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Intracellular metabolism of 2'-deoxy-4'-*C*-ethynyl-2-fluoroadenosine, a novel 4'-*C*-ethynyl nucleoside analog potent against multidrug-resistant HIV-1 variants

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Background

We previously reported that a series of 4'-*C*-ethynyl nucleoside analogs were active against a wide spectrum of HIV isolates including a variety of laboratory and clinical drug-resistant HIV strains *in vitro*. Among such analogs, 2'-deoxy-4'-*C*-ethynyl-2-fluoroadenosine (E2FdA), fluorine-substituted at 2-position of adenine, represents one of the most potent agents against HIV with IC_{50} values of ~1 nM. To further evaluate the characteristics of E2FdA, we examined the intracellular metabolism of E2FdA and its effects on the activity of human polymerases.

Methods

Intracellular metabolism of E2FdA

CEM cells were seeded at 10^6 cells/ml, incubated with 200 nM ^{3}H-E2FdA (specific radio activity : 5 Ci/mmol) or ^{3}H-3'-azido-2', 3'-dideoxythymidine (AZT) (specific radio activity : 12 Ci/mmol) for 6 h, and thoroughly washed to remove extracellular drugs. After further incubation, nucleosides/nucleotides within the cells were extracted with 60% methanol at various time points (2, 4, 8, 12, 24 h), and subjected to HPLC on an ion exchange Partisil 10-SAX column. Then one-minute fractions were collected and radioactivity was measured using liquid scintillation counter to determine the amounts of metabolites. In HPLC analysis, two elution buffers were used. Buffer A : 0.03 M ammonium phosphate, Buffer B : 9 parts 0.7 M ammonium phosphate and 1 part 100% ethanol.

Persistence of anti-HIV activity of E2FdA

MT-4 or MAGI-CCR5 cells were exposed to 0.1 or 1 μM E2FdA (or AZT) for 24 h, thoroughly washed to deplete extracellular drugs, cultured for various periods of time (0, 2, 6, 12, 24 and 24 h), then exposed to HIV, and further cultured for 5 days in MT-4 cells or 48 h in MAGI-CCR5 cells. Anti-HIV activity was monitored using p24 production or in MAGI assay.

Inhibition assay against DNA polymerases β and γ

In the inhibition assay, the following reaction mixture was used: 50 mM Tris, pH 8.0, 8 mM $MgCl_2$, 60 mM KCl, 10 mM DTT, 30 μg/ml BSA, 250 nM DNA 21/36mer or 0.1 mg/ml calf thymus DNA as DNA primer/template, 0.3 μM dATP (α-^{32}P labeled), 2.5 nM pol γ or 100 nM pol β, and various concentrations of ddATP or E2FdATP as inhibitor. The reaction was performed at 37°C for 30 min, and stopped by adding 0.5M EDTA. Then the reaction mixture was dotted onto DE81 filter paper. After washed with 0.5M sodium phosphate buffer for 3 times, the filter paper was exposed against phosphor imaging screen. The polymerase activity was measured by quantifying the amount of incorporated dAMP.

Results

Amounts of intracellular E2FdA-monophosphates (MP), -diphosphates (DP), and -triphosphates (TP) increased proportionately with increased E2FdA concentrations. When exposed to AZT, only AZTMP levels markedly increased compared to AZTDP and AZTTP. Intracellular $T_{1/2}$ of E2FdATP, an active form of E2FdA, was ~18 h in CEM cells, which was significantly greater than $T_{1/2}$ of AZTTP (~3 h). Similar results were obtained in MT-4 and MAGI cells. E2FdA (0.1 μM) protected ~50% of MT-4 and MAGI cells against the infection of HIV added 24 h after E2FdA removal from cultures. The IC_{50} value of E2FdATP to inhibit dATP (0.3 μM) incorporation mediated by DNA polymerase γ was 10 μM, which was significantly higher than that of ddATP (IC_{50} value: 0.2 μM)

Figure 1 Structure of 2'-deoxy-4'-*C*-ethynyl-2-fluoroadenosine (E2FdA)

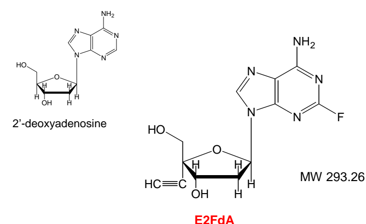
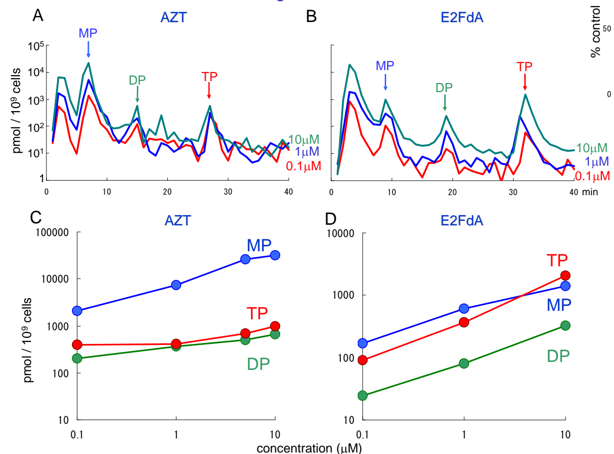


Table 1 Anti HIV activity of E2FdA

Compound	IC_{50} (μM) HIV$_{Ba-L}$ (R5)	HIV$_{NL4-3}$ (X4)	HIV$_{MDR(C)}$ (X4)	HIV$_{MDR(G)}$ (X4)	HIV$_{MDR(MM)}$ (R5)
E2FdA	0.0004	0.001	0.004	0.021	0.003
AZT	0.015	0.033	0.391	0.383	0.034
TFV	0.042	0.029	0.047	0.087	0.082
APV	0.025	0.014	0.282	0.248	0.431
SQV	0.008	0.003	0.037	0.026	0.221

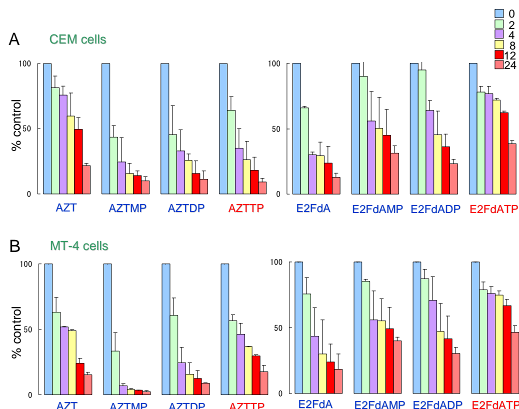
The IC_{50} values were determined using PHA-PBMC and the inhibition of p24 Gag protein production was used as an endpoint. All assays were conducted in triplicate. HIV$_{MDR(C)}$, HIV$_{MDR(G)}$ and HIV$_{MDR(MM)}$ were isolated from patients who received antiretroviral therapy for a long period of time and whose virus isolates showed a number of RT and PR mutations.

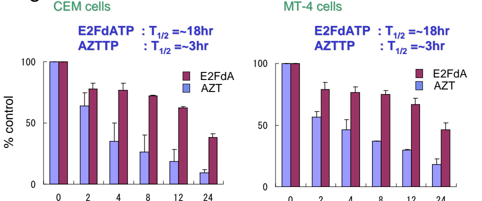
Figure 2 Changes in intracellular metabolites of AZT and E2FdA at different drug concentrations



CEM cells were seeded at 10^6 cells/ml, incubated with different concentrations (0.1, 1, 10 μM) of ^{3}H-E2FdA or ^{3}H-AZT for 6 h, washed, and nucleosides/nucleotides within the cells were extracted with 60% methanol. Subsequently the supernatants were subjected to HPLC. Panels (A) and (B) : One-minute fractions were collected until 40 min and radioactivity was determined for (A) AZT and (B) E2FdA. Panels (C) and (D) : Each value shown represents the sum of the activity for the peak fraction for MP, DP, or TP plus the activity for its flanking one-minute fraction (*i.e.*, the total of 3 one-minute fractions). Panels (C) and (D) are for AZT and E2FdA, respectively. In the formation of AZT metabolites, only AZTMP level increased with time roughly in proportion to higher input concentrations, however, in E2FdA, intracellular levels of all forms of phosphorylated metabolites (E2FdA-MP, -DP and -TP) increased.

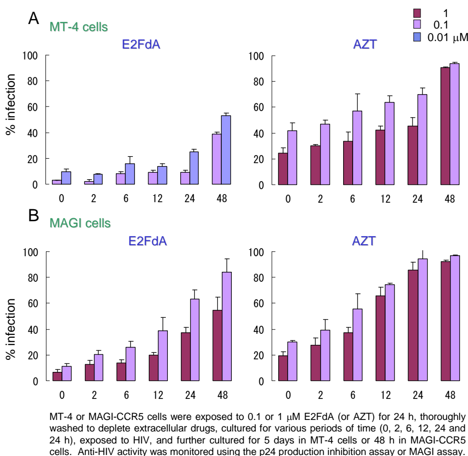
Figure 3 Intracellular retention of AZT and E2FdA metabolites





(A) CEM cells were seeded at 10^6 cells/ml, incubated with 0.2 μM of ^{3}H-E2FdA or ^{3}H-3-AZT for 5 h and washed to remove the extracellular drugs. After further incubation of the cells for 0, 2, 4, 8, 12 and 24 h, cell extracts were prepared and the amount of the radiolabeled metabolites were determined by HPLC analysis. The results shown denote arithmetic means of two or three independently conducted experiments. (B) The assays were similarly performed in MT-4 cells. (C) Decay of intracellular levels of AZTTP and E2FdATP in CEM and MT-4 cells.

Figure 4 Persistence of anti-HIV activity after removal of E2FdA and AZT from culture medium



MT-4 or MAGI-CCR5 cells were exposed to 0.1 or 1 μM E2FdA (or AZT) for 24 h, thoroughly washed to deplete extracellular drugs, cultured for various periods of time (0, 2, 6, 12, 24 and 24 h), exposed to HIV, and further cultured for 5 days in MT-4 cells or 48 h in MAGI-CCR5 cells. Anti-HIV activity was monitored using the p24 production inhibition assay or MAGI assay.

Table 2 Inhibitory effects of E2FdA against DNA polymerases β and γ

	pol β D21/36 mer	pol β Calf Thymus DNA	pol γ D21/36 mer	pol γ Calf Thymus DNA
E2FdATP	>100	>100	>100	10
ddATP	3	0.2	2	0.2

IC_{50} values of E2FdATP and ddATP to inhibit dATP (0.3 μM) incorporation by human DNA polymerases β and γ were determined using two different DNA primer / templates, DNA21/36mer and activated calf thymus DNA.

Conclusions

E2FdA possesses long intracellular $T_{1/2}$ of its TP form (~18 h) and exerts minimal inhibition to DNA polymerase γ. The present data suggest that once daily dosing schedule of E2FdA could be possible with few side effects and warrant that E2FdA be further developed as a potential therapeutic for HIV infection.