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Potent activity of a Nucleoside Reverse Transcriptase Inhibitor, 4’-Ethynyl-2'-Fluoro-2'-Deoxyadenosine, against HIV-1 infection in Hu-PBMC-NOD/SCID/JAK3null (NOJ) mouse model.

Running title: Protection of CD4+ cells by EFdA in vivo

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Abstract

4'-Ethynyl-2-fluoro-2'-deoxyadenosine (EFdA), a recently discovered nucleoside reverse transcriptase inhibitor (NRTI), exhibits a wide spectrum of wild-type and multi-drug-resistant clinical HIV-1 isolates (EC₅₀: 0.0001–0.001 μM). In the present study, we used human peripheral blood mononuclear cell (hu-PBMC)-transplanted human immunodeficiency virus type 1 (HIV)-infected, NOD/SCID, janus kinase-3-knock-out (NOJ) mice for in vivo evaluation of the anti-HIV activity of EFdA. Administration of EFdA decreased the replication and cytopathic effects of HIV-1 without identifiable adverse effects. In PBS-treated mice, the CD4⁺/CD8⁺ cell ratio in the spleen was low (median: 0.04, range 0.02–0.49), while that in mice receiving EFdA was increased (median: 0.65, range 0.57–1.43). EFdA treatment significantly suppressed the number of HIV-1 RNA (median: 9.0×10² copies/ml, range: 8.1×10²–1.1×10³; versus 9.9×10⁴ copies/ml, range: 8.1×10²–1.1×10³; P<0.001) and p24 level in plasma (2.5×10³ range: 8.2×10²–5.6×10³ pg/ml, versus 2.8×10² range: 8.2×10¹–6.3×10² pg/ml; p<0.001) and p24⁺ cells in the spleen (1.90 %, range: 0.33–3.68, versus median: 0.11 %, range: 0.00–1.00, p=0.003) in comparison with PBS-treated mice. These data suggest that EFdA is a promising candidate for a new age of HIV-1 chemotherapy and should be further developed as a potential therapy for individuals with multidrug-resistant HIV-1 variants.
Key Words: HIV-1, mouse model, nucleoside reverse transcriptase inhibitors (NRTI), 4’-ethynyl-2-fluoro-2’-deoxyadenosine (EFdA)
**Introduction**

Highly active antiretroviral therapy (HAART), combining two or more reverse transcriptase inhibitors and/or proteinase inhibitors, has been successful in reducing in the morbidity and mortality caused by human immunodeficiency virus type 1 (HIV-1) infection (6, 27). The limitations of antiviral therapy for AIDS are exacerbated by the development of drug-resistant HIV-1 variants, the existence of viral reservoirs (4, 5), and a number of inherent adverse effects (1, 31). Nucleoside reverse transcriptase inhibitors (NRTI), including zidovudine (AZT), didanosine (ddI), lamivudine (3TC), and stavudine (d4T), constitute the most important class of anti-retroviral compounds for the treatment of HIV-1 infection (9, 17). However, the application of these compounds is clinically limited due to their cytotoxicity through inhibition of the host DNA polymerase and the rapid emergence of drug-resistant viral strains (2, 16). Therefore, developing new compounds with reduced cytotoxicity and improved antiviral potency, especially against drug-resistant viral strains, has become an urgent therapeutic objective. Recently, a new antiviral agent, 4’-ethynyl-2-fluoro-2’-deoxyadenosine (EFdA), was created (21, 23, 24). EFdA shows potent antiviral activity (EC50 subscript=0.004μM) and good activity against NRTI-resistant strains (10). Interestingly, EFdA-triphosphate (active form of EFdA) showed higher intracellular stability (21) and generated a more persistent antiviral effect than other NRTI. In addition, EFdA is effective against human polymerases α, β, and γ, suggesting that
EFdA might serve as a suitable therapy for treating individuals with HIV-1 infection and AIDS (21).

Severe immunodeficient mice transplanted with human peripheral blood mononuclear cells (hu-PBMC-SCID) represent a useful model for AIDS research, including preclinical evaluation of antiretroviral agents and vaccine development. Although the initial SCID mouse model required many PBMC for engraftment and showed inconsistent efficacy (20), the recently introduced NK cell deficient mice show markedly improved engraftment efficiency. Here, we established human PBMC-transplanted HIV-1JR-FL-infected, nonobese diabetic (NOD)-SCID, janus kinase-3 (Jak-3)-knock-out (NOJ) mice, in which massive and systemic HIV-1 infection occurs, human CD4+/CD8+ cell ratios significantly decrease, and high levels of HIV-1 viremia are achieved. Using these mice, a novel anti-HIV-1 agent, EFdA, an NRTI, exerted potent anti-HIV-1 activity. Thus, our refined hu-PBMC-SCID mouse model is a powerful tool to evaluate antiretroviral activity and the adverse effects of new anti-HIV-1 agents.
Materials and Methods

**Antiviral agents.** 4′-Ethynyl-2-fluoro-2'-deoxyadenosine (EFdA) was synthesized as published elsewhere (21, 23, 24).

**Pharmacokinetic analysis of EFdA in Balb/c mice.** Pharmacokinetic analysis of EFdA in Balb/c mice was performed as previously described (22). In brief, plasma samples were collected periodically over 4 h, following a single EFdA administration at a dose of 20 mg/kg body weight dissolved in 250 µl phosphate-buffered saline (PBS). Each plasma sample (50 µl) was centrifuged at 10,000 rpm for 10 min, and the supernatant was injected into a high-performance liquid chromatography (HPLC) system. The eluent was monitored at 262 nm UV, and the EFdA concentration in plasma was determined.

To examine the adverse effects of high-dose EFdA treatment, EFdA was administered to Balb/c mice twice a day intraperitoneally at a dose of 5–50 mg/kg for 14 days, and we observed their status and body weight twice a week.

**Transplantation of human PBMC in NOJ mice.** NOD/SCID/JAK3null (NOJ) mice were established and maintained in the Center for Animal Resources and Development, Kumamoto University (Kumamoto, Japan)(26). The mice were 16 to 20 weeks old at the time of transferring human PBMC. Human PBMC-transplanted NOJ (hu-PBMC-NOJ) mice were generated by previously described methods (22). Briefly, NOJ mice were irradiated (1.8 Gy), and PBMC (1 × 10⁷) were freshly prepared from heparinized blood of a
single healthy HIV-1 seronegative donor by Ficol-Hypaque density gradient centrifugation, resuspended in PBS (0.1 ml), and infused intraperitoneally into each mouse. Peripheral blood was collected from healthy volunteers after obtaining their informed consent, according to the institutional guidelines approved by The Faculty of Medical and Pharmaceutical Sciences, Kumamoto University. All animal experiments were performed according to the guidelines of Kumamoto University, Graduate School of Medical Science.

**Treatment of HIV-1-infected hu-PBMC-NOJ mice with EFdA.** Five days after PBMC implantation, HIV-1JR-FL (25,000 50% tissue culture infectious doses) was intraperitoneally inoculated into each mouse in which PBMC engraftment was confirmed. Twenty-four hours after HIV-1 inoculation, EFdA (10 μg in 0.1 ml PBS/mouse, twice a day) or PBS was administered for 14 consecutive days (Fig. 3). On day 15, blood samples were collected from the mouse orbit, and then peritoneal cavity and spleen cells were harvested and resuspended in PBS.

**Flow cytometric analysis.** The reconstructed human PBMC proliferation recovered from mice was determined by flow cytometric analysis with allophycocyanin (APC)-Cy7-conjugated anti-mouse CD45 (BD PharMingen, San Diego, CA), Pacific Blue (PB) -conjugated anti-human CD45, APC-conjugated anti-human CD4 (Dako Cytomation, Glostrup, Denmark), phycoerythrin (PE)-Cy7-conjugated anti-human CD3 (e-Bioscience, San Diego, CA) and FITC-conjugated anti-human CD8 (Beckman Coulter, Fullerton, CA) monoclonal antibodies. The cells were treated with red cell
lysing buffer (155 mM NH₄Cl, 10 mM KHCO₃, and 0.1 mM EDTA) to lyse erythrocytes before staining. Single-cell suspensions were prepared in staining medium (PBS with 3% fetal bovine serum and 0.05% sodium azide) and stained with mAb as described above. After 30-minute incubation on ice, the cells were washed twice with washing medium, fixed in PBS with 0.1% paraformaldehyde for 20 min in the dark, and permeabilized in PBS with 0.01% saponin. After 10-minute incubation on ice, cells were stained with PE-conjugated anti-HIV-1 p24 monoclonal antibody (Beckman Coulter, Fullerton, CA) for 30-minute on ice. After staining, the cells were analyzed on a LSR II flow cytometer (BD Bioscience, San Jose, CA). Data were analyzed with FlowJo (Tree Star, San Carlos, CA) software.

**Quantification of murine plasma HIV-1 p24 and viral RNA copy numbers.**

The amount of p24 antigen in murine plasma was determined using the HIV-1 p24 Antigen ELISA kit (ZeptoMetrix Corp., Buffalo, NY). The plasma viral load was quantified with the AMPLICOR HIV-1 monitor test, version 1.5 (Roche Diagnostics, Branchburg, NJ).

**Statistical analysis.** Nonparametric statistical analyses were performed using the Mann-Whitney U test and StatView version 4.51.1 software (Abacus Concepts, Berkeley, CA). P values <0.05 were defined as significant.

**Results**

**Pharmacokinetics of EFdA in Balb/c mice.**

We examined the pharmacokinetics of EFdA in Balb/c mice by
intraperitoneally administering the compound at a dose of 20 mg/kg. Plasma samples were collected periodically for up to 4 h and subjected to HPLC analysis. As shown in Fig. 2, the concentration of EFdA reached the maximal concentration 10-30 min after intraperitoneal administration and then decreased rapidly. Although the initial blood concentration has high varieties, we found that the area under the blood concentration time curve (AUC) was similar among the four mice (4.18, 2.44, 6.10, and 7.23: mean = 4.99±1.68 mg/L h). Next, we administered EFdA to Balb/c mice twice a day intraperitoneally at a dose of 5-50mg/kg for 14 days to examine the adverse effect induced by high-dose EFdA treatment. Mice treated with EFdA at a dose of 5–50mg/kg did not show any body weight loss (data not shown). No acute and sub-acute whole body effects were observed in mice. Mice treated with 50mg/kg showed ruffled fur but the main organs of these mice appeared normal. These results suggest that even high doses of EFdA have few adverse effects in mice.

**Effects of EFdA on CD4+ and CD8+ cell counts in HIV-1-infected hu-PBMC-NOJ mice.**

The in vivo antiviral potency of EFdA was investigated in the hu-PBMC-NOJ mice model of HIV-1 infection. NOJ mice were intraperitoneally transplanted with human PBMC (1 x 10^7 cells /mouse) 5 days before inoculation with HIV-1 (JRFL: R5 strain). EFdA (10μg/mouse: 0.5 mg/kg) was intraperitoneally administered twice a day for 15 days (Fig.3). PBMC were recovered from murine peripheral blood, the peritoneal cavity, and the
spleen on day 16 after HIV-1 inoculation. Samples were stained with anti-mouse CD45-APC/Cy7, anti-human CD45-PB, CD3-PE/Cy7, CD4-APC, and CD8-FITC and subjected to flow cytometric analysis for the determination of CD4⁺/CD8⁺ cell ratios. As shown in Fig. 4A, distinct CD4⁺ cells as well as CD8⁺ cells were seen in PBMC recovered from uninfected PBMC transplanted mice. There were only a few CD4⁺ cells in PBMC recovered from HIV-1 JRFL infected, PBS-treated mice, resulting in a low CD4/CD8 ratio (median: 0.04, range 0.02–0.49); however, CD4⁺ cell frequency was increased by EFdA treatment (median: 0.65, range 0.57–1.43) up to the level of uninfected mice (median: 0.79, range 0.73–1.43) in the PBMC as well as the spleen and peritoneal cavity (Fig. 4B). The numbers of CD4⁺ cells in PBS-treated mice peripheral blood, spleen, and peritoneal cavity were significantly lower than in EFdA-treated ($P < 0.001$) or uninfected ($P < 0.005$) mice (Fig. 5), while there were no significant differences in CD8⁺ cell numbers between groups, indicating that EFdA is not toxic to lymphocytes. Thus, EFdA protects CD4⁺ T cells against HIV-1 infection-induced cell death.

**EFdA suppressed HIV-1 viremia in hu-PBMC-NOJ mice.**

The amount of HIV-1 p24 in plasma was also found to be very high in PBS-treated mice (median: $1.9 \times 10^3$ pg/ml, range: $8.3 \times 10^2$–$5.6 \times 10^3$). EFdA was found to significantly suppress the amount of plasma p24 as examined on day 15 (median: $2.1 \times 10^2$ pg/ml, range, $8.3 \times 10^1$–$6.3 \times 10^2$; $P < 0.001$) (Fig. 6A). We also determined the HIV-1 RNA copy number in infected,
PBS-treated mice and found that the median copy number was $9.9 \times 10^4$ (range: $1.3 \times 10^4$–$5.4 \times 10^5$) copies/ml on day 15 after HIV-1 inoculation; however, EFdA significantly suppressed viremia (median: $9.0 \times 10^2$ copies/ml, range: $8.1 \times 10^2$–$1.1 \times 10^3$; $P < 0.001$) on day 15.

**Effects of EFdA on intracellular p24 levels in HIV-1-infected hu-PBMC-NOJ mice.**

The number of p24$^+$ cells in human CD3$^+$ cells in the spleen, peripheral blood, and peritoneal cavity was analyzed by flow cytometric analysis. The frequency of p24$^+$ cells in the spleen was found to be high in PBS-treated mice (median: 1.90 %, range: 0.33–3.68). EFdA was found to significantly suppress the level of p24$^+$ cells (median: 0.11 %, range: 0.00–1.00; $P = 0.003$) (Fig. 7AB). The frequency of p24$^+$ cells in peripheral blood and the peritoneal cavity was also found to be high in PBS-treated mice and significantly suppressed after EFdA treatment. No apparent EFdA-associated adverse effects were seen throughout the study period.

**Discussion**

In the present study, we have demonstrated the potent activity of EFdA as an agent against HIV in hu-PMBC-NOJ mice. As demonstrated, this particular model is well suited to the study of therapeutic interventions in the HIV arena, providing information on the treatment effects on CD4$^+$ T cell count as well as viral markers, such as plasma p24, HIV-1RNA, and intracellular p24, which are important parameters in determining the
overall effectiveness of a treatment in HIV-1-positive patients.

Severe combined immunodeficient (SCID) mice implanted with human peripheral blood mononuclear cells (PBMC), which are known as hu-PBMC SCID mice, have been used as an animal model for investigating the pathogenesis of HIV infection (15, 18, 19); however, PBMC reconstitution of the SCID mouse varies considerably among transplantation methods, laboratories, experiments, graft sources, and even individual mice (20). PBMC transplantation into NOD/SCID animals resulted in a significant increase in the positive transplantation rate compared to identical treatment of SCID animals (7, 13). More recently, the introduction of mice with complete loss of NK cells, such as NOD/SCID/common γ−/− (NOG) mice (8, 32), Balb/c Rag-2−/−γ−/− mice (30), and NOD/SCID/JAK3−/− (NOJ) mice (26), markedly improved the engraftment of PBMC as well as human hematopoietic stem cells, and has enabled more stable and precise analysis (14, 22, 29). HIV-1 was challenged 2 weeks after PBL transplantation in the previous work (22, 28), since HIV-1 R5 virus is not adequately infective soon after transplantation (3). We optimized the time of viral infection, and found that JRFL could successfully infect and replicate in virus challenge as early as 5 days after PBL transplantation. Since the hu-PBL-NOJ mouse HIV-1 infected model needed a relatively smaller amount of human PBL and shorter duration of HIV-1 infection and replication than previous studies (7, 13, 22, 28), it could be a more useful instrument for analyzing the pathogenesis of HIV-1 infection and testing the efficacy of antiviral agents.
A number of 4′-ethynyl(4′-E)-2′-deoxynucleosides and their analogs (EdN) were synthesized, and a series of potent anti-HIV-1 compounds identified that blocked the replication of a wide spectrum of laboratory and clinical HIV-1 strains in vitro (11, 23, 25). By optimizing such 4′-E nucleoside analogs, EFdA has been found to have potent anti-HIV activity, including highly multidrug-resistant variants, with favorable in vitro cell toxicities (21, 24). EFdA shows unique anti-HIV-1 function and characteristics. EFdA-triphosphate shows greater intracellular stability and generates a more persistent anti-viral effect than other NRTI such as AZT or tenofovir. EFdA acts as a chain terminator upon incorporation at the primer-end; however, it showed no inhibition of cellular polymerases (21). In addition, unlike other adenosine-based NRTI, EFdA showed adenosine deaminase (ADA) resistance (10) and moreover, it had a very high selectivity index, and high-dose EFdA was not toxic to Balb/c mice (Fig. 1B). In the present study, hu-PBMC-NOJ AIDS model mice treated with EFdA maintained high levels of human CD4+ lymphocytes (Figs. 4, 5), suppressed plasma levels of p24 and HIV-1 RNA (Fig. 6), and reduced the number of infected (p24+) cells without apparent adverse effects. Although we cannot directly compare with previously studied anti-HIV-1 agents, our study suggests that EFdA is expected to be effective for clinical use and is a favorable anti-HIV-1 therapeutic agent. It is of note that determination of the precise pharmacokinetics and pharmacodynamics is awaited in clinical trials when EFdA is assessed in humans.
In summary, the data presented here provide strong evidence that the hu-PBMC-NOJ mouse is a valuable model for preclinical testing of new antiretroviral agents. Using this HIV-1 infection mouse model system, we have demonstrated that a new antiretroviral agent, EFdA, has potent anti-HIV-1 activity in vivo without apparent adverse effects. Since EFdA has unique functional properties, lower cytotoxicity, and superior persistence of antiviral activity, it is a promising candidate for a new age of HIV-1 chemotherapy.
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Figure Legends

Figure 1. Structure of 4’-ethynyl-2-fluoro-2’-deoxyadenosine (EFdA)

Figure 2. Pharmacokinetics
Pharmacokinetics of EFdA. Each mouse was administered EFdA intraperitoneally at a dose of 20 mg/kg, and blood samples were taken at 15, 30, 60, 120, and 240 min (n=4).

Figure 3. Protocol for drug administration

Figure 4. Effects of EFdA on the CD4+ /CD8+ cell ratio in HIV-1-infected hu-PBMC-NOJ mice.
A. PBMC recovered on day 16 after R5 HIV-1JRFL inoculation were subjected to flow cytometry. Representative flow cytometric analysis profiles are shown.
B. PBMC, spleen cells and peritoneal cavity cells recovered on day 16 after HIV-1 inoculation were subjected to flow cytometry. CD4/CD8 ratios of each mouse are shown.

Figure 5. Effects of EFdA on numbers of CD4+ and CD8+ cells.
PBMC, spleen cells, and peritoneal cavity cells recovered on day 16 after HIV-1 inoculation were counted and subjected to flow cytometry. Short bars
indicate the medians.

**Figure 6. Effects of EFdA on the amount of plasma p24 and HIV-1 RNA**

Blood samples were collected from mouse orbit on day 16 after HIV-1 inoculation. Short bars indicate the medians.

**Figure 7. Effects of EFdA on HIV-1 infected cells.**

A. PBMC recovered on day 16 after HIV-1 JRFL inoculation were stained with anti-p24-PE, anti-mouse CD45-APC/Cy7, anti-human (h)CD45-PB, anti-hCD4-PB, anti-hCD4-APC, anti-hCD3-PECy7, and anti-hCD8-FITC and subjected to flow cytometry. Representative flow cytometric analysis profiles of the mouseCD45^{+}hCD45^{+}hCD3^{+}hCD8^{+} gated fraction are shown.

B. PBMC, spleen cells, and peritoneal cavity cells recovered on day 16 after HIV-1 inoculation were subjected to flow cytometry. The percent of p24+ cells among CD4 T cells (CD45^{+}hCD45^{+}hCD3^{+}hCD8^{+} gated) is shown.

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Figure 1

MW 293.26

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[Chemical structure image]
Figure 2
Figure 3

hu-PBMC transplantation | HIV-1 infection | sacrifice
---|---|---
day -5 | 0 | 1 | 15

EFdA (0.5 mg/kg) \textit{ip, bid}
Figure 4

A. HIV-1 JRFL infected

PBS  EFdA  uninfected

CD4  CD8

B. Peripheral blood  Spleen  Peritoneal Cavity

CD4/CD8 ratio

PBS  EFdA  uninfected  PBS  EFdA  uninfected  PBS  EFdA  uninfected
Figure 5

**Peripheral blood**

- CD4
  - Cell number (/ml)
  - PBS: △, EFdA: ●, uninfected: ○
  - $P < 0.001$, N.S.
  - $P < 0.005$

- CD8
  - Cell number (/ml)
  - PBS: △, EFdA: ●, uninfected: ○
  - N.S.
  - N.S.

**Spleen**

- CD4
  - Cell number (/ Spleen)
  - PBS: △, EFdA: ●, uninfected: ○
  - $P < 0.005$
  - $P < 0.001$, N.S.

- CD8
  - Cell number (/ Spleen)
  - PBS: △, EFdA: ●, uninfected: ○
  - N.S.
  - N.S.

**Peritoneal Cavity**

- CD4
  - Cell number (/ mouse)
  - PBS: △, EFdA: ●, uninfected: ○
  - $P < 0.001$, N.S.
  - $P < 0.005$

- CD8
  - Cell number (/ mouse)
  - PBS: △, EFdA: ●, uninfected: ○
  - N.S.
  - N.S.
Figure 6

A

p24 (pg/ml)

10^4

10^3

10^2

10^1

PBS

EFdA

B

HIV-1 RNA (copies/ml)

10^6

10^5

10^4

10^3

10^2

PBS

EFdA

P < 0.001

P < 0.001
Figure 7

A

PBS

FEdA

CD4

1.9%

0.5%

p24

100 101 102 103 104

B

Spleen

Peripheral blood

Peritoneal Cavity

Intracellular p24 (%)

PBS

EFdA

PBS

EFdA

PBS

EFdA

P < 0.005

P < 0.005

P < 0.005