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Activity against Human Immunodeficiency Virus Type 1, Intracellular Metabolism, and Effects on Human DNA Polymerases of 4′-Ethynyl-2-Fluoro-2′-Deoxyadenosine▽

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We examined the intracytoplasmic anabolism and kinetics of antiviral activity against human immunodeficiency virus type 1 (HIV-1) of a nucleoside reverse transcriptase inhibitor, 4′-ethynyl-2-fluoro-2′-deoxyadenosine (EFdA), which has potent activity against wild-type and multidrug-resistant HIV-1 strains. When CEM cells were exposed to 0.1 μM [^{3}H]EFdA or [^{3}H]3′-azido-2′,3′-dideoxythymidine (AZT) for 6 h, the intracellular EFdA-triphosphate (TP) level was 91.6 pmol/10^9 cells, while that of AZT was 396.5 pmol/10^9 cells. When CEM cells were exposed to 10 μM [^{3}H]EFdA, the amount of EFdA-TP increased by 22-fold (2,090 pmol/10^9 cells), while the amount of [^{3}H]AZT-TP increased only moderately by 2.4-fold (970 pmol/10^9 cells). The intracellular half-life values of EFdA-TP and AZT-TP were ~17 and ~3 h, respectively. When MT-4 cells were cultured with 0.01 μM EFdA for 24 h, thoroughly washed to remove EFdA, further cultured without EFdA for various periods of time, exposed to HIV-1_{NL4-3}, and cultured for an additional 5 days, the protection values were 75 and 47%, respectively, after 24 and 48 h with no drug incubation, while those with 1 μM AZT were 55 and 9.2%, respectively. The 50% inhibitory concentration values of EFdA-TP against human polymerases α, β, and γ were >100 μM, >100 μM, and 10 μM, respectively, while those of ddA-TP were >100 μM, 0.2 μM, and 0.2 μM, respectively. These data warrant further development of EFdA as a potential therapeutic agent for those patients who harbor wild-type HIV-1 and/or multidrug-resistant variants.

Highly active antiretroviral therapy (HAART) has had a major impact on the AIDS epidemic in industrially advanced nations. However, eradication of human immunodeficiency virus type 1 (HIV-1) does not appear to be currently possible, in part due to the viral reservoirs remaining in blood and infected tissues. Moreover, a number of challenges have been encountered in the antiviral therapy of HIV-1 infection (7, 24, 25). Challenges include (i) various acute to long-term drug-related toxicities; (ii) only a partial restoration of immunologic functions is achieved once HIV-infected individuals develop AIDS; (iii) the development of various cancers as a consequence of survival prolongation with HAART; (iv) flare-ups of inflammation in individuals receiving HAART, i.e., the immune reconstruction syndrome (IRS); and (v) the increased cost of antiviral therapy.

Successful antiviral drugs, in theory, exert their virus-specific effects by interacting with viral receptors, virally encoded enzymes, viral structural components, or viral genes or their transcripts without disturbing cellular metabolism or function (19). However, at present, no antiretroviral drugs or agents are likely to be completely specific for HIV-1 or devoid of toxicity or adverse effects in the therapy of AIDS, which has been a critical issue because patients with AIDS and its related diseases will have to receive antiretroviral therapy for a long period of time, perhaps for the rest of their lives. Thus, the identification of a new class of antiretroviral drugs that have a unique mechanism(s) of action, that are highly potent to drug-resistant HIV-1 variants, that delay or do not allow the emergence of drug-resistant variants, and that produce no or minimal adverse effects remains an important therapeutic objective.

We recently designed and synthesized a number of 4′-ethynyl (4′-*E*)-2′-deoxynucleosides and their analogs (EdNs) and identified a series of potent anti-HIV-1 compounds which blocked the replication of a wide spectrum of laboratory and clinical HIV-1 strains in vitro (14, 21). These EdN analogs, unlike the existing Food and Drug Administration (FDA)-approved nucleoside reverse transcriptase inhibitors, possess a 3′-OH in their sugar moiety; however, they cause viral DNA chain termination, resulting in reverse transcriptase inhibition (14, 23). Through our optimization efforts of such 4′-*E* nucleoside analogs, we have now identified 4′-*E*-2-fluoro-2′-deoxyadenosine (EFdA), which exerts highly potent anti-HIV activity with favorable in vitro cell toxicities.

In the present study, we determined the profiles of antiviral activity and cell toxicity of EFdA and further examined its

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cellular uptake, intracytoplasmic anabolism, and kinetics of antiviral activity against various HIV-1 strains. The present data suggest that EFdA represents a potent anti-HIV-1 agent with the possibility of a once- or twice-a-day regimen and warrants further development as a potential therapeutic agent for those harboring wild-type HIV-1 and/or multidrug resistant variants.

MATERIALS AND METHODS

Antiviral agents and radiochemicals. EFdA was newly designed, synthesized, and tested for anti-HIV-1 activity in vitro. A method for the synthesis of EFdA will be published elsewhere. The structure of EFdA is illustrated in Fig. 1. 3′-Azido-2′,3′-deoxythymidine (AZT or zidovudine) was purchased from Sigma (St. Louis, MO). Saquinavir (SQV) and amprenavir (APV) were kindly provided by Roche Products Ltd. (Welwyn Garden City, United Kingdom) and Glaxo-SmithKline (Research Triangle Park, Durham, NC), respectively. Tenofovir (TDF) was obtained from the National Institutes of Health AIDS Research and Reference Reagent Program. [*methyl*-^{3}H]AZT (specific radioactivity, 12 Ci/mmol) and [8-^{3}H]EFdA (specific radioactivity, 5 Ci/mmol) were obtained from Moravek Biochemicals, Inc. (Brea, CA).

Cells and viruses. CEM and MT-4 cells were grown in RPMI 1640-based culture medium supplemented with 15% fetal calf serum (FCS; HyClone Laboratories, Logan, UT), 50 U of penicillin per ml, and 50 μg of streptomycin per ml. The HeLa-CD4-LTR-β-gal indicator cell line expressing human CCR5 (CCR5$^+$-MAGI [multinuclear activation of a galactosidase indicator]) (17) was a kind gift from Yosuke Maeda. CCR5$^+$-MAGI cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% FCS, 200 μg/ml G418, 100 μg/ml hygromycin B, and 100 μg/ml zeomycin. Peripheral blood mononuclear cells (PBMC) isolated from HIV-1-seronegative donors by using Ficoll-Hypaque were cultured in RPMI 1640-based culture medium containing 10% FCS and antibiotics with 10 μg of phytohemagglutinin (PHA-PBMC) for 3 days prior to drug susceptibility assays. HIV-1 strains used for the drug susceptibility assay (see below) were as follows: HIV-$1_{Ba\text{-}L}$, HIV-$1_{NL4\text{-}3}$, and three HIV-1 clinical isolates, HIV-$1_{MDR/C}$, HIV-$1_{MDR/G}$, and HIV-$1_{MDR/MM}$, which were originally isolated from patients with AIDS who had received from 9 to 11 anti-HIV-1 drugs over the previous 32 to 83 months and which were genotypically and phenotypically characterized as multidrug-resistant HIV-1 variants (15, 28).

Drug susceptibility assay. The susceptibilities of HIV-$1_{Ba\text{-}L}$, HIV-$1_{NL4\text{-}3}$, and the three multidrug-resistant primary HIV-1 isolates to various drugs were determined as previously described (28), with minor modifications. Briefly, PHA-PBMC (10^6 cells/ml) were exposed to 50 50% tissue culture infectious doses of HIV-$1_{Ba\text{-}L}$ or each primary HIV-1 isolate and cultured in the presence or absence of various concentrations of drugs in 10-fold serial dilutions in 96-well microculture plates (10^5 cells/well). On day 7 of culture, the supernatant was harvested, and the amount of p24 Gag protein was determined by using a fully automated chemiluminescent enzyme immunoassay system (Lumipulse F; Fujirebio, Inc., Tokyo, Japan) (20). The drug concentrations that suppressed the production of p24 Gag protein by 50% (50% effective concentrations [EC_{50}s]) were determined by comparison with the level of p24 production in drug-free control cell cultures. All assays were performed in triplicate. To determine the drug susceptibility of HIV-$1_{NL4\text{-}3}$, a methylthiazoletetrazolium (MTT) assay was used, employing MT-4 cells (10^4 cells/well) as target cells, as previously described (1). The cytotoxicity of each drug against MT-4 cells and PBMC was also determined using the MTT assay as previously described (1).

Uptake and intracellular metabolism of EFdA. CEM cells (10^6 cells/ml) were incubated with various concentrations (0.1, 1, and 10 μM) of [^{3}H]EFdA or [^{3}H]AZT for 6 h, followed by thorough washing to remove extracellular drugs. Subsequently, nucleosides/nucleotides within the cells were extracted with 60% methanol and subjected to high-performance liquid chromatography (HPLC) on an ion exchange Partisil 10-SAX column (Whatman International Ltd., Maidstone, United Kingdom). HPLC analysis was performed according to previously established procedures (18) using two elution buffers, buffer A (0.03 M ammonium phosphate) and buffer B (nine parts of 0.7 M ammonium phosphate and one part 100% ethanol). For elution, the following program was used: 5 min of buffer A, followed by 10 min of a highly convex gradient to 75% buffer A–25% buffer B, followed by 15 min of a slightly convex gradient to 100% buffer B, and finally followed by a 10-min isocratic elution with buffer B. One-minute elution fractions were collected, and the radioactivity of each fraction was measured using a liquid scintillation counter to determine the amount of metabolites.

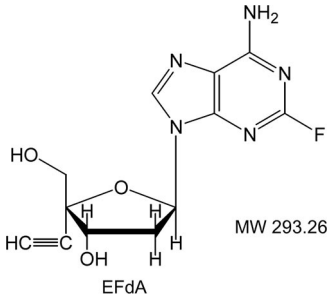

FIG. 1. Structure of EFdA.

Determination of the amount of EFdA and its metabolites within human CD4$^+$ T cells. Human CD4$^+$ CEM and MT-4 cells (10^6 cells/ml) were incubated with 200 nM [^{3}H]EFdA or [^{3}H]AZT for 6 h, thoroughly washed, and further cultured without the addition of EFdA or AZT, followed by the extraction of nucleosides/nucleotides with 60% methanol at various time periods of culture (2, 4, 8, 12, and 24 h). The amounts of intracellular metabolites were determined by HPLC analysis as described above.

In vitro persistence of anti-HIV activity of EFdA. MT-4 cells (10^5 cells/ml) were exposed to a concentration of 0.01, 0.1, or 1 μM EFdA, AZT, or TDF for 24 h, thoroughly washed to deplete extracellular drugs, cultured for various periods of time (0, 2, 6, 12, 24, and 48 h), exposed to HIV-$1_{NL4\text{-}3}$, and further cultured for 5 days. On day 5, the amount of p24 antigen produced in the culture medium was determined to monitor the anti-HIV-1 activity, as described above. The MTT assay was performed, employing PBMC (10^6 cells/ml) and HIV-$1_{Ba\text{-}L}$ under the same conditions as described above, and the activity of the drug to block the cytopathic effect of the virus was evaluated.

An additional assay was performed using CCR5$^+$-MAGI cells (the MAGI assay) under the same conditions as described above, using HIV-$1_{Ba\text{-}L}$ in place of HIV-$1_{NL4\text{-}3}$ and the final culture after 48 h instead of 5 days of culture. The MAGI assay was conducted as previously described (16). Briefly, CCR5$^+$-MAGI cells were plated (10^4 cells/well) and cultured in 96-well, flat-bottomed microculture plates. After 24 h of incubation, the cells were exposed to various concentrations of a test compound and HIV-1 in DMEM containing 15% FCS and were stained at 48 h of culture with chlorophenol red β-D-galactopyranoside. Supernatants were removed, and the cells were lysed with 100 μl of phosphate-buffered saline containing 1% Triton X-100. A solution (100 μl) containing 10 mM chlorophenol red β-D-galactopyranoside, 2 mM $MgCl_2$, and 0.1 M KH_2PO_4 was added to each well, the mixture was incubated at room temperature in the dark for 30 min, and the optical density (wavelength, 570 nm) was measured in a microplate reader (V_{max}; Molecular Devices, Sunnyvale, CA).

Determination of EFdA-TP effects on human DNA polymerases α, β, and γ. Human DNA polymerases α and β were obtained from Terasa Wang at Stanford University and Joann B. Sweasy at Yale University, respectively. Human DNA polymerase γ was purified by Anderson as previously described (2). In the steady-state enzymatic assay, a DNA primer/template of 21 and 36 nucleotides (D_{21}/D_{36}) and activated calf thymus DNA were employed. The sequences of D_{21}/D_{36} were 5′-TCA GGT CCC TGT TCG GGC GCC-3′ (primer) and 3′-CGA AAG TCC AGG GAC AAG CCC GCG GTG ACG ATC TCT-5′ (template), respectively.

In the assay of polymerase activity inhibition, the reaction mixture used was as follows: 50 mM Tris (pH 8.0), 8 mM $MgCl_2$, 60 mM KCl, 10 mM dithiothreitol, 30 μg/ml bovine serum albumin, 250 nM D_{21}/D_{36} or 0.1 mg/ml calf thymus DNA as the DNA primer/template, 0.3 μM dATP (α-^{32}P labeled), 1 unit DNA polymerase α (defined as the amount of polymerase α that incorporated 1 pmol of ^{32}P-labeled dATP into calf thymus DNA at 37°C in 30 min), 100 nM polymerase β or 2.5 nM polymerase γ, and various concentrations of EFdA-TP or ddA-TP as the inhibitor. The reaction was performed at 37°C for 30 min and was stopped by adding 0.5 M EDTA. Subsequently, the reaction mixture was dotted onto DE81 filter paper. After each dotted filter paper was washed three times with 0.5 M sodium phosphate buffer, the paper was subjected to phosphorimaging analysis, and the polymerase activity was determined by quantifying the amount of incorporated dAMP (27).

Determination of K_i values for EFdA-TP inhibition of human polymerase γ. For steady-state inhibition assays, a final concentration of 10 nM human polymerase γ catalytic subunit, 50 nM human polymerase γ accessory subunit, 2.5 mM $MgCl_2$, 1 μM D_{21}/D_{36}, and various concentrations of [α-^{32}P]dATP were used. These conditions were determined to be in the linear phase at steady state

TABLE 1. Anti-HIV-1 activity of EfdA

Drug	EC_{50} value (μM ± SD) for the strains shown (fold change)[a]					CC_{50} value (μM) for:	
	R5-HIV-1_{Ba-L}	R5-HIV-$1_{MDR/MM}$[b]	X4-HIV-1_{NL4-3}	X4-HIV-$1_{MDR/C}$[b]	X4-HIV-$1_{MDR/G}$[b]	MT-4 cells	PHA-PBMC cells
EFdA	0.0004 ± 0.00007 (1)	0.003 ± 0.0005 (8)	0.001 ± 0.0002 (1)	0.004 ± 0.001 (4)	0.021 ± 0.008 (21)	11	12
AZT	0.015 ± 0.004 (1)	0.34 ± 0.008 (22)	0.033 ± 0.007 (1)	0.39 ± 0.06 (12)	0.38 ± 0.09 (12)	>100	>100
TDF	0.032 ± 0.006 (1)	0.082 ± 0.01 (3)	0.0 ± 0.006 (1)	0.047 ± 0.01 (2)	0.087 ± 0.02 (3)	ND[c]	53
APV	0.025 ± 0.005 (1)	0.43 ± 0.03 (17)	0.024 ± 0.004 (1)	0.28 ± 0.02 (12)	0.25 ± 0.03 (10)	>100	>100
SQV	0.008 ± 0.004 (1)	0.22 ± 0.05 (28)	0.01 ± 0.005 (1)	0.037 ± 0.004 (4)	0.026 ± 0.008 (3)	26	78

[a] The EC_{50}s were determined with PHA-PBMC, by the inhibition of p24 Gag protein production by the drug as an end point. For HIV-1_{NL4-3}, MT-4 cells were exposed to the virus, and the EC_{50} values were determined using the MTT assay. All assays were conducted in duplicate or triplicate, and the data shown represent means ± 1 standard deviation (SD) derived from the results of two independent experiments. Numbers in parentheses represent changes in EC_{50}s for each isolate compared to the EC_{50}s for HIV-1_{Ba-L} and HIV-1_{NL4-3}.

[b] Strains HIV-$1_{MDR/C}$, HIV-$1_{MDR/G}$, and HIV-$1_{MDR/MM}$ were isolated from patients who had received antiretroviral therapy for long periods of time and whose virus strains had acquired a number of mutations in the RT- and PR-encoding HIV-1 genes. The amino acid substitutions identified for the RT-encoding regions of HIV-$1_{MDR/MM}$, HIV-$1_{MDR/C}$, and HIV-$1_{MDR/G}$ compared to the consensus type B sequences cited from the Los Alamos database include M41L, D67N, T69D, D123E, M184V, G196R, L210W, L214F and T215Y, M41L, E44D, D67N, T69D, A98S, D123E, D177E, M184V, L210W, R211K, L214F, T215F, K219Q and L228H, and D67G, S68G, T69D, K70R, V118I, E122K, I135T, M184V, Q197K, T125F, D218E, and K219Q, respectively.

[c] ND, not done.

after 2 min. Premixed solutions of dATP, $MgCl_2$, and various concentrations of an inhibitor were mixed with the enzyme and D_{21}/D_{36} to initiate the reaction. After 2 min, each reaction was quenched with a final concentration of 0.3 M EDTA. A constant amount of 5′-radiolabeled D_{36} was added at the end of the reaction to normalize for loading. Products were then analyzed by 20% polyacrylamide gel electrophoresis and analyzed on a Bio-Rad Molecular Imager FX. The amount of product was determined as the fraction of the elongated primer (D_{22}) divided by the radiolabeled template (D_{36}). Michaelis-Menten curves were generated, and the apparent V_{max} and K_m values for substrate in the presence of various concentrations of the inhibitor were determined. Nonlinear regression was then performed, fitting the curves for competitive inhibition to provide the K_i value for EFdA-TP (Table 2). The values of K_m for dATP and K_{cat} were also determined.

RESULTS

Anti-HIV-1 activity and cytotoxicity of EFdA. Certain 4′-ethynyl-2′-deoxynucleoside analogs exert potent activity against a wide spectrum of HIV-1 strains including the multidrug resistant (MDR) HIV-1 variants and the HIV-1 strains as previously described (14). In an attempt to optimize the anti-HIV-1 activity, we generated a number of 4′-ethynyl-containing congeners and identified EFdA as one of the most potent and least toxic nucleoside analogs. As shown in Table 1, in the evaluation of the activity of EFdA against HIV-1 variants, we employed three primary HIV-1 strains isolated from patients for whom existing anti-HIV-1 regimens had failed after they had received from 9 to 11 anti-HIV-1 drugs over 32 to 83 months (15, 28). These MDR strains contained amino acid substitutions in the reverse transcriptase- and protease-encoding regions, which have reportedly been associated with HIV-1 resistance. EFdA blocked the replication of five HIV-1 strains, (X4-HIV-1_{NL4-3}, X4-HIV-$1_{MDR/C}$, X4-HIV-$1_{MDR/G}$, R5-HIV-1_{Ba-L}, and R5-HIV-$1_{MDR/MM}$), with EC_{50} values ranging from 0.0004 to 0.021 μM (Table 1). The EC_{50} value change of EFdA (21-fold) against HIV-$1_{MDR/G}$ is greater than that of AZT (12-fold). However, it should be noted that against all MDR HIV-1 variants examined, the absolute EC_{50} values of EFdA remained the lowest compared to those of four representative FDA-approved antiviral agents (AZT, TDF, APV, and SQV). Although EFdA's 50% cytotoxicity concentration (CC_{50}, the concentration of a compound that reduces the number of cells by 50%) value was relatively low (11 μM for MT-4 cells and 12 μM for PHA-PBMC) compared to that of AZT and APV (both were >100 μM), the selectivity indices of EFdA with X4-HIV-1_{NL4-3} and R5-HIV-1_{Ba-L} were 11,000 and 27,500, respectively, indicating that EFdA had a relatively favorable cytotoxicity profile compared to those of AZT and APV, whose selectivity indices were >3,030 and >3,571 with X4-HIV-1_{NL4-3} and >6,667 and >4,000 with R5-HIV-1_{Ba-L}, respectively.

Intracellular metabolism of EFdA. In order to characterize the profile of intracellular metabolism of EFdA, we tritiated EFdA ([^{3}H]EFdA) and determined the amounts of intracellular metabolites of [^{3}H]EFdA in human CD4$^+$ CEM cells. CEM cells were cultured in the presence of 0.1, 1, and 10 μM [^{3}H]EFdA for 6 h, and intracellular nucleosides/nucleotides were extracted with 60% methanol as previously described (18). The extracted samples containing [^{3}H]EFdA metabolites were subjected to HPLC, and the radioactivity of each eluted sample was determined and plotted as a function of elution time. The identity of each peak of [^{3}H]EFdA metabolite was determined by comparison with the known elution times of unlabeled EFdA-MP, EFdA-DP, and EFdA-TP. The amount of the specific metabolite was determined as the sum of the radioactivity for its peak plus the activity for two flanking 1-min fractions.

First, we examined the uptake of EFdA into CEM cells when cultured in the presence of 0.1 μM [^{3}H]EFdA for 6 h by determining the area under the radioactivity curve, which added up to 1,731 pmol/10^9 cells. In contrast, the uptake level of AZT was greater than that of EFdA by approximately 2.5-fold (4,349 pmol/10^9 cells), when determined with CEM cells exposed to 0.1 μM [^{3}H]AZT.

Among the three distinct peaks, representing EFdA-MP, -DP, and -TP, seen with 0.1 μM EFdA exposure (Fig. 2A), the amount of EFdA-TP was comparable to that of EFdA-MP and greater than that of EFdA-DP. When the cells were exposed to higher concentrations (1 and 10 μM) of [^{3}H]EFdA, the amounts of all three EFdA phosphates increased proportionately (Fig. 2B). This profile of EFdA phosphates contrasted with that of AZT phosphates in cells exposed to 0.1 μM [^{3}H]AZT (Fig. 2C), in which the amount of AZT-MP was

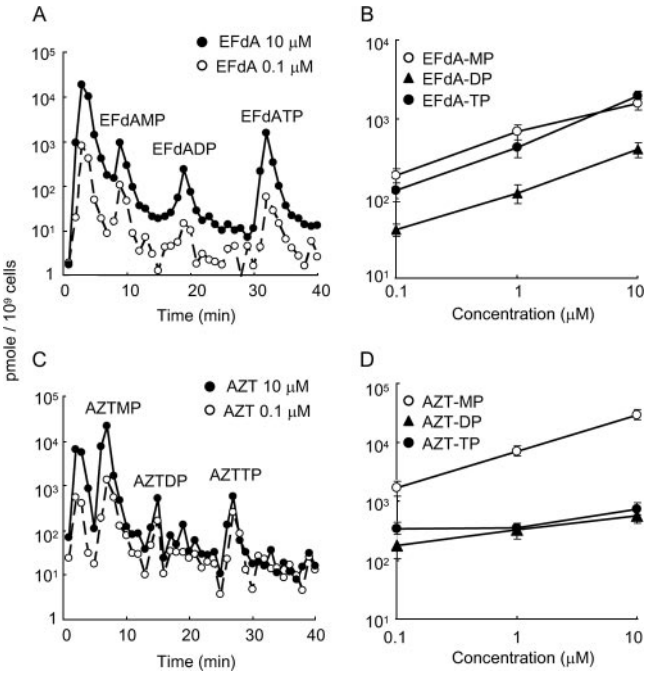

FIG. 2. Cellular uptake and intracellular phosphorylation profiles of EFdA. CEM cells (10^6 cells/ml) were incubated in the presence of various concentrations (0.1, 1, and 10 μM) of [^{3}H]EFdA or [^{3}H]AZT for 6 h and washed, and intracellular nucleosides/nucleotides were extracted with 60% methanol. The extracts were subjected to HPLC. One-minute fractions were collected until 40 min, and the radioactivity of the collected samples was determined with a scintillation counter for EFdA (A) and AZT (C). The radioactivity of the sample showing each peak and its flanking samples' radioactivity (total of 3-min fractions) were summed and plotted for EFdA (B) and AZT (D). For the sake of clarity, the profiles of [^{3}H]EFdA and [^{3}H]AZT metabolites in the cells exposed to 1 μM of each agent have been omitted from panels A and C. Determination of intracellular phosphorylation profiles of EFdA and AZT was conducted on two independent occasions, and comparable profiles were obtained in those two experiments. Shown in panels A and C are representative data. The data shown in panels B and D are geometric means ± 1 standard deviation.

greater than that of AZT-DP or AZT-TP, in agreement with previous observations by Furman et al. (8) and Balzarini et al. (3). When the cells were exposed to 1 and 10 μM [^{3}H]AZT, the amounts of AZT-MP increased disproportionately in comparison with those of AZT-DP and -TP. The increase of EFdA-TP level from 0.1 to 10 μM exposure was 22-fold, while that of the AZT-TP level was only 2.4-fold. The ratio of EFdA-TP to EFdA-MP with 10 μM exposure was 1.5, while that of AZT-TP to AZT-MP was 0.031. The high EFdA-TP/EFdA-MP ratio indicates that EFdA is highly efficiently phosphorylated to produce EFdA-TP and/or that EFdA-TP, once formed, is substantially stable intracellularly. The significant intracellular accumulation of AZT-MP and the small amount of AZT-TP are in agreement with previous observations that AZT-MP is a relatively poor substrate for human thymidylate kinase and that the transition of AZT-MP to AZT-DP is substantially delayed (8).

Intracellular persistence of phosphates of EFdA-TP. We therefore determined the intracellular stability of each species of EFdA phosphate. When CD4$^+$ CEM cells were cultured with 200 nM [^{3}H]EFdA for 6 h and the amount of each of the phosphates was determined at various time points following thorough washing of the cells, the intracellular levels of all forms of EFdA phosphates persisted longer than those of AZT phosphates (Fig. 3A). As determined at 8 h after EFdA removal, intracellular levels of EFdA-MP, -DP, and -TP had remained, with 50%, 45%, and 72%, respectively, of the values at 0 h, while intracellular levels of AZT-MP, -DP, and -TP rapidly decreased to 16%, 26%, and 26%, respectively. As determined at 24 h after drug removal, intracellular levels of AZT-MP, -DP, and -TP were 31, 23, and 38%, respectively, for EFdA and 10, 11, and 9%, respectively, for AZT. Moreover, the data showed that the half-life ($t_{1/2}$) values of EFdA-MP, -DP, and -TP were 8.1, 6.7, and 17.2 h, respectively, while those of AZT-MP, -DP, and -TP were 1.4, 1.6, and 2.8 h, respectively, indicating that the intracellular half-lives of all three EFdA phosphates are significantly longer than those of AZT phosphates. The profiles of EFdA and AZT phosphorylation determined with another human CD4$^+$ MT-4 T-cell line were similar to those obtained with CEM cells (Fig. 3B).

Persistent antiviral activity of EFdA after EFdA removal in culture. Since EFdA-TP was present persistently (at a $t_{1/2}$ of ~17 h) in the cytoplasm of human CD4$^+$ T cells, as described above, we asked whether the antiviral activity of EFdA against HIV-1 persisted after EFdA removal from the culture in comparison to the persistence activities of AZT and TDF. MT-4 cells were cultured in the presence of EFdA, AZT, or TDF for 24 h, thoroughly washed, and exposed to HIV-1_{NL4-3} for 0, 2, 6, 12, 24, and 48 h following EFdA removal, and the magnitude of HIV-1_{NL4-3} replication inhibition was determined using p24 amounts in the culture as a readout. As shown in Fig. 4A, when MT-4 cells were pretreated with 0.01 μM EFdA, representing an EFdA concentration 10-fold greater than its EC_{50} value with MT-4 cells (Table 1), the percentages of protection values in the cells exposed to HIV-1_{NL4-3} at post-24 and -48 h were 75 and 47%, respectively. To comparatively evaluate the persistence of the antiviral activity of EFdA as observed above, we tested the persistence of the activity of TDF, which can be administered once daily in clinical settings, under the same conditions. When MT-4 cells were pretreated with 1 μM TDF, representing a TDF concentration 34-fold greater than its EC_{50} value with MT-4 cells (Table 1), the percentages of protection values at post-24 and -48 h were 90 and 64%, respectively. In contrast, in MT-4 cells pretreated with 1 μM AZT, representing an AZT concentration 33-fold greater than its EC_{50} value (Table 1), the percentages of protection values in the cells exposed to HIV-1_{NL4-3} at post-24 and -48 h were 55 and 9.2%, respectively. When we examined the persistence of antiviral activity of EFdA using another target, PBMC or CCR5$^+$-MAGI cells, and HIV-1_{Ba-L}, the protection by EFdA also appeared to be more persistent than that of AZT (Fig. 4B and C). These results corroborated the longer intracellular persistence of EFdA-TP once formed in human CD4$^+$ T cells, as described above (Fig. 3).

Inhibitory effects of EFdA against human cellular DNA polymerases. The inhibition of human DNA polymerases by nucleoside reverse transcriptase inhibitors is known to be associated with critical adverse effects including lactic acidosis and peripheral neuropathy (6, 12). We therefore asked whether EFdA had inhibitory effects on human DNA poly-

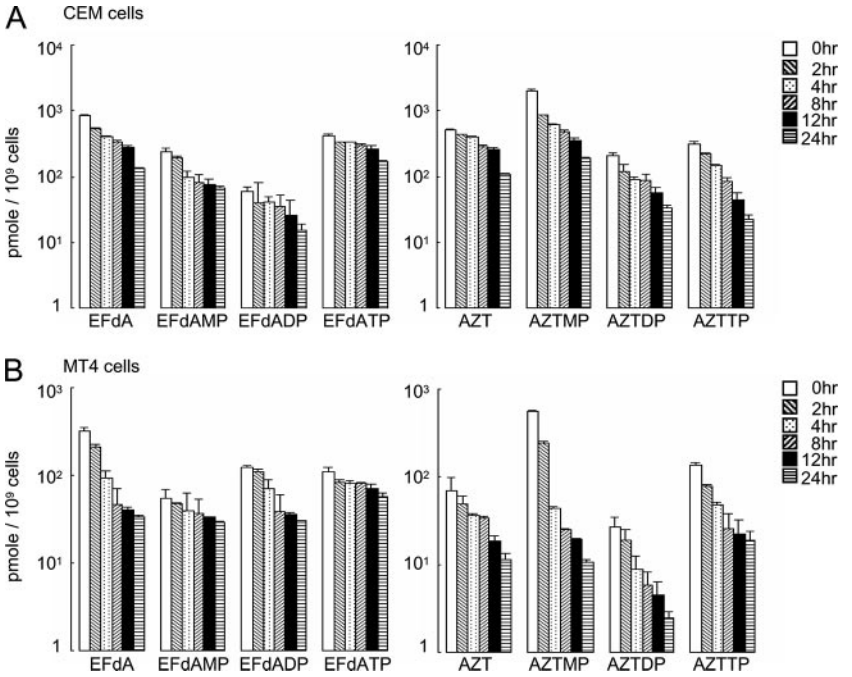

FIG. 3. Intracellular stability of AZT and EFdA metabolites. CEM (A) or MT-4 (B) cells (10^6 cells/ml) were incubated with 0.2 μM of [^{3}H]EFdA or [^{3}H]AZT for 6 h and thoroughly washed to remove the extracellular drug. After further incubation of the cells for 0, 2, 4, 8, 12, and 24 h, cell extracts were prepared and subjected to HPLC analysis. Results are shown as arithmetic means of two or three independently conducted experiments (± 1 standard deviation).

merases α, β, and γ. First we determined the 50% inhibitory concentration (IC_{50}) values of EFdA-TP and ddA-TP (an active metabolite of didanosine that is, like EFdA, an adenosine congener) against human DNA polymerases α, β, and γ by using calf thymus DNA or a DNA oligomer (D_{21}/D_{36}) as the primer/template. Both EFdA-TP and ddA-TP had virtually no inhibition against DNA polymerase α at a concentration of up to 100 μM, as determined with calf thymus DNA (Table 2). EFdA-TP exerted virtually no inhibition against polymerase β, as determined with D_{21}/D_{36} and calf thymus DNA (IC_{50} values were both >100 μM), while ddA-TP had a substantial inhibitory effect, with IC_{50} values of 3 and 0.2 μM, respectively. EFdA-TP also had virtually no inhibition against polymerase γ with D_{21}/D_{36} (IC_{50}, >100 μM); however, it was moderately inhibitory against polymerase γ, with an IC_{50} value of 10 μM with calf thymus DNA. In contrast, ddA-TP had substantial inhibitory effects on polymerase γ, using D_{21}/D_{36} or calf thymus DNA, with IC_{50} values of 2 and 0.2 μM, respectively. The K_i values of EFdA-TP and ddA-TP against DNA polymerase γ, as determined with D_{21}/D_{36}, were 24.4 ± 7.9 and 4.6 ± 1.7 μM, respectively. These data showed that EFdA-TP had significantly less inhibitory effects on human DNA polymerases than the TP form of the FDA-approved anti-HIV-1 drug, didanosine.

DISCUSSION

In the present study we demonstrated that EFdA exerts potent activity against a wide range of HIV-1 strains including laboratory and primary strains and highly multidrug-resistant variants, with reasonably low cytotoxicity, as tested in test tubes. In terms of the mechanism of antiviral activity of EFdA, previous reports of 4′-substituted-2′-deoxynucleosides, such as 4′-AZT, have shown that following intracellular anabolism to the 5′ triphosphate, HIV-1 reverse transcriptase (RT) efficiently incorporated the nucleotide, which prevented further chain elongation of the viral DNA (4, 5). Although the rate of incorporation for the 5′-triphosphate of 4′-AZT was quite low, HIV-1 RT was able to incorporate two consecutive molecules efficiently. The subsequent distortion of the growing primer brought about by this incorporation seems to prevent further DNA chain elongation, thus causing delayed chain termination (4, 5). Thus, the salient feature of RT inhibition by 4′-substituted-2′-deoxynucleosides, including EFdA, could be that they cause delayed chain termination, which occurs beyond the polymerase-active site. Indeed, we have recently solved the crystal structure of HIV-1 RT in complex with double-stranded DNA with EFdA-TP (A. Sawani et al., presented at the Retroviruses Conference, Cold Spring Harbor, NY, 22 to 27 May 2007). We found that HIV-1 RT can incorporate EFdA monophosphate at the 3′ end of DNA primers against thymidine. The incorporated EFdA-MP acts as a chain terminator at the point of incorporation, suggesting that RT-catalyzed extension from EFdA-MP primer termini is difficult despite the availability of a free 3′-OH at the inhibitor-terminated primer end. Structural analysis provided insights into unfavorable interactions between the 4′-ethynyl group of the inhibitor-terminated primer and RT residues that may cause inhibition of polymerization.

Considering that the complex antiviral regimens of HAART constitute the major causes of treatment failure and that recent results from multiple clinical trials have shown that a once-

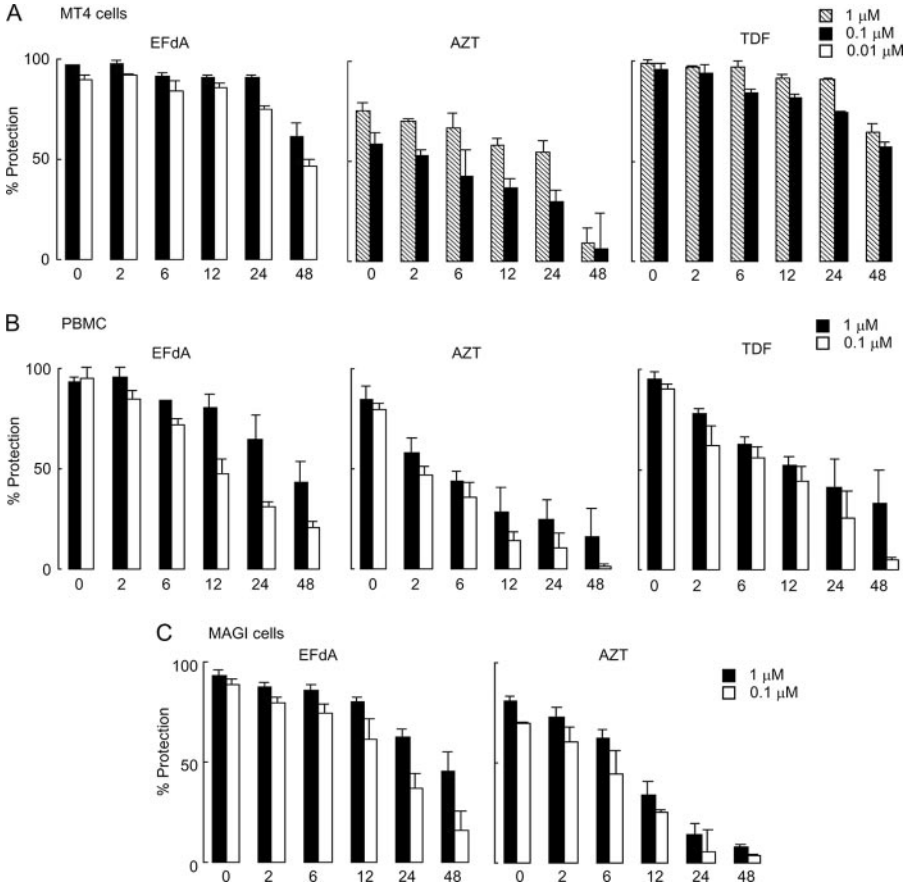

FIG. 4. Persistence of anti-HIV-1 activity after removal of EFdA, AZT, and TDF from culture media. MT-4 (A), PBMC (B), or CCR5$^+$-MAGI cells (C) were exposed to 0.01, 0.1, or 1 μM EFdA (or AZT) for 24 h, thoroughly washed to deplete extracellular drugs, cultured for various periods of time (0, 2, 6, 12, 24, and 48 h), exposed to HIV-1, and further cultured for an additional 5 days with MT-4 cells and PBMC or for an additional 48 h with CCR5$^+$-MAGI cells. Anti-HIV-1 activity was monitored using p24 production or with an MTT assay or a MAGI assay.

daily or twice-daily regimen has produced an improved prognosis (11, 13, 26), we examined whether the pharmacodynamics of EFdA potentially supported a once- or twice-a-day regimen by determining the profiles of anabolic phosphorylation of EFdA in human CD4$^+$ T cells. With regard to the use of these CD4$^+$ human T-cell lines, the intracellular metabolism of certain nucleosides is known to be considerably affected by the status of cells, depending upon proliferation rates, activation states, donors, and other factors (9, 10). It should be noted that the EC_{50} value of EFdA against HIV-$1_{Ba\text{-}L}$ determined with PBMC was 0.0004 μM, while that against HIV-$1_{NL4\text{-}3}$ determined with MT-4 cells was 0.001 μM. The difference between the values was only a factor of ~3. Thus, we assumed that the phosphorylation pattern and the ratios of EFdA-TP over its possible competitive counterpart, dATP, should be comparable, and we employed two human CD4$^+$ T-cell lines, CEM and MT-4 cells. The present data from these cell lines showed that EFdA efficiently underwent cellular uptake into the cytoplasm and was readily phosphorylated to EFdA-MP, -DP, and -TP (Fig. 2A and B). However, all EFdA phosphates persisted significantly longer than AZT phosphates. Indeed, in both CEM and MT-4 cells exposed to AZT,

TABLE 2. Inhibitory effects of EFdA against DNA polymerase α, β, and γ[a]

Primer/template	IC_{50} value (μM ± SD)					K_i value (μM ± SD) of polymerase γ (D_{21}/D_{36})
	Polymerase α (calf thymus DNA)	Polymerase β		Polymerase γ		
		D_{21}/D_{36}	Calf thymus DNA	D_{21}/D_{36}	Calf thymus DNA	
EFdA-TP	>100	>100	>100	>100	10 ± 2	24.4 ± 7.9
ddA-TP	>100	3 ± 0.3	0.2 ± 0.07	2 ± 0.3	0.2 ± 0.02	4.6 ± 1.7

[a] In steady-state kinetic assays, DNA primer/template of 21 and 36 nucleotides (D_{21}/D_{36}) or activated calf thymus DNA was employed. The IC_{50} values were determined in the presence of 0.3 mM dATP. The IC_{50} values and K_i values represent means ± standard deviations (SD) from two independent experiments. Values of K_m for dATP and K_{cat} were 0.55 ± 0.13 and 0.4 ± 0.03, respectively.

not only the intracellular levels of AZT-DP and AZT-TP but also that of the accumulated AZT-MP rapidly declined in comparison to EFdA phosphates (Fig. 3A and B). These data suggest that AZT phosphates are more vulnerable to intracellular catalysis than EFdA phosphates. The data also suggest that both AZT-MP and -DP get catalyzed without undergoing further phosphorylation. Indeed, the intracellular $t_{1/2}$ of EFdA-TP, an active metabolite of EFdA, was much greater, at 17.2 h, than that of AZT-TP (at 2.8 h) (Fig. 3). It is noteworthy that the intracellular $t_{1/2}$ of the triphosphate forms of d4T, ddC, 3TC, ddI, ABC, and TDF (PMPApp) were reportedly 3.5, 2.6, 10.5 to 15.5, 25 to 40, 3.3, and 15.4 h, respectively (22). Compared with the half-lives of these FDA-approved drugs, EFdA-TP's intracellular half-life (17.2 h) was relatively long, and these results suggest its favorable intracellular pharmacokinetics. We therefore asked whether the longer intracellular persistence of EFdA-TP resulted in more persistent anti-HIV-1 activity of EFdA as EFdA was removed from the culture medium. It was noted that when MT-4 cells were incubated with EFdA (0.1 μM) for 24 h, thoroughly washed to remove EFdA from the culture medium, cultured for various periods of time without adding EFdA, exposed to HIV-1, and further cultured for 5 days, substantial levels of antiviral activity (at post-24- and -48 h, protection values were 91 and 61%, respectively) were seen. The post-24 and -48 h protection values of TDF (0.1 μM), an FDA-approved once-daily anti-HIV-1 drug, were 74 and 57%, respectively (Fig. 4A). In contrast, substantially lower levels of antiviral activity were observed for AZT than for EFdA. When the cells were preincubated with 0.1 μM AZT, only 6% protection was seen with MT-4 cells (Fig. 4). This relatively poor protective activity of AZT should stem from the relatively short $t_{1/2}$ of AZT-TP (8). Thus, the present data that indicate EFdA-TP has a substantially long $t_{1/2}$ of 17 h, in addition to the observed in vitro persistence of antiviral activity, suggest that a once-daily or twice-daily regimen of EFdA is possible.

In regard to the in vitro selection of HIV-1 variants resistant to EFdA, we previously reported that the 3TC resistance-conferring M184V substitution in reverse transcriptase is the major substitution that reduces anti-HIV-1 activity of 4′-ethynyl analogs, although the EC_{50} value change with the M184V substitution was only approximately sixfold (14). In the present work, when we examined MDR HIV-1 variants containing a number of mutations including M184V, the level of resistance was similarly moderate, with changes in their EC_{50} values ranging from 4- to 21-fold (Table 1). However, against such MDR HIV-1 variants, the absolute EC_{50} values remained lowest for EFdA compared to those of four representative FDA-approved antiviral agents (AZT, TDF, APV, and SQV) (Table 1). Thus, it is possible that the "genetic barrier" to HIV-1 acquisition of EFdA resistance can be substantially higher than at least the agents examined in the present study.

It was noted that when cells were exposed to high concentrations (1 and 10 μM) of [^{3}H]EFdA, the amounts of EFdA phosphates increased proportionately (Fig. 2B). This profile of EFdA phosphates contrasted with those of AZT phosphates, which showed that levels of AZT-TP increased only slightly when the cells were exposed to higher concentrations of AZT (Fig. 2D). This phosphorylation profile of AZT stems from the fact that thymidylate kinase has a good affinity for AZT-MP (K_m of ~8 μM), comparable to that of dT-MP (K_m of ~4 μM), while AZT-MP has an extremely low V_{max} value (only 0.3% relative to the V_{max} of dT-MP) (8), resulting in the accumulation of AZT-MP and low levels of AZT-TP. These data suggest that the intracellular anabolic phosphorylation of EFdA to EFdA-TP is substantially efficient, which explains the reason that EFdA exerts such a potent and persistent anti-HIV-1 activity.

As noted above, EFdA was efficiently converted to its active form, EFdA-TP, whose intracellular $t_{1/2}$ was substantially longer (as long as ~17 h) than that of AZT-TP (Fig. 3). However, there was a concern that the long intracellular persistence of EFdA-TP might cause cellular DNA damages, particularly since EFdA retains a 3′-OH group, which may get incorporated into the growing cellular DNA chain, resulting in human DNA chain termination. All the currently available nucleoside reverse transcriptase inhibitors (NRTI) are not devoid of adverse effects such as lactic acidosis and peripheral neuropathy, which are thought to be associated with the interactions of NRTI-TP and cellular DNA polymerases. Therefore, we examined the effects of EFdA-TP on DNA polymerases α, β, and γ, using ddA-TP, the active form of ddI, as a control. EFdA-TP had virtually no significant inhibition against DNA polymerases α and β, although it had moderate inhibitory effects against DNA polymerase γ, with an IC_{50} value of 10 μM when calf thymus DNA was used as a template/primer. The K_i value of EFdA-TP, determined using D_{21}/D_{36} as the template/primer, was 24.4 μM, while that of ddA-TP was 4.6 μM. The anti-HIV-1 drug ddI is known to cause damages in DNA polymerase γ-mediated mitochondrial DNA synthesis, and one can be concerned about the possibility that EFdA may also cause mitochondrial DNA damages since the K_i value (24.4 μM) of EFdA-TP with DNA polymerase γ was only 5.3-fold less than that of ddA-TP (4.6 μM). However, EFdA is much more potent, with an EC_{50} value (~0.0004 μM with PHA-PBMC exposed to HIV-1_{Ba-L} [Table 1]) higher than that of ddI (EC_{50}, ~1.5 μM in PHA-PBMC exposed to HIV-1_{Ba-L}) (28), and indeed, the ratio of the K_i value to the IC_{50} value for EFdA is as great as 61,000. Thus, EFdA could produce more potent antiviral effects with fewer adverse effects when used as a therapeutic agent for HIV-1 infection and AIDS, although it is important that the antiviral effects and safety of experimental agents be determined only through rigorously controlled preclinical and clinical trials.

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