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 [3H]EFdA or [3H]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 [3H]EFdA, the amount of EFdA-TP increased by 22-fold (2,090 pmol/10^9 cells), while the amount of [3H]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-1NL4-3, 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 reconstitution 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...
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'-deoxycytidine (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. [3H]-AZT (specific radioactivity, 5 Ci/mmol) and [8-3H]-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) and human CD4 (-MAGI) (multinuclear activation of a galactosidase indicator) (12) 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 zeocin. 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-1Ba-L, HIV-1NL4-3, and three multidrug-resistant primary HIV-1 isolates to various drugs were determined by comparison with the level of p24 production in drug-free cultures. 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 (106 cells/ml) and HIV-1d21/D36 under the same conditions as described above, and the activity of the drug to block the cytopathic effect of the virus was evaluated.

In vitro persistence of anti-HIV activity of EFdA. MT-4 cells (106 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-1NL4-3, 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 (106 cells/ml) and HIV-1d21/D36, 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-1d21/D36 in place of HIV-1NL4-3, 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 (106 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 MgCl2, and 0.1 mM KH2PO4 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 (Multiskan 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. Sweany 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 (D21/D36) and activated calf thymus DNA were employed. The sequences of D21/D36 were 5'-TCA GGT CCC TGT TCG GGC GCC-3‘ and 3’-CCA AAT AGG GAC AGG CCC GCG GTG ACG 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 MgCl2, 60 mM KCl, 10 mM dithiothreitol, 30 μg/ml bovine serum albumin, 250 mM D21/D36 or 0.1 mg/ml calf thymus DNA as the DNA primer/template, 0.3 μM dATP (α-32P labeled), 1 unit DNA polymerase α (defined as the amount of polymerase α that incorporated 1 pmol of [32P]-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 Ki 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 MgCl2, 1 μM D21/D36, and various concentrations of [α-32P]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       | EC50 value (µM ± SD) for the strains shown (fold change) | CC50 value (µM) for: |
|------------|---------------------------------------------------------|----------------------|
|            | R5-HIV-1_{Ba-L} | R5-HIV-1_{MDR/MM} | X4-HIV-1_{NL4-3} | X4-HIV-1_{MDR/C} | X4-HIV-1_{MDR/G} | 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) | 0.034 ± 0.008 (22) | 0.003 ± 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* | 53 |
| APV        | 0.025 ± 0.005 (1) | 0.43 ± 0.13 (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 EC50s were determined with PHA-PBMC, by the inhibition of p24 Gag protein production by the drug as an end point. For HIV-1_{Ba-L}, MT-4 cells were exposed to the virus, and the EC50 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 EC50 for each isolate compared to the EC50 for HIV-1_{Ba-L} and HIV-1_{MDR}.

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, D124N, T148I, L159F, Y163F, and K173R.

c ND, not done.

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 EC50 values ranging from 0.0004 to 0.021 µM (Table 1). The EC50 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 EC50 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 (CC50) 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 >5,030 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 titrated EFdA ([3H]EFdA) and determined the amounts of intracellular metabolites of [3H]EFdA in human CD4+ CEM cells. CEM cells were cultured in the presence of 0.1, 1, and 10 µM [3H]EFdA for 6 h, and intracellular nucleosides/nucleotides were extracted with 60% methanol as previously described (18). The extracted samples containing [3H]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 [3H]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 [3H]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 [3H]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 EFdA-DP, which contains an ethynyl group (16). The ethynyl group may be responsible for the increased affinity of EFdA to its target as compared to that of AZT and APV, which contain an acetylene group (16).
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 [3H]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 1.5, while that of AZT-TP level was only 2.4-fold. The ratio of EFdA-TP to AZT-TP was 0.031. The high EFdA-TP/EFdA-MP ratio 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-
merases α, β, and γ. First we determined the 50% inhibitory concentration (IC50) 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 (D21/D36) 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 D21/D36 and calf thymus DNA (IC50 values were both >100 μM), while ddA-TP had a substantial inhibitory effect, with IC50 values of 3 and 0.2 μM, respectively. EFdA-TP also had virtually no inhibition against polymerase γ with D21/D36 (IC50 >100 μM); however, it was moderately inhibitory against polymerase γ, with an IC50 value of 10 μM with calf thymus DNA. In contrast, ddA-TP had substantial inhibitory effects on polymerase γ, using D21/D36 or calf thymus DNA, with IC50 values of 2 and 0.2 μM, respectively. The Ki values of EFdA-TP and ddA-TP against DNA polymerase γ, as determined with D21/D36 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 terminus 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-
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 EC50 value of EFdA against HIV-1Ba-L determined with PBMC was 0.0004 μM, while that against HIV-1NL4-3 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,

![Graph](image)

**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.

| Primer/template | IC<sub>50</sub> value (μM ± SD) | K<sub>i</sub> value (μM ± SD) of polymerase γ<sup>a</sup> (D<sub>21</sub>/D<sub>36</sub>)<sup>b</sup> |
|-----------------|---------------------------------|---------------------------------|
| EFdA-TP         | >100                            |                                 |
| ddA-TP          | >100                            |                                 |
|                 | D<sub>21</sub>/D<sub>36</sub>      | Calf thymus DNA                 |
|                 | 3 ± 0.3                          | 0.2 ± 0.07                      |
|                 | D<sub>21</sub>/D<sub>36</sub>      | Calf thymus DNA                 |
|                 | >100                            | 10 ± 2                          |
|                 | 2 ± 0.3                          | 0.2 ± 0.02                      |
|                 |                                 | 4.6 ± 1.7                       |

<sup>a</sup> In steady-state kinetic assays, DNA primer/template of 21 and 36 nucleotides (D<sub>21</sub>/D<sub>36</sub>) or activated calf thymus DNA was employed. The IC<sub>50</sub> values were determined in the presence of 0.3 mM dATP. The IC<sub>50</sub> values and K<sub>i</sub> values represent means ± standard deviations (SD) from two independent experiments. Values of K<sub>i</sub> for dATP and K<sub>i</sub> for EFdA were 0.55 ± 0.13 and 0.4 ± 0.03, respectively.

![Graph](image)

**TABLE 2.** Inhibitory effects of EFdA against DNA polymerase α, β, and γ<sup>a</sup>
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 EC50 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 EC50 values ranging from 4- to 21-fold (Table 1). However, against such MDR HIV-1 variants, the absolute EC50 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 [3H]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 dTMP ($K_{m}$ of ~4 μM), while AZT-MP has an extremely low $V_{\text{max}}$ value (only 0.3% relative to the $V_{\text{max}}$ of dTMP) (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 IC50 value of 10 μM when calf thymus DNA was used as a template/primer. The $K_{i}$ value of EFdA-TP, determined using D2/D36 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 EC50 value (~0.0004 μM with PHA-PBMC exposed to HIV-1Ba.L [Table 1]) higher than that of ddI (EC50 ~1.5 μM in PHA-PBMC exposed to HIV-1Ba.L) (28), and indeed, the ratio of the $K_{i}$ value to the IC50 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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