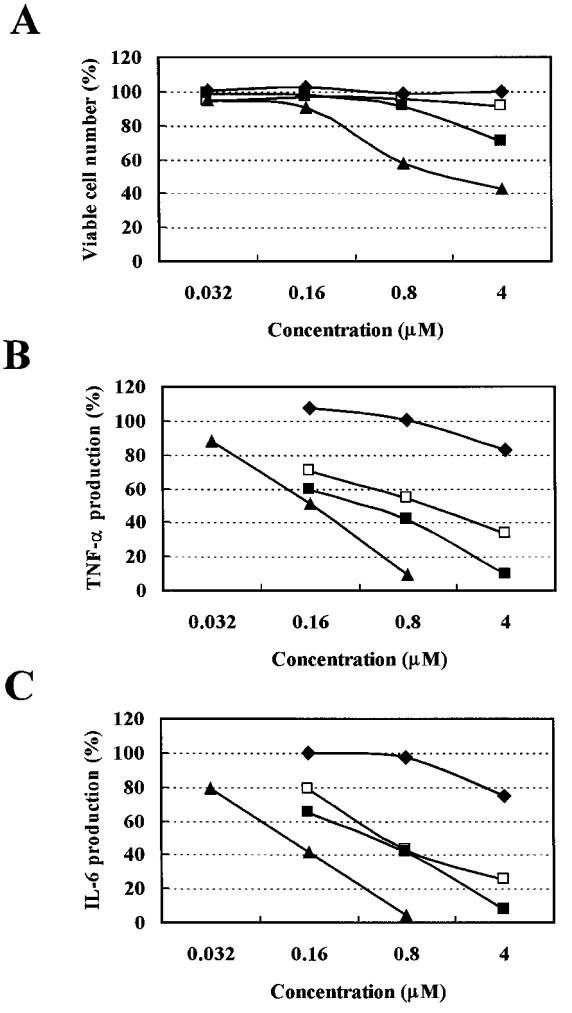

Fig. 4. Inhibitory effects of fluoroquinoline derivatives on A, viable cell number; B, TNF-α production; and C, IL-6 production in PBMCs. The cells were stimulated with PHA (2 μg/ml) and cultured in the presence of various concentrations of K-12 (□), K-37 (■), K-38 (▲), and K-42 (♦). After a 24-hr incubation, the culture supernatants were examined for their TNF-α and IL-6 concentrations. At the same time, the viable cell number was determined by the MTT method. Data are expressed as the percent of control (no compound). All experiments were carried out in duplicate, and mean values are shown.

duced *trans*-activation (Fig. 3). Thus, we assume that the fluoroquinoline derivatives target the cellular factors cooperatively working or interacting with Tat.

Another interesting finding is that all of the fluoroquinoline derivatives were less inhibitory of HIV-1 replication and more cytotoxic in MT-4 cells than in PBMCs (Tables 1 and 3). Similarly, the Tat inhibitors Ro5–3335 and Ro24–7429 did not display any selective inhibition of HIV-1 in MT-2 and MT-4 cells (Witvrouw *et al.*, 1992, data not shown). This may be because of the high level expression of NF-κB in these cell lines (Luznik *et al.*, 1995). Alternatively, the expression of HTLV-I Tax may modulate the regulation of HIV-1 gene expression, because MT-2 and MT-4 cells are persistently infected with HTLV-I (Miyoshi *et al.*, 1982). Accordingly, the fluoroquinoline derivatives proved to be more potent and selective inhibitors of HIV-1 replication in MOLT-4 cells (HTLV-I-free T-lymphoblastoid cell line) than in MT-4 cells (Table 2).

It has recently been reported that BTC and some flavonoids selectively inhibit TNF-α-induced HIV-1 expression in OM-10.1 cells (Butera *et al.*, 1995; Critchfield *et al.*, 1996). Similar to the fluoroquinoline derivatives, these compounds prevented HIV-1 mRNA synthesis but did not affect NF-κB activation or Tat functions. More recently, Critchfield *et al.* (1997) have demonstrated that both BTC and chrysin (one of the flavonoids) inhibit the activity of human recombinant casein kinase II. They postulate that casein kinase II may regulate HIV-1 transcription by phosphorylating cellular proteins involved in HIV-1 *trans*-activation. Therefore, we have also examined whether the fluoroquinoline derivatives inhibit the activity of casein kinase II using a synthetic peptide and a recombinant IκBα as substrates (Janosch *et al.*, 1996). Even the most potent derivative, K-38, did not affect the enzyme activity at concentrations up to 20 μM, which indicates that the fluoroquinoline derivatives are not inhibitors of casein kinase II, whereas BTC could reduce the phosphorylation of IκBα by casein kinase II (data not shown).

The future prospects of fluoroquinoline derivatives as anti-HIV-1 agents are still unclear. Although cellular factors are considered potential targets for inhibition of HIV-1 replication (Baba, 1997), inhibition of such factors may be accompanied by substantial cytotoxicity or unexpected biological activities. In fact, the present study has revealed that there is a close correlation between the anti-HIV-1 activity and cytotoxicity of the fluoroquinoline derivatives. Among the derivatives, K-38 was the most active but the most cytotoxic. Furthermore, some of the fluoroquinoline derivatives could inhibit the production of TNF-α and IL-6 in PHA-stimulated PBMCs and the TNF-α-induced expression of ICAM-1 in HUVECs (Fig. 4 and 5). Again, K-38 was the most potent inhibitor of cytokine production and ICAM-1 expression. Several cytokines, including TNF-α and IL-6, are strong inducers of HIV-1 replication, and they are overexpressed in the lymphoid tissues of HIV-1-positive individuals (Fauci, 1993). In addition, these cytokines are produced from brain macrophages and microglia and seem to play a considerable role in the pathogenesis of HIV-1-associated central nervous system disorders (Merrill and Chen, 1991; Epstein and Gendelman, 1993). From this point of view, the fluoroquinoline derivatives' inhibition of cytokine production may have benefit for the prophylaxis and treatment of AIDS-associated dementia. On the other hand, K-42, a more specific inhibitor of HIV-1 replication, may have an advantage over other fluoroquinoline derivatives in terms of possible side effects.

In conclusion, the novel fluoroquinoline derivatives presented here have a certain improvement in either anti-HIV-1 activity (K-37 and K-38) or specificity (K-42), compared with K-12. However, their target molecule and *in vivo* toxicity profiles remain to be elucidated before the compounds are recognized as promising candidates for the treatment of HIV-1-infection in humans.

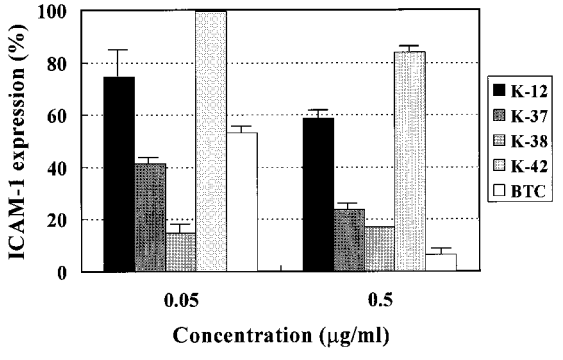

Fig. 5. Inhibitory effects of fluoroquinoline derivatives on ICAM-1 expression in HUVECs. Confluent cells were stimulated with TNF-α (100 ng/ml). After a 4-hr incubation, the cells were examined for their ICAM-1 expression, according to the procedures described in Materials and Methods. The levels of ICAM-1 expression are expressed as the percent of control (no compound). All experiments were carried out in triplicate, and mean values ± standard deviation are shown.

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Send reprint requests to: Dr. Masanori Baba, Division of Human Retroviruses, Ctr for Chronic Viral Disease, Faculty of Medicine, Kagoshima University, 8–35-1, Sakuragaoka, Kagoshima 890, Japan. E-mail: baba@med3.kufm.kagoshima-u.ac.jp