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Oral Administration of the Nucleoside EFdA (4′-Ethynyl-2-Fluoro-2′-Deoxyadenosine) Provides Rapid Suppression of HIV Viremia in Humanized Mice and Favorable Pharmacokinetic Properties in Mice and the Rhesus Macaque

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Like normal cellular nucleosides, the nucleoside reverse transcriptase (RT) inhibitor (NRTI) 4′-ethynyl-2-fluoro-2′-deoxyadenosine (EFdA) has a 3′-hydroxyl moiety, and yet EFdA is a highly potent inhibitor of human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) replication with activity against a broad range of clinically important drug-resistant HIV isolates. We evaluated the anti-HIV activity of EFdA in primary human cells and in HIV-infected humanized mice. EFdA exhibited excellent potency against $HIV_{JR\text{-}CSF}$ in phytohemagglutinin-stimulated peripheral blood mononuclear cells (PBMCs), with a 50% inhibitory concentration of 0.25 nM and a selectivity index of 184,000; similar antiviral potency was found against 12 different HIV clinical isolates from multiple clades (A, B, C, D, and CRF01_AE). EFdA was readily absorbed after oral dosing (5 mg/kg of body weight) in both mice and the rhesus macaque, with micromolar levels of the maximum concentration of drug in serum (C_{max}) attained at 30 min and 90 min, respectively. Trough levels were at or above 90% inhibitory concentration (IC_{90}) levels in the macaque at 24 h, suggesting once-daily dosing. EFdA showed reasonable penetration of the blood-brain barrier in the rhesus macaque, with cerebrospinal fluid levels at approximately 25% of plasma levels 8 h after single oral dosing. Rhesus PBMCs isolated 24 h following a single oral dose of 5 mg/kg EFdA were refractory to SIV infection due to sufficiently high intracellular EFdA-triphosphate levels. The intracellular half-life of EFdA-triphosphate in PBMCs was determined to be >72 h following a single exposure to EFdA. Daily oral administration of EFdA at low dosage levels (1 to 10 mg/kg/day) was highly effective in protecting humanized mice from HIV infection, and 10 mg/kg/day oral EFdA completely suppressed HIV RNA to undetectable levels within 2 weeks of treatment.

Nucleoside/nucleotide reverse transcriptase (RT) inhibitors (NRTIs) are highly effective for both first-line therapy and preexposure prophylaxis (PrEP) of human immunodeficiency virus (HIV) infection. There are seven FDA-licensed single NRTIs, including the nucleoside emtricitabine (FTC) and the nucleotide tenofovir (TFV). Long-term use of these drugs has resulted in the emergence of drug-resistant variants in treated patients as well as in treatment-naive individuals due to transmission of these drug-resistant variants (1, 2). New, more-potent compounds with activity against NRTI-resistant strains are needed. All currently approved anti-HIV NRTIs lack the 3′-hydroxyl group and therefore inhibit further DNA polymerization by HIV RT through immediate chain termination after incorporation into the nascent DNA. The absence of a 3′-OH, however, imparts unfavorable properties to these NRTIs by negatively impacting their binding affinity for RT compared to the natural deoxynucleoside triphosphate (dNTP) substrates and by reducing their binding affinity for the cellular nucleoside/nucleotide kinases responsible for intracellular phosphorylation of NRTIs to the active triphosphates (3). A new class of 4′-substituted NRTIs has been developed whose members retain the 3′-hydroxyl group and are more potent, with a higher *in vitro* selectivity index (SI) than any currently licensed NRTI (3–8). The most potent of these analogs, EFdA (4-ethynyl-2-fluoro-2′-deoxyadenosine), also has a halogen substitution at the 2-position of the adenine ring which confers resistance to degradation by adenosine deaminase, resulting in improved intracellular half-lives (3, 8, 9).

The potency of EFdA stems in part from a novel mechanism of action, translocation-defective RT inhibition, described by Michailidis et al. (3, 10), involving inhibition of primer translocation following EFdA-monophosphate (EFdA-MP) incorporation. HIV RT can use EFdA-5′-triphosphate (EFdA-TP) as a substrate more efficiently than the natural dATP substrate, and, despite the presence of the 3′-hydroxyl group, the incorporated EFdA-MP acts as a *de facto* terminator of further RT-catalyzed DNA synthesis because of the diffi-

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culty of RT translocation on the nucleic acid primer attached to 3′-terminal EFdA-MP. Importantly, EFdA retains significant potency against a broad range of clinically important drug-resistant HIV isolates (5, 8, 11). The favorable properties of EFdA led us to assess the *in vitro* activity of EFdA against HIV clades A, B, C, D, and E in human peripheral blood mononuclear cells (PBMCs) and its *in vivo* activity in three different humanized mouse models (SCID-hu Thy/Liv, NSG-hu Thy/Liv, and NSG-BLT).

The SCID-hu Thy/Liv mouse model of HIV infection is a useful platform for the preclinical evaluation of antiviral efficacy *in vivo*. The human thymus (Thy) implant in these mice supports long-term differentiation of human T cells, and the model has been standardized and validated with four classes of licensed antiretrovirals for the evaluation of antiviral drugs against HIV (12, 13). One important advantage of SCID-hu Thy/Liv mice for studies of HIV prophylaxis is their high (essentially 100%) susceptibility to HIV infection after injection of the virus directly into the thymus/liver (Thy/Liv) implant. The major advantage of the NSG-hu Thy/Liv and NSG-BLT mouse models is the robust peripheral reconstitution of human leukocytes and establishment of systemic HIV infection and plasma viremia after HIV challenge by multiple routes; the models' major drawbacks are variability in HIV susceptibility (14) between mice and a high incidence of graft-versus-host disease (GvHD) (35% by 22 weeks) (15, 16).

We also carried out preliminary pharmacokinetic (PK) analyses of orally administered EFdA in two animal species, humanized mice and the rhesus macaque.

MATERIALS AND METHODS

Drugs and viruses. EFdA was provided by Yamasa Corporation (Chiba, Japan) and was also obtained by custom synthesis (Life Chemicals Inc., Burlington, ON, Canada). [8-^{3}H]EFdA was prepared by custom tritiation of EFdA (Moravek Biochemicals, Brea, CA). The following reagents were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: tenofovir (TFV), tenofovir disoproxil fumarate (TDF), and emtricitabine [(−) FTC] and HIV molecular clones pYK-JRCSF (R5) from Irvin S. Y. Chen and Yoshio Koyanagi (17–19); pNL4-3 (X4) from Malcolm Martin (20); HIV type 1 (HIV-1) 92RW008 and 98IN022 from The UNAIDS Network for HIV Isolation and Characterization; HIV-1 KNH1088 (catalog no. 11244) and HIV-1 A08483M1 (catalog no. 11262) and AF484516 (catalog no. 11263) from Victoria Polonis (21); HIV-1 US1 (GS 004), HIV-1 US4 (GS 007), HIV-1 CM235 (GS 020), and HIV-1 CM240 (GS 022) from Nelson Michael (22–24); and simian immunodeficiency virus (SIV) Delta/B670 from M. Murphey-Corb (25, 26). Infectious supernatants of the molecular clones were prepared in HEK 293T cells by Lipofectamine 2000 transfection, and primary isolates were expanded in phytohemagglutinin (PHA)-stimulated PBMCs. Stock virus titers were determined in PHA-stimulated PBMCs by endpoint dilution with assessment of supernatant p24 by enzyme-linked immunosorbent assay (ELISA) after 7 days, and 50% tissue culture infective doses ($TCID_{50}$) were calculated using the Reed-Muench method.

***In vitro* antiviral assays.** Cryopreserved PHA-stimulated PBMCs, pooled from 6 donors, were thawed and inoculated in bulk the next day with HIV at a multiplicity of infection (MOI) of 0.001 for 2 h at 37°C in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 5 U per ml of human recombinant interleukin-2 (rIL-2). A total of 100,000 HIV-infected PBMCs in 100 μl were seeded in triplicate into round-bottom 96-well plates and treated with 100 μl of serially diluted EFdA, TFV, FTC, or medium alone and cultured for 7 days. Supernatants were assayed for p24 at a 1:800 dilution by quantitative HIV-1 p24 ELISA (PerkinElmer), and cytotoxicity determinations were performed in parallel in drug-treated uninfected PBMCs by incubation with (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide (MTT) on day 7. Fifty percent inhibitory concentrations (IC_{50}) and 50% cytotoxic concentrations (CC_{50}) were determined by a 4-parameter fit model (SOFTmax PRO 3.0, Molecular Devices). At day 7, untreated virus control wells had mean HIV-1 p24 concentrations of 35 to 130 ng/ml.

SCID-hu Thy/Liv mice. The University of California, San Francisco, (UCSF) is a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the UCSF Institutional Animal Care and Use Committee. SCID-hu Thy/Liv mice were generated by implantation of 6-to-8-week-old male C.B17 scid mice (model CB17SC-M, C.B-*Igh-1*^{b}/IcrTac-*Prkdc*^{scid}; Taconic) with 1-mm^3 pieces of human fetal thymus and liver from a single donor under the kidney capsule as described previously (12, 27). Sulfamethoxazole-trimethoprim-treated food pellets (SCIDS MD; Bio-Serv) were added to the cages to prevent opportunistic infections, and pellets were removed from mouse cages 2 days before experimental treatment initiation. Implants of anesthetized mice were inoculated by direct injection 21 weeks after implantation with 50 μl of HIV_{NL4-3} (1,000 $TCID_{50}$) or RPMI 1640 medium (mock infection). EFdA was dissolved in sterile phosphate-buffered saline (PBS) and administered to 7 mice per group twice daily by subcutaneous injection in 200 μl at a range of dosage levels (0.3, 1, 3, and 10 mg/kg of body weight/day) beginning the day before HIV inoculation. TDF plus FTC (Truvada) was dissolved in 0.5% medium-viscosity sodium carboxymethylcellulose (Sigma) and administered to 7 mice twice daily by oral gavage in 200 μl at a total dosage of 100 mg/kg/day (60 mg/kg day TDF plus 40 mg/kg/day FTC). Treatment was initiated the day before HIV inoculation and continued until Thy/Liv implant collection.

The Thy/Liv implants were collected from euthanized mice 21 days after inoculation, when HIV_{NL4-3} replication was expected to peak in the implants. Single-cell suspensions were made by placing the implant into a sterile nylon mesh bag, submerging the bag in phosphate-buffered saline (PBS)–2% FBS in a 60-mm-diameter tissue culture dish, and dispersing the tissue between the nylon layers with forceps, as described previously (12, 27, 28). For the branched DNA assay, dry pellets of 5×10^6 implant cells were frozen and stored at −80°C. Cells were disrupted with sterile disposable pestles and a cordless motor grinder (Kontes) in 8 M guanidine HCl with 0.5% sodium N-lauroylsarcosine. The RNA was extracted with 0.5 ml 100% ethanol and pelleted at 12,000 × *g* for 20 min at 4°C. Supernatants were aspirated to remove DNA, and RNA pellets were washed with 0.5 ml 70% ethanol, placed on dry ice, and digested with reagents supplied by the manufacturer (Versant HIV-1 RNA 3.0 assay; Siemens Healthcare Diagnostics). Levels of implant HIV RNA are expressed as numbers of copies per 10^6 implant thymocytes, and the $\log_{10}$ values were used for calculation of geometric means. The limit of detection was $10^{1.48}$ RNA copies per 10^6 cells, and this lower-limit value was used for calculation of means for implants with undetectable viral RNA. For p24 ELISA, pellets of 2.5×10^6 cells were resuspended in 400 μl of p24 lysing buffer (1% Triton X-100, 0.5% sodium deoxycholate, 5 mM EDTA, 25 mM Tris Cl, 250 mM NaCl, and 1% aprotinin), rotated overnight at 4°C, and stored at −20°C. Thawed samples were transferred into HIV-1 p24 antibody-coated microplates (PerkinElmer) for quantitative ELISA. A standard curve was generated with the kit-supplied standards, and the results were calculated as pg p24 per 10^6 implant cells. The limit of detection of the p24 ELISA was 5 pg p24 per 10^6 cells.

NSG-hu Thy/Liv and NSG-BLT mice. NSG-hu Thy/Liv mice were generated as described above for SCID-hu Thy/Liv mice by implanting 6-to-8-week-old female nonobese diabetic (NOD)-scid IL-2Rγ$^{-/-}$ (NSG) mice (stock no. 005557, NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ; The Jackson Laboratory) with human fetal thymus and liver under the kidney capsule. One cohort of NSG-BLT mice was generated by tail vein injection of cryopreserved human CD34$^+$ hematopoietic stem progenitor cells (HSPC) (500,000 cells per mouse) isolated from the autologous fetal liver. These mice were not irradiated prior to cell injection because we have found that this conditioning step is not necessary for robust human leu-

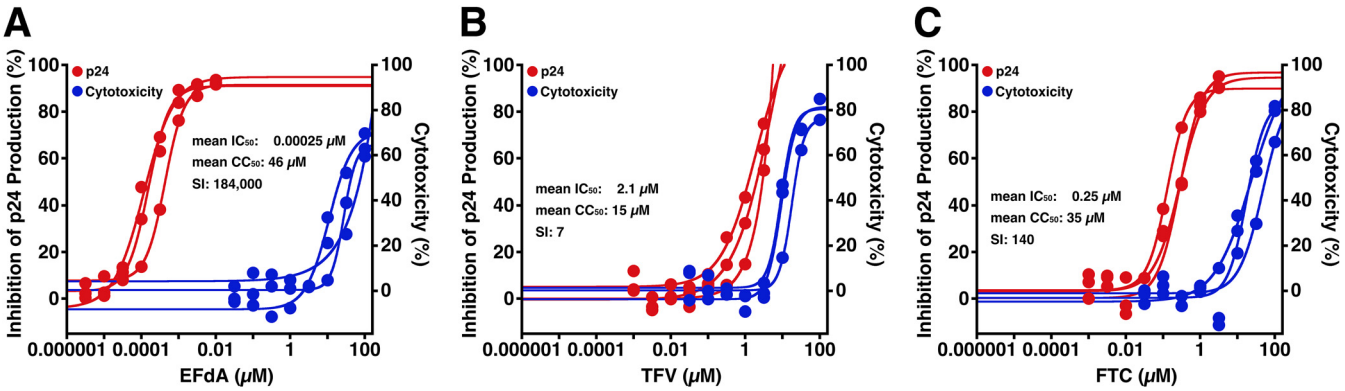

FIG 1 (A) EFdA has extremely potent and highly selective *in vitro* antiviral activity against $HIV_{JR\text{-}CSF}$ in PHA-stimulated PBMCs. PBMCs were inoculated at an MOI of 0.001 and incubated for 7 days for determination of inhibition of supernatant HIV p24 production and MTT reduction for cytotoxicity evaluation. Data from three independent assays are shown with mean IC_{50}, mean CC_{50}, and selectivity index (SI) (ratio of CC_{50}/IC_{50}). (B and C) TFV was 8,400 times less potent than EFdA, with an SI of 7 (B), and FTC was 1,000 less potent than EFdA, with an SI of 140 (C). At day 7, untreated virus control wells had mean p24 concentrations of 120 ng/ml.

kocyte reconstitution. In addition, irradiation has systemic toxic effects on many cell types and impairs HSPC and stromal cell function. NSG-hu mice were inoculated intraperitoneally (i.p.) with 10,000 $TCID_{50}$ of $HIV_{JR\text{-}CSF}$ 12 to 26 weeks after Thy/Liv implantation. EFdA or TDF plus FTC was administered by once- or twice-daily oral gavage (200 μl) at the indicated dosage levels beginning either the day before inoculation or after viremia had been established for 5 or 6 weeks. For the pharmacokinetic study, NSG-hu Thy/Liv mice were treated with a single oral administration of 5 mg/kg, and blood was collected after 30 min, 1 h, 2 h, and 12 h into blood collection tubes containing lithium heparin (Sarstedt). Each data point represents the blood from a single animal. Tubes were inverted 10 times, and plasma was removed after low-speed centrifugation for 5 min at room temperature and stored at −80°C.

Rhesus macaque. A 10-kg adult male rhesus macaque (*Macaca mulatta*) was implanted with an inline vascular catheter under the supervision of a veterinarian experienced in primate surgery, using a jacketed tether system to avoid undue stress on the animal during the frequent blood withdrawals. The animal was maintained in accordance with the NIH *Guide to the Care and Use of Laboratory Animals* under the approval of the University of Pittsburgh Institutional Animal Care and Use Committee. The University of Pittsburgh is accredited by the AAALAC. The same animal was used for two separate studies, carried out 1 month apart. Fifty milligrams of EFdA (the dose equivalent of 5 mg/kg) was dissolved in 40 ml grape juice immediately before use and delivered orally via a 50-ml needleless syringe. The animal consumed the dose within 30 s. Blood samples (2 ml) were taken at baseline 1 h before EFdA administration and at multiple time points thereafter over a 24-h period and placed into tubes containing EDTA. Red blood cells were removed by low-speed centrifugation, and the plasma was stored at −80°C until analysis. In addition, 20-ml blood draws were performed 24 h before dosing with EFdA and 8 h and 24 h after dosing. PBMCs were isolated from these samples by the use of Ficoll-Paque and stored at −80°C for subsequent infectivity analysis. In one study, the animal was sedated with ketamine 8 h after EFdA administration to obtain a sample of cerebrospinal fluid (CSF).

Rhesus PBMCs were thawed, washed to remove the dimethyl sulfoxide (DMSO)-containing storage buffer, and then exposed to $SIV_{DeltaB670}$ at an MOI of 0.03 for 1 h at 37°C. The cells were then washed to remove SIV and were cultured in medium containing IL-2 (10 U/ml) for 24 h. PHA was added to reach a final concentration of 10 μg/ml, and the cells were incubated for an additional 3 days, washed to remove the PHA, and resuspended in medium containing IL-2. At various times, aliquots of supernatant were removed and replenished with fresh medium containing IL-2, and SIV p27 concentrations were determined by ELISA as previously described (29).

EFdA pharmacokinetics. Plasma (or CSF) was mixed vigorously with three vol of acetonitrile and stored at 4°C for 2 h, followed by removal of insoluble material by centrifugation (15,000 × *g*). Supernatants were removed and stored at 4°C for an additional 16 h and then centrifuged

TABLE 1 Antiviral activity of EFdA against multiple HIV clades in human PBMCs[a]

Virus	Clade	Coreceptor usage	IC_{50}(s) (nM) (no. of assays)	IC_{90}(s) (nM) (no. of assays)
HIV-1 KNH1088	A	R5	0.26, 0.38	3.6, >10
HIV-1 92/RW/008	A	R5	0.10, 0.12	1.1, 0.95
HIV-1 NL4-3	B	X4	0.16 ± 0.03 (4)	2.4 ± 1.1 (4)
HIV-1 JR-CSF	B	R5	0.18 ± 0.04 (7)	2.5 ± 0.61 (7)
HIV-1 US1 (GS 004)	B	R5	0.26, 0.27	>10, 7.6
HIV-1 US4 (GS 007)	B	R5	0.29, 0.44	>10, >10
HIV-1 98/IN/022	C	R5	0.58, 0.20	6.8, >10
HIV-1 MW/93/965 (301965)	C	R5	0.39, 0.28	6.1, >10
HIV-1 A08483M1	D	R5	0.96	>10
HIV-1 J32228M4	D	R5	0.75	>10
HIV-1 CM235 (GS 020)	CRF01_AE	R5	0.77, 0.98	>10, >10
HIV-1 CM240 (GS 022)	CRF01_AE	R5	0.31, 0.54	>10, 4.0

[a] IC_{50} and IC_{90} values were determined by a 4-parameter fit model (SOFTmax PRO 3.0; Molecular Devices). Either individual values (for 1 or 2 independent assays) or means ± standard errors of the means (SEM) (for 4 or 7 independent assays, as shown in parentheses) are shown. At day 7, untreated virus control wells had mean p24 concentrations of 35 to 130 ng/ml.

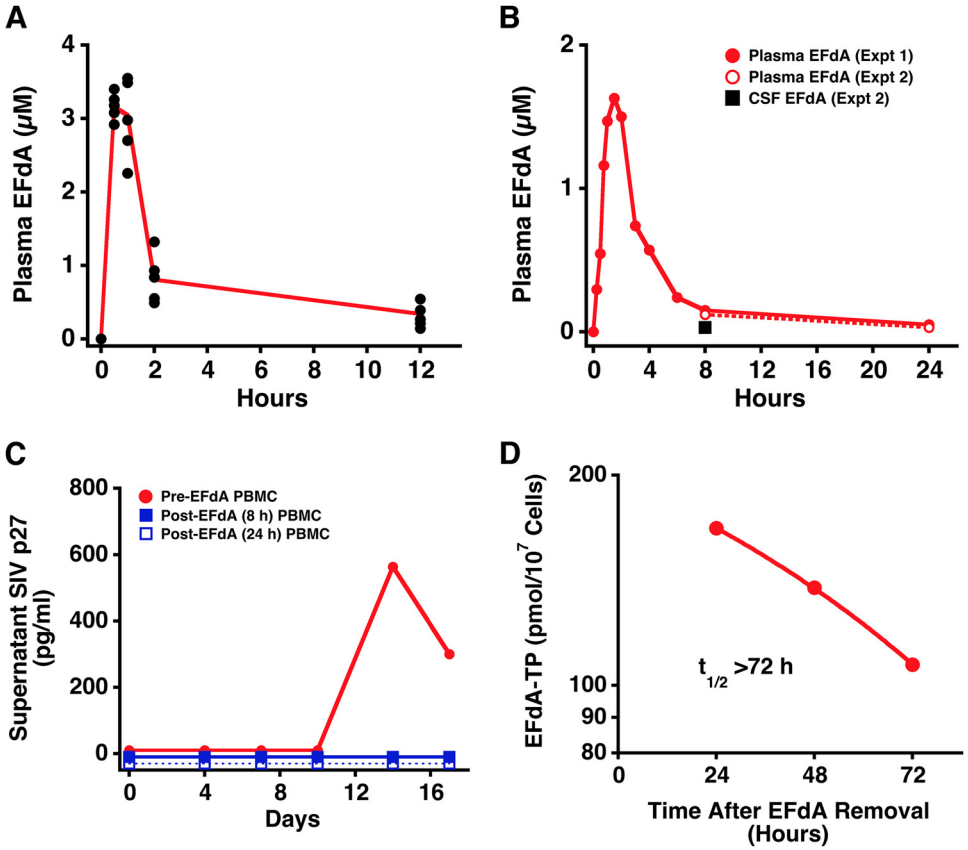

FIG 2 Pharmacokinetics of oral EFdA. (A and B) Plasma levels of EFdA in humanized mice (A) and plasma and CSF levels of EFdA in a rhesus macaque (B) after a single oral dose of 5 mg/kg. (C) Resistance to $SIV_{DeltaB670}$ infection of PBMCs isolated from the rhesus macaque following a single oral dose of EFdA. (D) The intracellular half-life of EFdA-triphosphate in human PBMCs was >72 h following a single exposure to EFdA *in vitro*.

(15,000 × *g*) again to remove any additional particulates. Clarified supernatants were taken to dryness *in vacuo*, and the dried samples were stored at −20°C until analysis. Dried samples were dissolved in a known volume of 10 mM potassium phosphate–5% acetonitrile (pH 7.4) for quantification of EFdA content by high-performance liquid chromatography (HPLC). Recovery of EFdA by this extraction procedure was evaluated by adding known amounts of EFdA to control mouse or rhesus plasma before analysis. The average recovery was 68% ± 5%, and all reported values were corrected for recovery. EFdA levels were quantified by C_{18} reverse-phase isocratic HPLC (Alltech Altima C18-HP) (3-μm particle size, 150 mm by 2.1 mm) at ambient temperature (20 to 22°C) using a mobile phase of 5% acetonitrile–10 mM potassium phosphate (pH 7.4), a flow rate of 0.2 ml/min, UV detection at 260 nm, and Gilson UniPoint LC System software for control and analysis. The average retention time for EFdA under these conditions was 23 min, and the limit of quantification was 5 pM. Pharmacokinetic parameters were calculated using the PK function add-in for Microsoft Excel (developed and provided by J. Usansky, A. Desai, and D. Tang-Liu, Allergan, Irvine, CA).

TABLE 2 Pharmacokinetic properties of orally administered EFdA

Parameter[a]	Value	
	Rhesus (n = 1)	Humanized mouse (n = 25)
C_{max} (nmol/ml)	1.6	3.2
T_{max} (h)	1.5	0.5
Elimination half-life (h)		
Initial	1.9	3.8
Secondary	~6.9[b]	
AUC (nmol/h/ml)	6.5[c]	9.5[d]

[a] Pharmacokinetic parameters were calculated from the data shown in Fig. 2A (mouse) and Fig. 2B (rhesus macaque) using the PK function add-in for Microsoft Excel (developed and provided by J. Usansky, A Desai, and D. Tang-Liu, Allergan, Irvine, CA). C_{max}, maximum concentration of drug in serum; T_{max}, time to maximum concentration of drug in serum; AUC, area under the concentration-time curve.
[b] The elimination profile of EFdA in rhesus plasma suggested at least two components. The value for the secondary, more-prolonged-elimination component represents an estimation, as there were too few data points to calculate this value with precision.
[c] AUC_{0-24} (area under the concentration-time curve from 0 to 24 h).
[d] AUC_{0-12}.

Serum protein binding of EFdA was carried out using an ultrafiltration approach (30). Briefly, [^{3}H]EFdA was incubated in 95% FBS for 30 min at 37°C, and then the mixture was subjected to centrifugal ultrafiltration through a 10-kDa-cutoff microfilter (Millipore). The amount of EFdA in the ultrafiltrate was compared to that in the starting material using liquid scintillation spectrometry.

Intracellular pharmacokinetics of EFdA-TP. Cryopreserved PHA-stimulated PBMCs, pooled from multiple donors, were thawed and activated by exposure to 5 μg/ml PHA and 10 U/ml IL-2 for 3 days and then isolated, washed, and resuspended in RPMI 1640 medium–15% FBS. The cells were exposed to 10 μM [^{3}H]EFdA for 4 h and then pelleted, washed twice with phosphate-buffered saline, and resuspended in RPMI–15% FBS with IL-2. At various time points, aliquots with known numbers of cells were removed, and the cells were pelleted by centrifugation. Cell pellets were vigorously mixed with ice-cold 70% methanol and stored

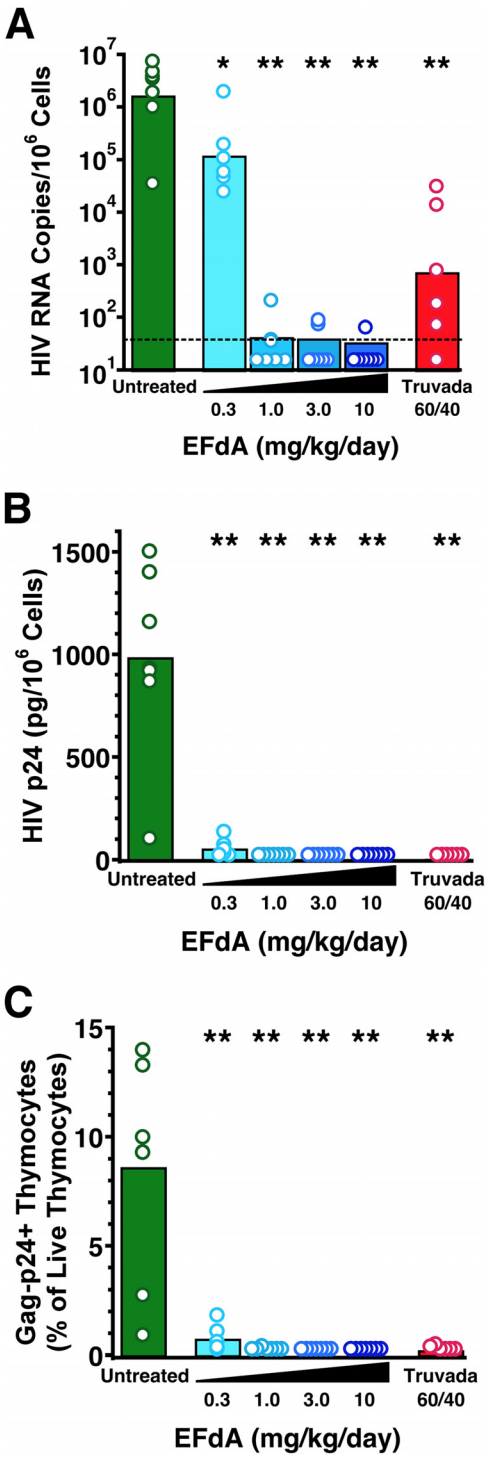

FIG 3 EFdA protects SCID-hu Thy/Liv mice from HIV_{NL4-3} infection at low dosage levels. Mice were treated with the indicated amounts of EFdA by twice-daily subcutaneous injection and with 60 mg/kg TDF plus 40 mg/kg emtricitabine (Truvada) by twice-daily oral gavage beginning the day before virus inoculation and continuing until implant collection 21 days after virus (or mock) inoculation. Antiviral efficacy was assessed by determining levels of HIV RNA (A) and p24 (B) and percentages of Gag-$p24^+$ thymocytes (C) for treated versus untreated mice. The columns represent the means, and the open circles represent individual mice from the same cohort 21 days after inoculation. *, $P < 0.05$; **, $P < 0.01$ (compared to untreated HIV-infected mice by the Mann-Whitney U test).

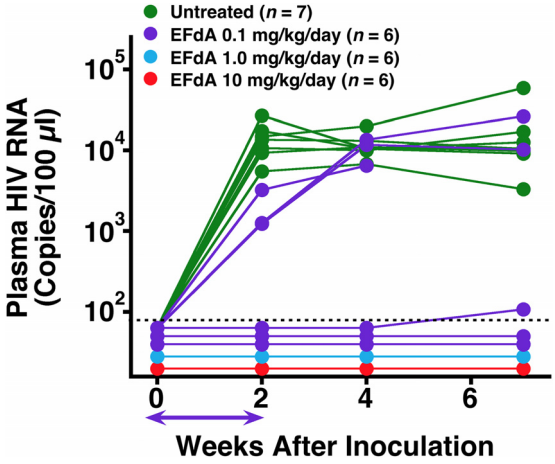

FIG 4 EFdA protects NSG-hu Thy/Liv mice from HIVJR-CSF infection at low dosage levels. NSG-hu Thy/Liv mice were treated for 2 weeks (indicated by horizontal arrow) with EFdA by twice-daily oral gavage beginning the day before intraperitoneal inoculation with HIV_{JR-CSF}. Antiviral efficacy was assessed by longitudinally determining HIV RNA levels in mouse plasma. Mice were completely protected from HIV infection at doses of 1 and 10 mg/kg/day, and 3 of 6 mice were protected at 0.1 mg/kg/day. The dotted line indicates the limit of HIV RNA detection (75 copies per 100 μl of mouse plasma).

at −20°C overnight. Extracts were clarified by centrifugation, and the clarified supernatant was dried *in vacuo*.

Dried extracts were reconstituted in a small volume of 5% acetonitrile–10 mM potassium phosphate (pH 7.4) and subjected to chromatography analysis using reverse-phase HPLC (Alltima HP C_{18}; 150 mm by 2.1 mm) with a flow rate of 0.1 ml/min. EFdA-TP eluted at 9.5 to 11.5 min under these conditions and was quantified by collecting 0.5-min fractions followed by counting by liquid scintillation spectrometry.

Flow cytometry. Thy/Liv implant cells were fixed and permeabilized with 1.2% paraformaldehyde and 0.5% Tween 20, stained with fluorescein isothiocyanate-conjugated anti-p24 (Beckman Coulter), and analyzed on an LSR II instrument (BD Biosciences). Percentages of Gag-p24-positive ($p24^+$) thymocytes were determined by collecting 100,000 total cell events and gating on singlets in a live lymphoid cell population identified by forward- and side-scatter characteristics, as described previously (13).

RESULTS

Potent anti-HIV activity by EFdA in human PBMCs. In agreement with a previous study (3), EFdA inhibited HIV replication in PHA-stimulated PBMCs with a mean IC_{50} of 0.25 nM and low cytotoxicity (CC_{50}, 46 μM), resulting in an *in vitro* SI of 184,000 (Fig. 1A). This is considerably better than that shown by either of the NRTI components of TDF plus FTC (Truvada), the current recommended clinical NRTI drugs. The nucleotide tenofovir (TFV) was 8,400 times less potent (IC_{50}, 2.1 μM), with an SI of 7 (Fig. 1B), and the nucleoside emtricitabine (FTC) was 1,000-fold less potent than EFdA (IC_{50}, 0.25), with an SI of 140 (Fig. 1C).

In vitro PBMC assays with 12 different HIV isolates from multiple clades (A, B, C, D, and CRF01_AE) showed that EFdA had IC_{50}s ranging from 0.10 to 1.0 nM and IC_{90} values of 2.4 and 2.5 nM for HIV_{NL4-3} and HIV_{JR-CSF}, respectively (Table 1). EFdA was most potent against the clade A and B isolates (IC_{50}, 0.1 to 0.4 nM), with slightly higher values against the clade D and CRF01_AE isolates (IC_{50}, 0.3 to 1.0 nM). As noted previously (3), these data confirm that EFdA is the most potent nucleoside inhibitor of HIV replication in primary human cells described to date.

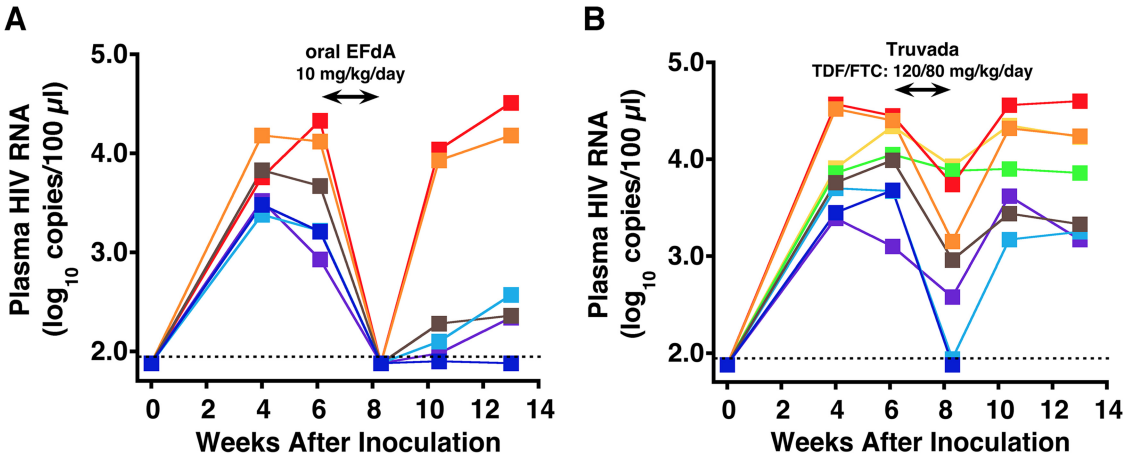

FIG 5 Reduction of plasma HIV RNA and viral rebound in HIV_{JR-CSF}-infected NSG-hu Thy/Liv mice treated with EFdA (A) or TDF plus FTC (Truvada) (B) by twice-daily oral gavage for 2 weeks (indicated by horizontal arrows), after which treatment was discontinued. Individual mice are indicated by different colors, and the dotted lines indicate the limit of HIV RNA detection (101.88 copies per 100 μl mouse plasma).

Pharmacokinetics of oral EFdA in NSG-hu Thy/Liv mice. Plasma was collected from NSG-hu Thy/Liv mice 30 min, 1 h, 2 h, and 12 h after a single oral dose of 5 mg/kg, and EFdA concentrations were measured by quantitative HPLC as described in Materials and Methods. Peak plasma levels of EFdA of 3.2 μM were noted 30 min after administration (Fig. 2A), with an elimination half-life of 3.8 h (Table 2). Importantly, plasma levels of 0.3 μM 12 h after dosing were at least 10 times the IC_{90} for most of the HIV-1 strains and clades evaluated (Table 1).

Pharmacokinetics of oral EFdA in rhesus macaque. A detailed pharmacokinetic study of oral EFdA was carried out using a male rhesus macaque. EFdA was rapidly absorbed, with a peak plasma level of ~1.6 μM 90 min after ingestion (Fig. 2B and Table 2), and the terminal elimination half-life was 1.9 h. The plasma level of EFdA 24 h after a single oral dosing was approximately 10 nM, at or above the IC_{90} for most HIV strains and clades tested. More importantly, PBMCs isolated from the animal 24 h after dosing were refractory to SIV infection in the absence of exogenous drug (Fig. 2C), suggesting that intracellular levels of the active EFdA-TP remained high enough to provide an effective barrier to SIV infection. Preliminary intracellular pharmacokinetic analysis suggests that the intracellular half-life of EFdA-TP in human PBMCs is >72 h following a single exposure to EFdA *in vitro* (Fig. 2D). Furthermore, unlike most NRTIs, EFdA was able to cross the blood-brain barrier, with levels of EFdA in CSF that were 25% of the plasma levels 8 h after dosing (Fig. 2B). Finally, there was essentially no serum protein binding of EFdA, with greater than 98% free EFdA noted after incubation in serum (data not shown).

Antiviral efficacy of EFdA in SCID-hu Thy/Liv mice. In a pilot study performed in SCID-hu Thy/Liv mice, EFdA was administered by subcutaneous injection beginning the day before HIV_{NL4-3} inoculation (1,000 $TCID_{50}$ in 50 μl) by direct implant injection. Groups of 6 or 7 mice were treated with a range of EFdA dosage levels (0.3 to 10 mg/kg/day), and their Thy/Liv implants were collected 21 days after inoculation, when HIV_{NL4-3} replication normally peaks in the implants. As little as 0.3 mg/kg/day EFdA reduced HIV RNA by >10-fold (from a mean of $10^{6.2}$ HIV RNA copies per 10^6 cells in untreated mice to $10^{5.2}$ copies in EFdA-treated mice), and 15 of 21 mice treated with 1 to 10 mg/kg/day EFdA had no detectable implant HIV RNA (<$10^{1.5}$ copies per 10^6 cells) (Fig. 3A). By comparison, oral TDF plus FTC given at a much higher dosage level (60 mg/kg/day TDF plus 40 mg/kg/day FTC) reduced viral RNA to undetectable levels in only one of six treated mice, and the mean for the group was $10^{2.9}$ copies per 10^6 cells. All 21 mice treated with 1 to 10 mg/kg/day EFdA had no detectable HIV p24 in their implants (Fig. 3B), while 2 of the 6 mice treated with TDF plus FTC had p24 levels just above the level of detection (8 and 21 pg per 10^6 cells). The dramatic reductions in implant p24 levels determined by ELISA were confirmed by the detection of few (<1%) Gag-$p24^+$ thymocytes by flow cytometry in mice treated with either 1 to 10 mg/kg/day EFdA or TDF plus FTC, whereas the mean percentage of Gag-$p24^+$ thymocytes in untreated mice was 7% (Fig. 3C). EFdA-treated mice exhibited no significant body weight loss or any other signs of drug toxicity.

Antiviral efficacy of EFdA in NSG-hu Thy/Liv mice. In preparation for EFdA suppression experiments in HIV-viremic humanized mice, we first evaluated the activity of EFdA in NSG-hu Thy/Liv mice with treatment beginning the day before i.p. HIV_{JR-CSF} inoculation. Groups of 6 mice each were treated by twice-daily oral gavage for 2 weeks beginning the day before inoculation, and plasma HIV RNA levels were followed longitudinally in individual mice by serial blood collection for 5 weeks after treatment cessation. Further confirming the potency of this NRTI, all 12 mice treated for 2 weeks with 1 and 10 mg/kg/day EFdA remained nonviremic after HIV challenge, while 3 of 6 mice (50%) were protected at 0.1 mg/kg/day (Fig. 4). The 7 untreated mice all became viremic by 2 weeks after HIV_{JR-CSF} inoculation, with sustained plasma HIV RNA levels of $10^{3.5-4.5}$ copies per 100 μl.

Antiviral efficacy of EFdA in NSG-hu Thy/Liv and NSG-BLT mice with established infection. Next, a cohort of NSG-hu Thy/Liv mice with established HIV_{JR-CSF} infection was treated to determine whether EFdA could suppress viremia to undetectable levels, as has been reported for combination therapy with TDF plus FTC plus the HIV integrase inhibitor raltegravir in BLT mice (31). Oral administration of 10 mg/kg/day EFdA beginning 6 weeks after HIV_{JR-CSF} inoculation reduced viremia to undetectable levels (<$10^{1.88}$ copies per 100 μl) in all six mice within 2

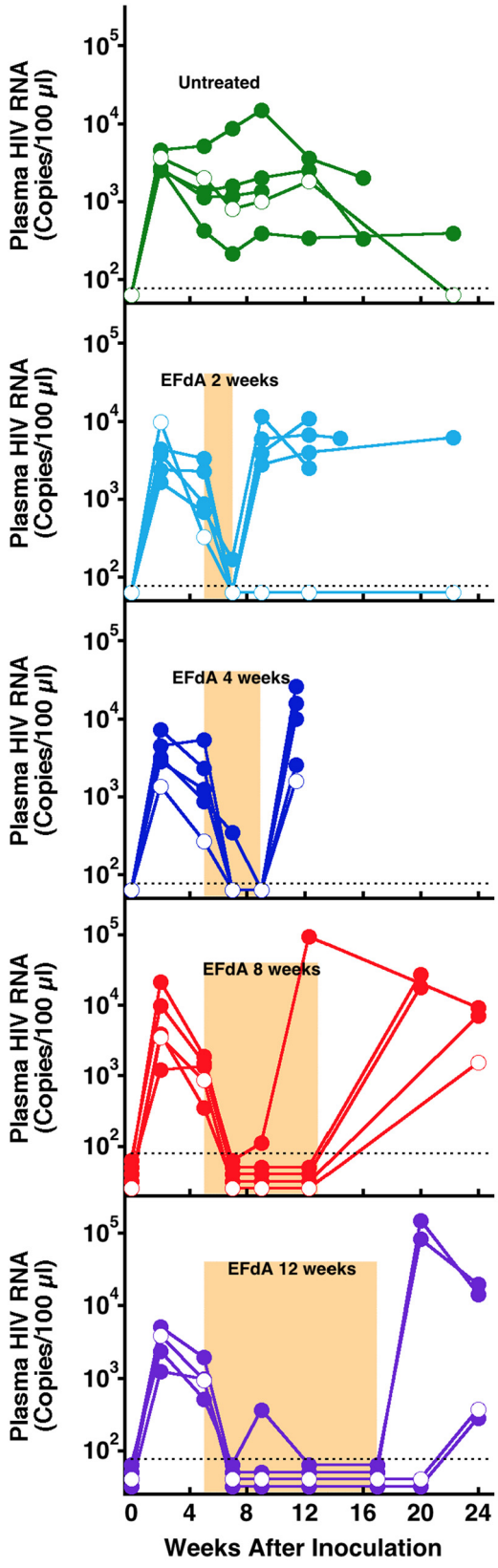

FIG 6 Reduction of plasma HIV RNA and viral rebound in $HIV_{JR\text{-}CSF}$-infected NSG-BLT mice (closed symbols) treated with 10 mg/kg/day EFdA by once-daily oral gavage for 2, 4, 8, or 12 weeks, after which treatment was discontinued. Data for individual mice (all from the same cohort) are shown, and the treatment period is indicated by shading. The dotted line indicates the limit of HIV RNA detection (75 copies per 100 μl mouse plasma). The open symbols indicate NSG-hu Thy/Liv mice (one per treatment group) that were not injected with $CD34^+$ hematopoietic stem progenitor cells 3 weeks after Thy/Liv implantation. The mouse that rebounded while on EFdA treatment in the 8-week group had HIV RNA with M184V in RT predominant in plasma collected at the last time point.

weeks of EFdA treatment (Fig. 5A). In stark contrast, only one of eight mice treated with a much higher dosage level of oral TDF plus FTC (120 mg/kg/day TDF plus 80 mg/kg/day FTC) had undetectable viremia after 2 weeks of treatment (Fig. 5B). The plasma viral load of all eight mice treated with TDF plus FTC rebounded to and plateaued at pretherapy levels within 2 weeks of treatment cessation. Interestingly, the four EFdA-treated mice with the lowest pretherapy viremia levels also rebounded, but to much lower setpoints, and one of these mice did not rebound at all during the 5-week posttreatment observation period.

We surmised that periods of EFdA treatment longer than 2 weeks might lead to even less HIV rebound in EFdA-treated mice, so we performed a follow-up experiment with groups of $HIV_{JR\text{-}CSF}$-viremic NSG-BLT mice treated with EFdA for 2, 4, 8, or 12 weeks (Fig. 6). Similarly to our previous study, HIV viremia did not rebound in 1 of 5 mice treated with EFdA for 2 weeks, but this effect was not seen with longer periods of EFdA treatment; all mice treated with EFdA for 4, 8, and 12 weeks experienced rebounds in plasma viremia after treatment cessation, and one mouse treated for 8 weeks rebounded to a high level (10^5 HIV RNA copies per 100 μl) before treatment was stopped. Genotyping of plasma collected 12 weeks after discontinuation of EFdA therapy showed that the rebound virus had the M184V mutation in the RT gene, which has been reported to confer resistance to EFdA (11). A second mouse treated with EFdA for 8 weeks that remained fully suppressed while on treatment had M184I in RT at the last time point. No other mutations in RT were detected in humanized mice treated with EFdA for 8 or 12 weeks.

DISCUSSION

NRTIs are the cornerstone of current first-line combination antiretroviral therapy (cART). The NRTIs are also the only antiretroviral drug class with FDA approval for preexposure prophylaxis (PrEP) in adults at high risk for sexually acquired HIV-1. Current NRTIs require daily dosing at levels of at least 300 mg.

Our results with EFdA in human PBMCs clearly demonstrate the striking antiviral potency and breadth of activity of this novel NRTI (19). EFdA was 8,400 times more potent than TFV and 1,000 times more potent than FTC against $HIV_{JR\text{-}CSF}$ in a side-by-side comparison, and its low cytotoxicity resulted in a very high SI (184,000). In addition, EFdA had IC_{50}s of 0.1 to 1.0 nM against 12 different HIV isolates from multiple clades (A, B, C, D, and CRF01_AE), which further highlights the drug's potential for HIV treatment worldwide. EFdA is clearly one of the most potent anti-HIV compounds described to date (3).

Our preliminary pharmacokinetic studies of orally administered EFdA in the mouse and the rhesus macaque showed that EFdA is rapidly absorbed and achieves plasma drug concentrations far in excess of those needed to inhibit HIV replication in $CD4^+$ T cells. While the initial plasma half-life of EFdA was not prolonged (2 to 4 h, depending on the species), PBMCs isolated

from the rhesus macaque 24 h after dosing were completely refractory to SIV infection *in vitro*, suggesting that the intracellular longevity of the antiviral EFdA-TP in PBMCs is sufficient to prevent any HIV infection, despite the low levels of plasma EFdA. In agreement with these observations, preliminary intracellular pharmacokinetic analysis showed an intracellular half-life for EFdA-TP of >72 h in human PBMCs following a single 4-h exposure to EFdA. This prolonged intracellular half-life of the active form of EFdA suggests that single daily dosing of EFdA should suffice for maintaining good virus control because drug-exposed cells may remain refractory to infection for prolonged periods after initial drug dosing. These data also suggest that EFdA has excellent potential for use in prevention modalities such as preexposure prophylaxis.

Oral treatment with as little as 1.0 mg/kg/day EFdA completely protected SCID-hu Thy/Liv and NSG-hu Thy/Liv mice from developing plasma viremia after HIV challenge, and monotherapy with 10 mg/kg/day EFdA reduced plasma HIV RNA in viremic mice to undetectable levels within 2 weeks. The potent antiviral activity we observed is similar to that shown for EFdA by Hattori et al. (4) in human PBMC-reconstituted nonobese diabetic (NOD)/SCID/Janus kinase 3 (Jak3) knockout (NOJ) mice treated i.p. with 1 mg/kg/day EFdA beginning the day after i.p. $HIV_{JR\text{-}FL}$ inoculation. In addition, Murphey-Corb et al. (29) treated two SIV-infected macaques with advanced simian AIDS by twice-daily subcutaneous injection of EFdA (0.4 mg/kg per injection). Both animals showed plasma viral RNA levels with 3-to-4-log_{10} decreases within 1 week of initiation of EFdA therapy that eventually became undetectable, clinical signs of SIV disease resolved within the first month of treatment, and no drug toxicity was detected over a 6-month treatment period. We now show that low-dose EFdA is readily available in plasma following oral dosing in both mice and rhesus macaque and that the plasma levels of EFdA attained are quite sufficient to block HIV replication. Furthermore, EFdA is able to penetrate the blood-brain barrier in the rhesus macaque, as levels of the drug in CSF were at least 25% of the plasma level 8 h following oral administration. The preliminary pharmacokinetic data we present provide a basis to understand the excellent *in vivo* potency of EFdA noted here and in previous studies (29).

As seen in EFdA-treated macaques, HIV viremia rebounded in humanized mice after treatment cessation, but viral rebound was substantially slower in EFdA-treated mice than in mice treated with TDF plus FTC, which may reflect the greater level of suppression achieved by EFdA during the treatment period. We surmised that prolonged EFdA treatment might further reduce the rate of viral rebound after treatment cessation, but this was not the case in a follow-up NSG-hu mouse study in which all of the mice treated with EFdA for up to 12 weeks rebounded, most of them to pretherapy levels.

Since one of our EFdA-treated mice experienced outgrowth of HIV with M184V in RT and one mouse experienced outgrowth of M184I, it will be of key importance to monitor this resistance pathway in any future clinical trials of this drug. It should be noted that, while the M184V mutation is the major mutation for resistance to EFdA, the degree of resistance conferred *in vitro* is low (<10-fold). Furthermore, we previously showed that the rebound virus in the macaque was primarily M184V, and yet this virus remained suppressed during treatment with very low doses of EFdA (0.4 mg/kg twice daily) (29).

In summary, we have demonstrated both in primary human and rhesus macaque cells and in HIV-infected humanized mice the highly potent and selective anti-HIV activity of EFdA. These results strongly encourage further preclinical and clinical development of EFdA and also provide support for future humanized studies designed to study suppression of viremia and rebound from viral reservoirs in the mice after treatment cessation.

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