ment within a host expressing the relevant HLA class I allele. Indeed, while RC may be somewhat compromised compared to that of wild type, escape mutant viruses able to fully or partially evade CTL detection *in vivo* are almost certainly more virulent in the HLA-matched host environment, leading to increased virion production and thereby enhanced pathogenesis. The context dependency of viral fitness is similarly illustrated by antiretroviral resistance mutations such as M184V within reverse transcriptase (86–91): viruses harboring this mutation display relative *in vitro* replicative defects but are certainly more fit than their wild-type counterparts in the presence of lamivudine both *in vivo* and *in vitro*.

It is notable that viremia remained relatively unchanged over the study period. One potential explanation for this observation is the existence of two conflicting processes that offset each other: on the one hand, an increase in virus production (manifested as pVL) as a result of viral adaptation to its host and, on the other, a concomitant decrease in RC as a result of the fitness costs of this adaptation. To investigate this hypothesis, we conducted a multivariate analysis to examine the relationship between pVL and the year of diagnosis when conditioned on RC; however, no apparent trend was observed (data not shown); larger-scale studies will therefore be necessary to further investigate the interplay between these factors.

It was surprising to see such a clear decline in viral RC over the relatively short study period, especially in a country where HIV incidence and prevalence are low (HIV prevalence in Japan is <0.1%; [HIV and AIDS Data Hub for Asia-Pacific, http://www.aidsdatahub.org/]). However, Japan is relatively ethnically homogeneous and its population exhibits a far narrower HLA frequency spectrum than the populations of Western countries (92). This more limited HLA diversity may facilitate the rapid accumulation of CTL escape mutations in circulating HIV-1 sequences (93), most notably, those restricted by common Japanese HLA class I alleles (30, 31).

Intriguingly, lower viral RC was observed in HLA-A*24$^+$ patients than HLA-A*24$^-$ patients in earlier but not later periods of the Japanese epidemic. We propose the following interpretation for this observation: regardless of epidemic stage, viruses from HLA-A*24-positive patients carry A*24 escape mutations; therefore, no differences in RC are observed between early and late isolates from A*24-positive individuals. However, if single (or combinations of) A*24 escape mutants, some of which carry modest replicative costs, are transmitted to A*24-negative individuals and if some of these escape mutants persist in A*24-negative individuals, despite these modest replicative costs, it is possible that such mutations will increase in frequency in the general population over the course of the epidemic. If so, it is conceivable that the RC in A*24-negative individuals will concomitantly decline to levels similar to those in A*24-positive individuals over time. Although we did not observe any differences in the prevalence of particular amino acids within known A*24-restricted CTL epitopes in Gag and Protease in early versus later sequences, accumulation of A*24 CTL escape mutations within Nef in circulating viruses in Japan has been reported (31), suggesting that a similar phenomenon could be occurring in Gag. The link between A*24-associated immune pressure and temporal reductions in RC therefore remains speculative; future studies will be required to define CTL escape mutations for HLA alleles frequently observed in Japanese populations and demonstrate the accumulation of such mutations during the HIV epidemic.

Despite the limited statistical power of the present study to detect such associations, we performed an exploratory analysis to identify specific amino acid residues that could explain observed reductions in viral RC. Although we identified a number of putative amino acids that were associated with lower RCs at a $P$ value of $<0.05$ and whose frequency appeared to increase over the study period, these associations did not remain significant after correction for multiple comparisons, and thus, our results should be interpreted with caution. Larger studies aimed at identifying amino acids associated with temporal alterations in RC are therefore warranted.

Another caveat is that only Gag-Protease-associated viral RC was evaluated in the present study; as such, our results may not be representative of RC of whole virus isolates. The reasons for specifically investigating Gag-Protease-associated RC are 2-fold. First, our primary purpose was to investigate temporal changes in viral RC potentially attributable to HLA-associated immune pressures in Gag, a critical target of CTL responses. Whole-virus assays would have been confounded by the autologous envelope sequence, which is a major determinant of viral fitness (57) but is highly sensitive to infection stage due to shifts in coreceptor usage (94–96). Second, the generation and evaluation of large numbers of viruses require higher-throughput methods. Whole-virus isolates are traditionally replicated in primary CD4$^+$ T cells, but the laborious and costly nature of these assays precludes their application to large sample panels such as that used in the present study.

Despite these limitations, the present study sheds light on the replicative costs of HIV-1 adaptation to its human host. Additional, larger studies spanning greater durations of the HIV epidemic undertaken in different geographic areas and host populations, as well as studies elucidating the clinical implications of alterations in viral RC, are warranted, to determine whether this is a local or global phenomenon.

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INTERNAL MEDICINE

□ CASE REPORT □

# Hemophagocytic Syndrome in an Acute Human Immunodeficiency Virus Infection

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## Abstract

Hemophagocytic syndrome (HPS) is an unrecognized complication occurring in the acute stage of human immunodeficiency virus (HIV) infection that is seldom reported in the literature. We herein present a case of HPS that occurred during the seroconversion stage of HIV infection. In this case, pancytopenia and liver dysfunction related to HPS recovered after the initiation of antiretroviral therapy (ART). This report indicates the importance of early recognition of HPS and suggests that prompt initiation of ART has the potential to control HPS in the acute stage of HIV infection.

Key words: hemophagocytic syndrome, human immunodeficiency virus, acute HIV infection, antiretroviral therapy

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## Introduction

Virus-associated hemophagocytic syndrome (VAHS) is a rare but potentially life-threatening disease. The Epstein-Barr virus (EBV) is the leading trigger of VAHS, and human immunodeficiency virus (HIV) is an additional causative agent of hemophagocytic syndrome (HPS). Thus far, most cases of HIV-associated HPS have been reported in patients with advanced HIV stages, during which the patients also suffered from concomitant infections or malignant diseases. The mortality rate of HPS patients in the pre-antiretroviral therapy (ART) era was reported to be 50-100% (1, 2). Even in the highly active anti-retroviral therapy (HAART) era, the death rate of HPS patients with advanced stages of HIV reaches 31%. The common causes of HPS in advanced stages are opportunistic infections such as tuberculosis or cytomegalovirus (1). Early recognition is essential in order to obtain a favorable outcome for patients with HPS associated with HIV infection. However, the entire picture of HPS associated with HIV, in particular, HPS associated with acute HIV infection, is incomplete. The direct role of HIV as a causative agent of HPS remains to be determined. We herein report the case of an HPS patient with acute HIV infection who completely recovered from HPS following ART. This case provides a clue to the pathophysiology of HPS associated with acute HIV infection and suggests that the early initiation of ART is associated with a favorable outcome.

## Case Report

In mid-September 2010, a 48-year-old Japanese man visited a local hospital due to fever, chills and a sore throat lasting five days. He presented with a non-pruritic erythematous rash on the trunk and extremities. Empirical antibiotic therapy with cefotaxime sodium was administered; however, the fever persisted. The patient developed leukocytopenia, thrombocytopenia and abnormal liver function tests. He was referred to our hospital for a work-up fifteen days after the first visit to the local hospital. At admission, the patient developed oral candidiasis. However, he was apparently well oriented, and there were no remarkable findings on his physical examination, except for erythematous oral pharynx. The patient had no significant hepatosplenomegaly. His

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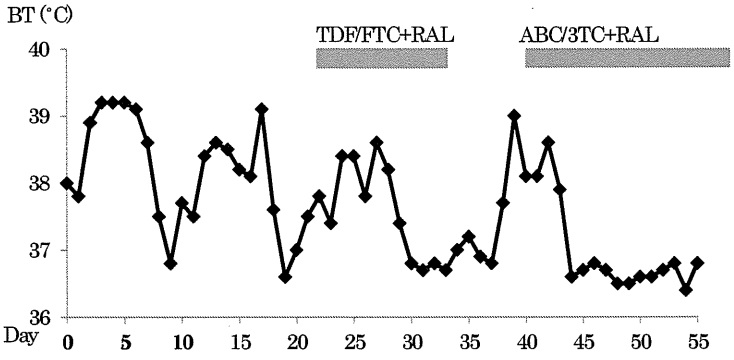


| Laboratory data/Day | D0 | D10 | D15 | D19 | D25 | D30 | D36 | D40 | D50 | D111 |
|---|---|---|---|---|---|---|---|---|---|---|
| WBC(/μL) | 3,750 | 1,890 | | 2,540 | 12,050 | 7,200 | 5,450 | 2,570 | | 3,830 |
| Hb(g/dL) | 12.1 | 12.9 | | 10 | 8.0 | 8.5 | 8.7 | 7.9 | | 13.2 |
| Plt(×104/μL) | 7.7 | 14.7 | | 8.8 | 33.3 | 23.7 | 30.9 | 24.4 | | 19.6 |
| AST(IU/L) | 68 | 181 | | 438 | 29 | 77 | 41 | 93 | | 23 |
| ALT(IU/L) | 50 | 124 | | 162 | 49 | 109 | 38 | 89 | | 21 |
| LDH(IU/L) | 963 | 1,077 | | 2,772 | 339 | 474 | 327 | 272 | | 180 |
| Ferr (ng/mL) | 2,112 | | | 34,192 | 1,939 | | 1,365 | | | |
| Cre(mg/dL) | 0.9 | 1.0 | | 1.7 | 1.0 | 5.3 | 1.5 | 1.3 | | 0.9 |
| BUN(mg/dL) | 9.1 | 5.3 | | 18.4 | 10.7 | 47.4 | 14.9 | 14.6 | | 15.8 |
| PT (%) | 109 | | | 122 | 97 | 57 | 90 | | | |
| TG(mg/dL) | 130 | | | 332 | | 266 | | | | 120 |
| CD4(/μL) | 98 | | 90 | | | 689 | | 147 | 290 | 547 |

Figure 1. The entire clinical course of the present patient. TDF/FTC: tenofovir/emtricitabine, RAL: raltegravir, ABC/3TC abacavir/lamivudine, WBC: white blood cell count, Hb: hemoglobin, Plt: platelet, AST: aspartate aminotransferase, ALT: alanine aminotransferase, LDH: lactate dehydrogenase, Ferr: ferritin, Cre: creatinine, BUN: blood urea nitrogen, PT: prothrombin time, TG: triglyceride, CD4: CD4 positive T lymphocyte count. D indicates days after admission.

chest X-ray was unremarkable with no identified foci of infection. No lymphadenopathies were noted on computed tomography (CT). At that time, the skin rash had already disappeared. The patient's body temperature was 38.0℃. The laboratory findings were as follows: white blood cell (WBC) count: 3,750/μL with 78% neutrophils, 18% lymphocytes and 3% monocytes; red blood cell (RBC) count: $383\times10^4$/μL; hemoglobin (Hb): 12.1 g/dL; platelet count (Plt): $7.7\times10^4$/dL; aminotransferase (AST): 68 IU/L; alanine aminotransferase (ALT): 50 IU/L; lactate dehydrogenase (LDH): 963 mg/dL; alkaline phosphatase (ALP): 228 IU/L; γ-glutamyltranspeptidase (γ-GTP): 42 IU/L; creatinine (Cre): 0.9 mg/dL; blood urea nitrogen (BUN): 9.1 mg/dL; ferritin: 2,112 mg/dL; and soluble interleukin-2 receptor (IL-2R): 1,090 U/mL. Neither pleocytosis nor elevated protein concentrations were found on a cerebrospinal fluid examination. The patient denied intravenous drug use but admitted to having occasional unprotected sexual intercourse with men one month prior to presentation. Screening for the HIV antigen/antibody test was positive, and a Western-blot (WB) analysis showed positivity for p24 proteins only and was scored as indeterminate. The patient's HIV-RNA level, CD4 T-cell count and CD8 T-cell count were 3,000,000 copies/mL, 98 cells/μL and 276 cells/μL, respectively. The presentation was consistent with that of a primary infection of HIV. Since acute HIV infection is generally a self-limited illness, we did not administer any specific treatments and instead carefully observed the patient's condition. The patient's entire clinical course is shown in Fig. 1. On the 13th day of hospitalization, the patient complained of right lower quadrant abdominal pain with tenderness, and the high-grade fever (39℃) relapsed. Radiological examinations performed on that day revealed no specific findings. The laboratory results obtained on the 20th day of hospitalization showed that the leukocytopenia, mild anemia, thrombocytopenia and levels of ALT, LDH and ferritin were progressively elevated (Fig. 1). The patient's fasting triglyceride level was also elevated to 332 mg/dL. A bone marrow biopsy was performed as one of the diagnostic procedures. A pathological examination of the bone marrow revealed normal cellularity with histiocyte infiltration and hemophagocytosis; however, no evidence of malignant cells was observed (Fig. 2). The hemoglobin level decreased to 8.0 g/dL. The findings (fever, bicytopenia, hypertriglyceridemia, hyperferritinemia and hemophagocytosis in the bone marrow) fulfilled the diagnostic criteria for HPS (3). The results of serological tests for other viral infections are shown in Table. The results showed that the patient had been previously in-

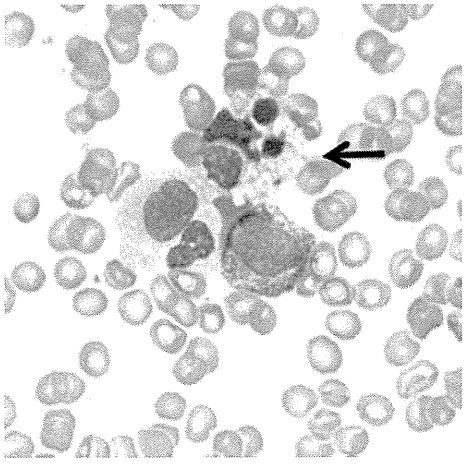

Figure 2. The microscopic findings of the bone marrow aspiration smear show mildly hypocellular marrow with activated macrophages and hemophagocytosis (arrow).

Table. Serological Tests for Other Viral Infections

| | | | |
|---|---|---|---|
| IgG VCA EBV | + | IgG HAV | - |
| IgM VCA EBV | - | IgM HAV | - |
| IgG EBNA | + | HCV Ab*2 | - |
| IgG EBV EA-DR | - | HBs Ag | - |
| IgG CMV | + | IgG HBs | - |
| IgM CMV | - | IgM HBc | - |
| CMV antigenemia*1 | - | IgG HBc | - |
| IgG HSV | - | IgG ParvoB19 | + |
| IgM HSV | - | IgM ParvoB19 | - |

VCA: viral capsid antigen, EBNA: EBV nuclear antigen, EA-DR: early antigen-diffuse restricted antibody, CMV: cytomegalovirus, HSV: herpes simplex virus, HAV: hepatitis A virus, HCV: hepatitis C virus, Ab: antibody, HBs: hepatitis B surface antigen, HBc: hepatitis B core antigen

*1 CMV antigenemia was performed 4 times during 2 months

*2 HCV Ab test was performed twice at 9-month intervals

fected with EBV and cytomegalovirus (CMV). However, he was not likely to have a reactivation of EBV or CMV because the tests for IgG EB early antigen-diffuse restricted antibodies and CMV antigenemia, markers of reactivation with immunodeficiency, were both negative. We did not perform a quantitative assay for human herpes virus (HHV)-8 DNA. From these findings, we diagnosed the patient with HPS complicated by an acute HIV infection. On the 20th day of hospitalization, the patient promptly received ART consisting of tenofovir/emtricitabine (TDF/FTC) and raltegravir (RAL) to prevent the progression of HPS. The patient's clinical condition subsequently improved and all symptoms, including the right lower abdominal pain, resolved. All blood laboratory test results returned to normal (Fig. 1). However, the patient developed acute renal failure one week after the initiation of ART. The liver failure was suspected to have been caused by tenofovir (Fig. 1). We subsequently discontinued the ART and carefully observed the patient's condition. Ten days after the discontinuation of ART, the high-grade fever relapsed and ART consisting of abacavir/lamivudine (ABC/3TC) and RAL was resumed. The patient's fever promptly subsided. The CD4 T-cell count rapidly increased to 689 cells/μL after four weeks of ART and remained stable thereafter. The HIV-RNA level showed a progressive decline and became undetectable six months after the initiation of ART. The patient has remained on ART and has never exhibited any clinical signs of HPS recurrence. Eleven months after the initial examination, the results of HIV WB, which showed the reactive bands of p 24, gp120 and gp160, were found to be positive.

## Discussion

Hemophagocytic syndrome, also called hemophagocytic lymphohistiocytosis (HLH), is a rare disease caused by a dysfunction of cytotoxic T-cells and natural killer (NK) cells. Impaired activity of NK cells and cytotoxic T-cells leads to cytokine dysregulation with proliferation and activation of histiocytes. Among the causes of secondary HPS, viral infection is the leading trigger for the abnormal functioning of macrophages. Any virus can cause HPS, although EBV is the most common causative agent. HIV itself can also be a causative agent of secondary HPS.

Since acute HIV infection is generally a self-limited illness, most clinicians do not administer any specific treatments for acute HIV infection, although controversy regarding this issue still exists. Persistent fever, splenomegaly and cytopenias, which are included in the diagnostic criteria of HPS, are often observed in HIV-infected patients in the seroconversion stage. Therefore, HPS is often underdiagnosed in acute HIV patients. High-dose corticosteroids are commonly used in the HIV-negative HPS setting; however, the use of high-dose corticosteroids to treat acute HIV infections remains highly controversial, as these drugs can promote HIV replication. In the present case, we were confronted with rapid disease progression and we made a decision to initiate ART. Subsequent to the administration of ART, the patient improved substantially. We would therefore like to propose that the administration of ART is viable a treatment option for HPS patients with acute HIV infections.

To the best of our knowledge, only seven cases of HPS associated with acute HIV infection have so far been reported in the English literature (4-8). In contrast to the later stages of HIV infection, the prognosis of HPS patients with acute HIV infections seems favorable. Indeed, all of the eight patients reported previously achieved complete recovery from HPS after the administration of ART or supportive therapy such as intravenous immunoglobulin or steroids. Of eight cases, four involved the administration of ART during acute HIV infection. The significant difference observed between the prognosis of HPS patients with acute HIV infection and that of HPS patients in advanced stages of HIV might be related to the CD4 T-cell count at the time of HPS diagnosis, as worse outcomes are observed in patients with CD4 T-cell counts less than 200 cells/μL (1).

Although HPS itself can be the cause of acute renal failure, in this case, the renal dysfunction was strongly suspected to be associated with TDF (9). The patient's renal dysfunction quickly improved following a temporal discon-

tinuation of ART and did not recur after resuming ART with an alternative regimen that did not include TDF. Among seven previously reported acute HIV-HPS cases, only one was complicated by acute renal failure, and that patient did not receive ART.

Six of the eight reported cases of acute HIV-HPS occurred in East Asia (Taipei, Korea and Japan (our case)). Of note, EBV-associated HPS also has a high incidence in the Far East. Although the precise mechanism underlying this phenomenon remains unclear, the presence of a pathogenic EBV strain in these countries is suspected (10). A specific polymorphism related to tumor necrosis factor (TNF)-α has been reported to be associated with increased susceptibility to secondary HPS in the Korean population (11). Regarding HIV strains, the presence of pathogenic HIV strains associated with HPS in these countries is unconfirmed. The fact that identical HIV strains were isolated from an acute HIV-**HPS patient** and an asymptomatic partner suggests that host genetic factors, rather than viral pathogenesis, may affect the occurrence of HPS in patients with acute HIV infections (5).

In this report, we highlight HIV as a causative agent of HPS and propose that ART may be effective in controlling acute HIV-HPS. Clinicians should therefore bear in mind that the prompt initiation of appropriate ART has the potential to control acute HIV-HPS.

The authors state that they have no Conflict of Interest (COI).

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# Balancing Antiviral Potency and Host Toxicity: Identifying a Nucleotide Inhibitor with an Optimal Kinetic Phenotype for HIV-1 Reverse Transcriptase

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## ABSTRACT

Two novel thymidine analogs, 3′-fluoro-3′-deoxythymidine (FLT) and 2′,3′-didehydro-3′-deoxy-4′-ethynylthymidine (Ed4T), have been investigated as nucleoside reverse transcriptase inhibitors (NRTIs) for treatment of HIV infection. Ed4T seems very promising in phase II clinical trials, whereas toxicity halted FLT development during this phase. To understand these different molecular mechanisms of toxicity, pre–steady-state kinetic studies were used to examine the interactions of FLT and Ed4T with wild-type (WT) human mitochondrial DNA polymerase γ (pol γ), which is often associated with NRTI toxicity, as well as the viral target protein, WT HIV-1 reverse transcriptase (RT). We report that Ed4T-triphosphate (TP) is the first analog to be preferred over native nucleotides by RT but to experience negligible incorporation by WT pol γ, with an ideal balance between high antiretroviral efficacy and minimal host toxicity. WT pol γ could discriminate Ed4T-TP from dTTP 12,000-fold better than RT, with only an 8.3-fold difference in discrimination being seen for FLT-TP. A structurally related NRTI, 2′,3′-didehydro-2′,3′-dideoxythymidine, is the only other analog favored by RT over native nucleotides, but it exhibits only a 13-fold difference (compared with 12,000-fold for Ed4T) in discrimination between the two enzymes. We propose that the 4′-ethynyl group of Ed4T serves as an enzyme selectivity moiety, critical for discernment between RT and WT pol γ. We also show that the pol γ mutation R964C, which predisposes patients to mitochondrial toxicity when receiving 2′,3′-didehydro-2′,3′-dideoxythymidine to treat HIV, produced some loss of discrimination for FLT-TP and Ed4T-TP. These molecular mechanisms of analog incorporation, which are critical for understanding pol γ-related toxicity, shed light on the unique toxicity profiles observed during clinical trials.

## Introduction

Since the development of AZT in 1985 (Mitsuya et al., 1985), NRTIs have been vital in the treatment of HIV infection. NRTIs inhibit RT by serving as nucleoside mimics; after phosphorylation by cellular kinases, the inhibitors are incorporated into DNA during reverse transcription. Currently available NRTIs lack a 3′-hydroxyl group, and incorporation causes immediate termination of polymerization. Despite the success of NRTIs, limitations include acquired resistance to

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**ABBREVIATIONS:** AZT, zidovudine; NRTI, nucleoside reverse transcriptase inhibitor; FLT, 3′-fluoro-3′-deoxythymidine; Ed4T, 2′,3′-didehydro-3′-deoxy-4′-ethynylthymidine; EFdA, 4′-ethynyl-2-fluoro-2′-deoxyadenosine; WT, wild-type; RT, reverse transcriptase; pol γ, DNA polymerase γ; exo$^+$ pol γ, exonuclease-competent DNA polymerase γ; dT, thymidine; dA, adenine; TP, triphosphate; MP, monophosphate; FDA, U.S. Food and Drug Administration; d4T, 2′,3′-didehydro-2′,3′-dideoxythymidine; $k_{exo}$, excision rate; $k_{pol}$, nucleotide incorporation rate; ddA, 2′,3′-dideoxyadenosine; ddC, 2′,3′-dideoxycytidine.

RT and host toxicity, which can manifest as neuropathy, lactic acidosis, and hepatotoxicity (Apostolova et al., 2011).

Although some toxicity can be attributed to mechanisms such as inhibition of phosphorylation (Apostolova et al., 2011), the primary cause of NRTI toxicity is inhibition of human mitochondrial DNA polymerase γ (pol γ) (Kohler and Lewis, 2007; Koczor and Lewis, 2010). Pol γ, which replicates the human mitochondrial genome, is a heterotrimer containing a catalytic monomer, with polymerization and exonuclease domains, and an accessory homodimer, which improves incorporation efficiency and processivity (Johnson et al., 2000; Lee et al., 2009). Pol γ is a member of the A family of DNA polymerases, which are most similar to RT because of fold commonalities and conserved active site residues (Bienstock and Copeland, 2004). Therefore, pol γ is more prone to insert NRTIs during replication, and side effects are often indicators of mitochondrial toxicity (Brinkman et al., 1999).

The dT-analog NRTIs AZT and d4T (stavudine) have been approved by the FDA for treatment of HIV infection but are plagued by toxicity (Lee et al., 2003), which highlights the need for safer NRTIs. Two dT analogs [i.e., FLT and Ed4T (festinavir)] have been under investigation (Fig. 1). In vitro work showed that FLT inhibited viral replication more effectively than did AZT (Kong et al., 1992), with slow evolution of FLT-resistant mutations in RT (Kim et al., 2001). However, FLT-treated rats experienced mitochondrial DNA depletion (Venhoff et al., 2009). Patient toxicity, including two deaths that resulted from hepatic failure, halted clinical trials in phase II (Flexner et al., 1994). Interest in FLT was renewed somewhat with reports that FLT is effective at lower, less toxic doses (De Clercq, 2010). Phase II clinical trials with Ed4T have proven more promising despite the compound's structural similarity to d4T (which lacks the 4′-ethynyl group), a relatively toxic NRTI that inhibits pol γ (Johnson et al., 2001; Bailey et al., 2009). In vitro studies showed that Ed4T is 5 times more potent and significantly less toxic than d4T (Dutschman et al., 2004; Paintsil et al., 2007), and development of RT resistance is slow (Yang et al., 2009).

Despite documented mitochondrial toxicity for FLT, and the potential for mitochondrial toxicity for Ed4T because of structural similarity to d4T, studies directly probing their interactions with pol γ in relation to RT are limited. Often NRTI potency comes at the expense of toxicity resulting from increased WT pol γ inhibition; therefore, both limited analog incorporation by WT pol γ and efficient incorporation by RT are vital. The ideal balance of potency and toxicity involves more efficient analog incorporation compared with native nucleotides by RT and negligible analog incorporation by WT pol γ. However, this has not yet been achieved.

Previous steady-state studies showed that FLT-TP and Ed4T-TP interact with RT and WT pol γ. Unfortunately, steady-state experiments provide information only on the rate-limiting step of catalysis. For these two polymerases, the rate of polymerization is masked by the slow product-release step. Complete characterization of the analog kinetic profiles was needed, including the analog affinity and incorporation rates for both enzymes and the rates of analog removal by pol γ. Pre–steady-state kinetic data were required to generate these molecular mechanisms of toxicity, which correspond well to the degree of mitochondrial toxicity observed among patients (Johnson et al., 2001). We also examined the R964C pol γ mutant, because patients with this mutation, which is located in the polymerase domain of the pol γ catalytic subunit, exhibit higher rates of mitochondrial toxicity when using d4T (Yamanaka et al., 2007).

We found that WT pol γ was 1400-fold better at distinguishing Ed4T-TP from dTTP than distinguishing FLT-TP from dTTP, and this specificity was moderately impaired in R964C pol γ. Conversely, RT preferred Ed4T-TP to FLT-TP and dTTP. It is noteworthy that Ed4T-TP is the first analog to show this exemplary balance of preferred incorporation by RT and negligible incorporation by WT pol γ. We propose that the didehydro ring of Ed4T (and d4T) is important for achieving impaired discrimination in RT, whereas the 4′-ethynyl group serves as an enzyme selectivity moiety that supports the high level of discrimination by WT pol γ. The unique kinetic mechanisms of interaction for FLT and Ed4T help explain the high levels of toxicity observed for FLT and predict lower levels of toxicity for Ed4T.

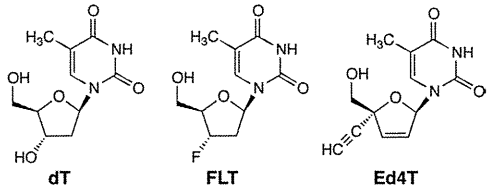


**Fig. 1.** Structures of dT and its analogs.

## Materials and Methods

**Reagents.** dTTP was purchased from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). Triphosphate versions of FLT and Ed4T were prepared as described previously (Ray et al., 2002c). The DNA oligonucleotides D22 (5′-GCCTCGCAGCCGTCCAACCAAC-3′) and D45 (3′-CGGAGCGTCGGCAGGTTGGTTGAGTTGGAGCTAG-GTTACGGCAGG-5′) were purchased from Integrated DNA Technologies, Inc. (Coralville, IA) and were purified on 20% polyacrylamide denaturing gels. T4 polynucleotide kinase (New England Biolabs, Ipswich, MA) was used to label the D22 oligonucleotide at the 5′ terminus with [$\gamma$-$^{32}$P]ATP (PerkinElmer Life and Analytical Sciences, Waltham, MA). This D22 primer was then annealed to the D45 template, as described previously (Ray et al., 2002a), to generate the DNA primer/template substrate.

**Enzymes.** The recombinant WT accessory subunit of pol γ was expressed and purified as described previously (Johnson et al., 2000). All pol γ catalytic subunits used contained an N-terminal hexa-histidine tag. The recombinant, exonuclease-deficient, WT catalytic subunit (i.e., WT pol γ) was expressed and purified as described previously (Graziewicz et al., 2004), with minor modifications in the strategy for chromatography. Specifically, WT pol γ was eluted from a nickel column by using a linear gradient of 20 to 400 mM imidazole. The recombinant exonuclease-competent pol γ catalytic subunit (exo$^+$ pol γ) was expressed and purified as described previously (Longley et al., 1998; Lim et al., 2003; Kasiviswanathan et al., 2010). Site-directed mutagenesis was used to generate the R964C pol γ construct, and the recombinant protein was expressed and purified as detailed elsewhere (Kasiviswanathan et al., 2010).

The recombinant RT (p66/p51 heterodimer) clone was kindly provided by Drs. Stephen Hughes and Andrea Ferris (Frederick Cancer Research and Development Center, Frederick, MD). The C-terminal hexa-histidine-tagged RT was purified as described previously (Kerr and Anderson, 1997; Kim et al., 2012). The purity of all proteins, as judged through SDS-polyacrylamide gel electrophoresis with Coomassie staining, was >90%.

**Single-Nucleotide Incorporation Assays.** Single-nucleotide incorporation experiments were performed by using a KinTek RQF-3

rapid chemical quench apparatus (KinTek Corp., Austin, TX) operated at 37°C. To determine the active-site concentration of each of the enzymes used, dTTP incorporation into the D22/D45 primer/template substrate was examined under burst conditions, as described previously (Murakami et al., 2003). Single-turnover conditions were used to examine the rates of dTTP, FLT-TP, and Ed4T-TP incorporation. For incorporation by WT pol γ and R964C pol γ, 100 nM pol γ catalytic subunit (active-site concentration) was preincubated with an excess of pol γ accessory subunit (4-fold higher concentration than the total, non–active-site, concentration of the catalytic subunit). Holoenzyme (heterotrimer) at 100 nM and 25 nM DNA primer/template substrate in pol γ reaction buffer (50 mM Tris, pH 7.8 at 37°C, 100 mM NaCl) were rapidly mixed with 2.5 mM $MgCl_2$ and varying concentrations of dTTP or analog. Single-nucleotide incorporation experiments with RT were performed in the same manner as the pol γ experiments except that 100 nM RT was used instead of pol γ holoenzyme. Reported concentrations are final (after mixing). After pre–steady-state time periods, the reactions were quenched with 0.3 M EDTA, pH 8.0, and the products were separated on 20% polyacrylamide denaturing gels (0.4 mm thick; 8 M urea). After quantification through filmless autoradiographic analysis with a Bio-Rad Molecular Imager FX (Bio-Rad, Hercules, CA), Kaleidagraph software (Synergy Software, Reading, PA) was used to fit plots of product concentrations versus time to a single exponential equation: product concentration = $A[1 - \exp(-k_{obs}t)]$, where $A$ is the amplitude, $k_{obs}$ is the observed first-order rate constant for dTTP or analog incorporation, and $t$ is the reaction time. The $k_{obs}$ values were plotted against nucleotide concentrations to generate the maximal nucleotide incorporation rate ($k_{pol}$) and $K_d$ (i.e., the dissociation constant for the nucleotide at the enzyme-primer/template substrate complex) through fitting to a hyperbolic equation: $k_{obs} = (k_{pol} \times \text{dNTP concentration})/(K_d + \text{dNTP concentration})$, with Kaleidagraph software.

**Excision Reactions.** To incorporate FLT-TP into the 5′-radiolabeled D22/D45 primer/template substrate, 8.0 μM FLT-TP, 5.0 μM DNA primer/template substrate, 1 μM WT pol γ holoenzyme, and 10 mM $MgCl_2$ were incubated for 3 h in pol γ reaction buffer at 37°C. For Ed4T-TP incorporation, 30 μM Ed4T-TP, 5.0 μM DNA primer/template substrate, 1.0 μM RT, and 10 mM $MgCl_2$ were incubated for 2 h in RT reaction buffer (50 mM Tris, pH 7.8 at 37°C, 50 mM NaCl) at 37°C. The reactions were optimized such that the analog incorporation reaction achieved completion. The remaining experimental parameters were the same for both FLT-MP and Ed4T-MP. The incubation mixtures were purified on 20% denaturing polyacrylamide sequencing gels (8 M urea) and, by using filmless autoradiographic analysis, the radiolabeled band, corresponding to D22 with the analog incorporated at position 23 (D22-FLT-MP or D22-Ed4T-MP), was removed. The DNA was extracted from the gel through gentle mixing overnight at room temperature, in a solution containing 0.5 M ammonium acetate, 20 mM magnesium acetate, 1 mM EDTA, and 0.1% SDS. After ethanol extraction (with a 75% ethanol solution for 4 h at −80°C) and drying, the D22-analog primer was rephosphorylated and reannealed as described previously, to generate the DNA primer-analog/template substrate (Ray et al., 2002a). An incubation of 150 nM exo$^+$ pol γ holoenzyme and 112 nM DNA primer-analog/template substrate in pol γ reaction buffer was manually mixed with 5 mM $MgCl_2$ to initiate the excision reaction at 37°C, under single-turnover conditions. All reported concentrations are final (after mixing). Aliquots of the mixture were removed at various time points and quenched with 0.3 M EDTA, pH 8.0. A 20% denaturing polyacrylamide sequencing gel (0.4 mm thick, 8 M urea) was used to separate the products, and filmless autoradiographic analysis was used to quantify the loss of substrate. A plot of percent substrate versus time was fit to a single exponential decay equation to generate the excision rate ($k_{exo}$). For Ed4T-MP excision, the plot was fit to a Boltzman sigmoidal equation, and the inflection point concentration was used to determine the half-life, which is proportional to $k_{exo}$ under pseudo-first-order kinetics.

## Results

**FLT-TP and Ed4T-TP Discrimination by WT pol γ.** A critical component of the in vitro toxicity profiles for novel NRTIs is the extent of incorporation by WT pol γ. Previous data on the interactions of these inhibitors with WT pol γ were limited to $IC_{50}$ of 2.7 μM (Cheng et al., 1987) and $K_i$ of 50 nM (Wińska et al., 2010) for FLT-TP and $IC_{50}$ of 100 μM for Ed4T-TP [100-fold higher than that for d4T-TP (Yang et al., 2007)]. Steady-state kinetic data reflect only the rate-limiting step of catalysis, which is product release for many polymerases, including WT pol γ. Therefore, rate constants associated with polymerization cannot be determined from such studies. We used pre–steady-state kinetic analyses to determine the $k_{pol}$ and $K_d$ for FLT-TP and Ed4T-TP incorporation, relative to dTTP.

Under single-turnover conditions, WT pol γ holoenzyme and the DNA primer/template substrate were rapidly mixed with $MgCl_2$ and varying concentrations of dTTP, FLT-TP, or Ed4T-TP for varying times. Triphosphate versions of the drugs were used because the cellular kinases required to phosphorylate the NRTI prodrugs were not present in these in vitro studies. After reaction quenching, the products were separated through gel electrophoresis, and filmless autoradiographic analysis allowed quantification of incorporation of the single correct nucleotide to form the D23 product. The amount of product formed was plotted against the reaction time. This was fit to a single exponential equation to generate the observed rate of nucleotide incorporation, $k_{obs}$, at each concentration (Fig. 2). These $k_{obs}$ values were then plotted against nucleotide incorporation and fit to a hyperbola to generate $k_{pol}$ and $K_d$ values (Fig. 3).

WT pol γ was able to incorporate dTTP with very high efficiency. The $k_{pol}$ for FLT-TP incorporation by WT pol γ was ~260-fold slower than that for dTTP incorporation (Table 1). However, the affinity for FLT-TP was actually 7.5-fold greater than that for dTTP, which means that WT pol γ preferred the nucleoside analog on the basis of $K_d$ alone. Because of this finding, the overall efficiency of FLT-TP incorporation was only 35-fold lower than that of dTTP incorporation, which indicates that FLT-TP may serve as a substrate for WT pol γ in vivo to a modest but significant extent. This kinetic profile of FLT-TP interaction with WT pol γ supports the evidence of mitochondrial toxicity in vitro (de Baar et al., 2007) and in vivo (Venhoff et al., 2009).

Ed4T-TP demonstrated a different kinetic mechanism of interaction with WT pol γ, compared with that seen for FLT-TP. The $k_{pol}$ for Ed4T-TP incorporation was 2100-fold and 7.9-fold slower than those for dTTP and FLT-TP, respectively (Table 1). Ed4T-TP also showed superiority to FLT-TP in its weak affinity for WT pol γ, with 3- and 23-fold increases in $K_d$, compared with dTTP and FLT-TP, respectively. Overall, impressive 6200-fold and 180-fold decreases in incorporation efficiency were seen for Ed4T-TP relative to dTTP and FLT-TP, respectively. This high level of discrimination against Ed4T-TP indicates that incorporation of this inhibitor in the physiological nucleotide milieu, where individual dNTP concentrations range from low micromolar to submillimolar levels in mammalian (rat) mitochondria (Song et al., 2005; Wheeler and Mathews, 2011), probably is a rare event. This is supported by the findings of low toxicity in vitro (Haraguchi et al., 2003; Dutschman et al., 2004; Tanaka et al., 2005).

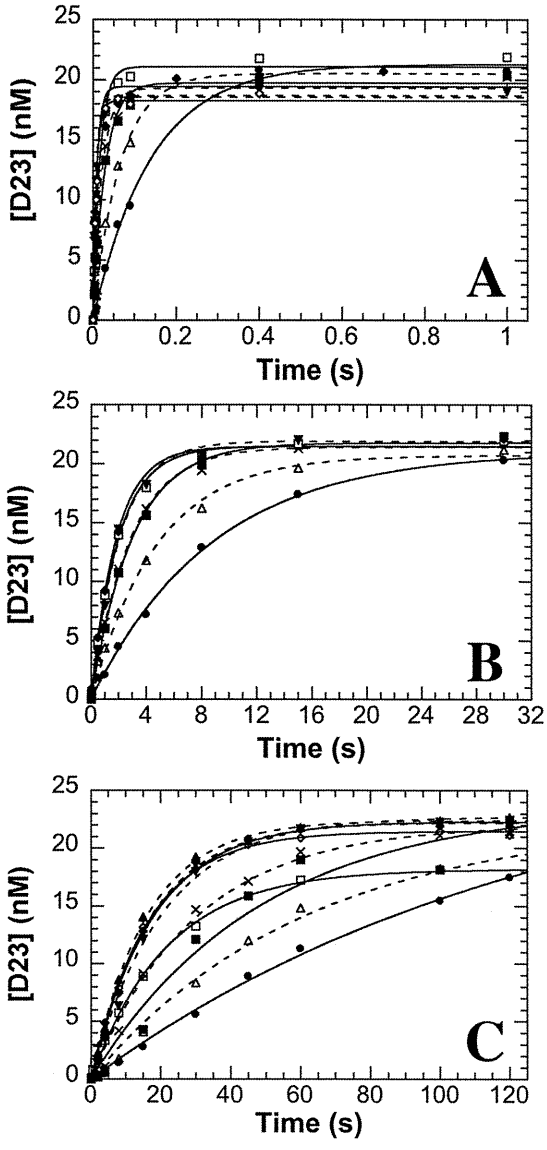


**Fig. 2.** Observed rates of incorporation by WT pol γ of dTTP or dTTP analogs opposite dA. Each point in the plot represents a single observation, and single exponential equations were used to fit the kinetic traces at varying concentrations of dTTP or dTTP analog. These six to eight experiments all contributed to the $k_{pol}$ and $K_d$ rate constant values. A, incorporation of dTTP. Concentrations of dTTP are denoted as follows: ●, 0.20 μM; △, 0.40 μM; ■, 0.80 μM; ×, 1.5 μM; □, 3.0 μM; ▼, 6.0 μM; ♦, 8 μM; +, 13 μM; ◇, 20 μM. B, incorporation of FLT-TP. Concentrations of FLT-TP are denoted as follows: ●, 0.10 μM; △, 0.25 μM; ■, 0.50 μM; ×, 1.0 μM; □, 2.0 μM; ▼, 5.0 μM; ♦, 8.0 μM. C, incorporation of Ed4T-TP. Concentrations of Ed4T-TP are denoted as follows: ●, 1.5 μM; △, 2.5 μM; ■, 5.0 μM; ×, 8.0 μM; □, 10 μM; ▼, 20 μM; ♦, 30 μM; +, 40 μM; ◇, 50 μM; ▲, 75 μM.

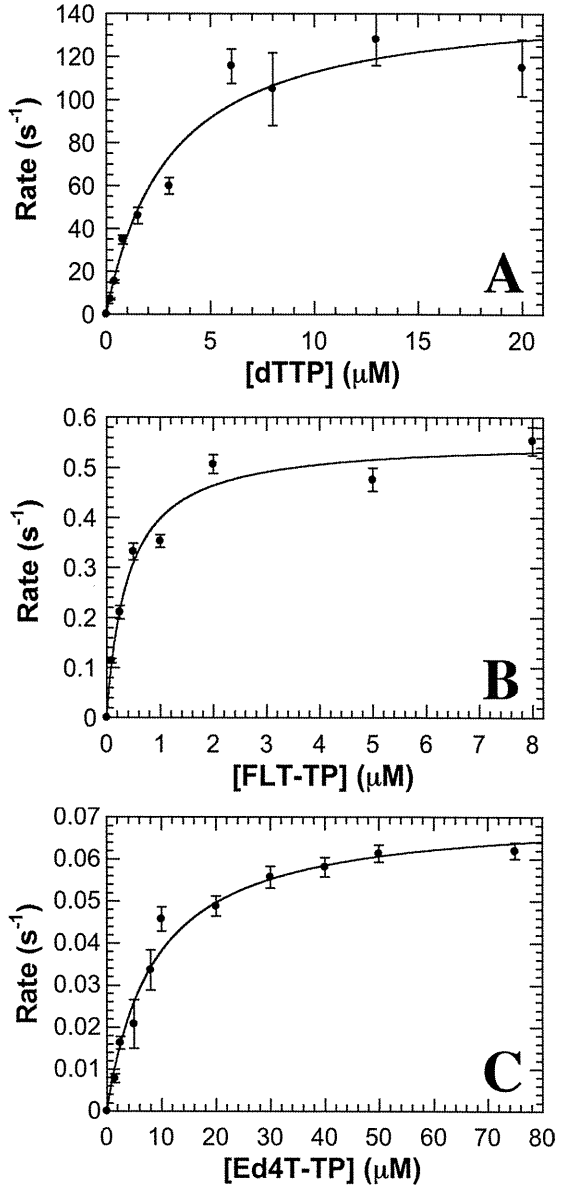


**Fig. 3.** Nucleotide concentration dependence of the observed rate of WT pol γ incorporation of dTTP analogs opposite dA. Hyperbolic equations were used to fit plots of the observed rate constants (generated from Fig. 2) versus dTTP analog concentrations to obtain $k_{pol}$ and $K_d$ values. Each point represents the observed rate generated from Fig. 2, and the S.E. is the deviance from the hyperbolic fits. Values for $k_{pol}$, $K_d$, and efficiency are presented in Table 1. A, incorporation of dTTP. B, incorporation of FLT-TP. C, incorporation of Ed4T-TP.

**FLT-TP and Ed4T-TP Discrimination by RT.** The characterization of the interactions of these analogs with RT is limited, with previous findings including a $K_i$ value of 5 nM for the inhibition of RT by FLT-TP (Cheng et al., 1987) and pre–steady-state studies showing that RT preferred dTTP 1.9-fold over Ed4T-TP, compared with a 4.5-fold preference for dTTP over d4T (Yang et al., 2008). We undertook pre–steady-state, single-turnover experiments in which RT and the DNA-primer/template substrate were rapidly mixed with $MgCl_2$ and varying concentrations of dTTP, FLT-TP, or Ed4T-TP (Fig. 4).

A 2.2-fold decrease in $k_{pol}$ and a 2-fold increase in $K_d$ were observed for FLT-TP incorporation by RT, relative to dTTP, which resulted in 4.2-fold greater efficiency for dTTP, compared with FLT-TP (Fig. 5 and Table 1). Although there was some preference for the native nucleotide, the incorporation efficiency, which was similar to that for dTTP, indicated that RT would incorporate FLT-TP at significant rates in vivo. The $k_{pol}$ for Ed4T-TP incorporation was identical to that of dTTP. It is noteworthy that the binding affinity was greater, as evidenced by the 2-fold decrease in $K_d$ relative to dTTP, which indicates that RT incorporated Ed4T-TP 2.0-fold more efficiently than dTTP (Fig. 5 and Table 1).

The discrimination by WT pol γ (Table 1) compared favorably with that of RT. RT showed a 8.3-fold loss of discrimi-

TABLE 1
Kinetic parameters for WT pol γ, RT, and R964C pol γ
Efficiency indicates $k_{pol}/K_d$. Discrimination indicates efficiency$_{dTTP}$/efficiency$_{analog}$.

| Enzyme and dTTP Analog | $k_{pol}$ | $K_d$ | Efficiency | Discrimination |
|---|---|---|---|---|
| | $s^{-1}$ | $\mu M$ | $\mu M^{-1} s^{-1}$ | |
| WT pol γ | | | | |
| dTTP | 147 ± 12 | 3.0 ± 0.8 | 49 | N.A. |
| FLT-TP | 0.56 ± 0.03 | 0.40 ± 0.08 | 1.4 | 35 |
| Ed4T-TP | 0.071 ± 0.003 | 9 ± 1 | 0.0079 | 6200 |
| RT | | | | |
| dTTP | 1.25 ± 0.06 | 3.0 ± 0.4 | 0.42 | N.A. |
| FLT-TP | 0.58 ± 0.05 | 6 ± 1 | 0.1 | 4.2 |
| Ed4T-TP | 1.25 ± 0.05 | 1.5 ± 0.2 | 0.83 | 0.51 |
| R964C pol γ | | | | |
| dTTP | 129 ± 8 | 3.7 ± 0.7 | 35 | N.A. |
| FLT-TP | 0.51 ± 0.03 | 0.23 ± 0.06 | 2.2 | 16 |
| Ed4T-TP | 0.11 ± 0.01 | 7 ± 2 | 0.02 | 1800 |

N.A., not applicable.

nation for FLT-TP incorporation, relative to WT pol γ, and a striking 12,000-fold decrease in discrimination was seen when Ed4T-TP incorporation by RT was compared with that by WT pol γ. RT is much more likely to incorporate these inhibitors than is WT pol γ. These findings can explain the low toxicity observed to date for Ed4T-TP, although additional clinical testing is needed.

**Excision of FLT-MP and Ed4T-MP by exo$^+$ pol γ.** An important consideration in the in vitro toxicity profile regarding NRTI incorporation by pol γ is the enzyme's inherent proofreading capability. Nucleoside analog incorporation by pol γ can be essentially negated if excision is efficient. On the basis of nucleoside analog incorporation efficiency, either WT pol γ or RT was used to incorporate a single FLT-TP or a single Ed4T-TP into the DNA primer/template substrate. The D22-FLT-MP/D45 or D22-Ed4T-MP/D45 primer/template was then used as a substrate in the excision reaction. Under single-turnover conditions, exo$^+$ pol γ holoenzyme and the DNA primer-analog/template substrate were manually mixed with $MgCl_2$, and the reaction was quenched after various times. After separation of the products through gel electrophoresis and quantification of the substrate band, the proportion of substrate was plotted versus time (Fig. 6) and the data were fit to a single exponential equation to obtain $k_{exo}$ values, as well as a sigmoidal equation in the case of Ed4T-MP excision (Fig. 6B, inset).

FLT-MP and Ed4T-MP were excised at similar modest rates, although Ed4T-MP excision was slower with the sigmoidal fit (Fig. 6). Half-lives for the exo$^+$ pol γ-D22-analog/D45 complex were 8.8 and 9.6 min for FLT and Ed4T, respectively. These rates of excision were approximately 34-fold slower, on average, than the excision rate for a correctly inserted dT for this primer/template substrate (0.042 $s^{-1}$) (Feng et al., 2001). However, both analogs were removed more efficiently than ddC and ddA, which have $k_{exo}$ values of 0.0003 $s^{-1}$ (Hanes and Johnson, 2008) and 0.0005 $s^{-1}$ (Johnson et al., 2001), respectively.

**FLT-TP and Ed4T-TP Discrimination by R964C pol γ.** It was established that d4T-treated patients with the R964C pol γ mutation have higher rates of mitochondrial toxicity (Yamanaka et al., 2007), primarily because of moderate defects in activity and lower levels of nucleoside analog discrimination (Bailey et al., 2009). To determine whether such alterations in pol γ activity were extended to other dTTP analogs, the single-turnover, pre–steady-state experiments performed with WT pol γ were repeated with the R964C pol γ holoenzyme (Fig. 7). Such studies are important to determine whether patients with the R964C pol γ mutation who receive FLT or Ed4T are at higher risk for mitochondrial toxicity.

The incorporation efficiency of dTTP decreased only slightly (1.4-fold) relative to WT pol γ (Table 1), which is consistent with the 1.5-fold loss of efficiency measured previously (Bailey et al., 2009). R964C pol γ was able to incorporate FLT-TP with slightly (1.6-fold) higher efficiency than WT pol γ, and Ed4T-TP was incorporated 2.5-fold more efficiently by R964C pol γ than by WT pol γ (Table 1). When overall discrimination (which takes into account defects in dTTP incorporation) was considered, R964C pol γ showed 2.2-fold and 3.4-fold decreases in discrimination for FLT-TP and Ed4T-TP, respectively, relative to WT pol γ (Table 1).

## Discussion

The interactions of FLT-TP and Ed4T-TP with WT pol γ and RT were unique, which clarified the reported differences in toxicity. The incorporation of FLT-TP by WT pol γ was 35-fold slower than that of dTTP, similar to findings for the more toxic NRTIs on the market, compared with the 1000- to nearly 1,000,000-fold slower incorporation for other low-toxicity inhibitors (Table 2). This indicates that some toxicity results from FLT-TP incorporation. On the basis of affinity alone, FLT-TP was preferred over dTTP by WT pol γ. Studies examining the potential for toxicity have focused on pol γ with the analog as a substrate, but we propose that this low $K_d$ indicates that the mitochondrial toxicity observed for FLT may result from direct inhibition. It is likely to be more complex than simple competition, because it was shown that FLT-TP is a noncompetitive inhibitor of pol γ (Wińska et al., 2010). It was proposed that mitochondrial toxicity results in part from competitive inhibition of host thymidine kinase 2 by FLT (Wang et al., 2011), and it is likely that many factors contribute to FLT toxicity.

Ideally, discrimination against dNTP analogs by WT pol γ would be high, whereas discrimination by RT would be low. This would indicate that WT pol γ could readily distinguish among NRTIs and native nucleotides, whereas RT could not. There is only a slight preference for dTTP over FLT-TP with RT, which compares favorably to data for many FDA-approved NRTIs and inhibitors under development (Table 2).

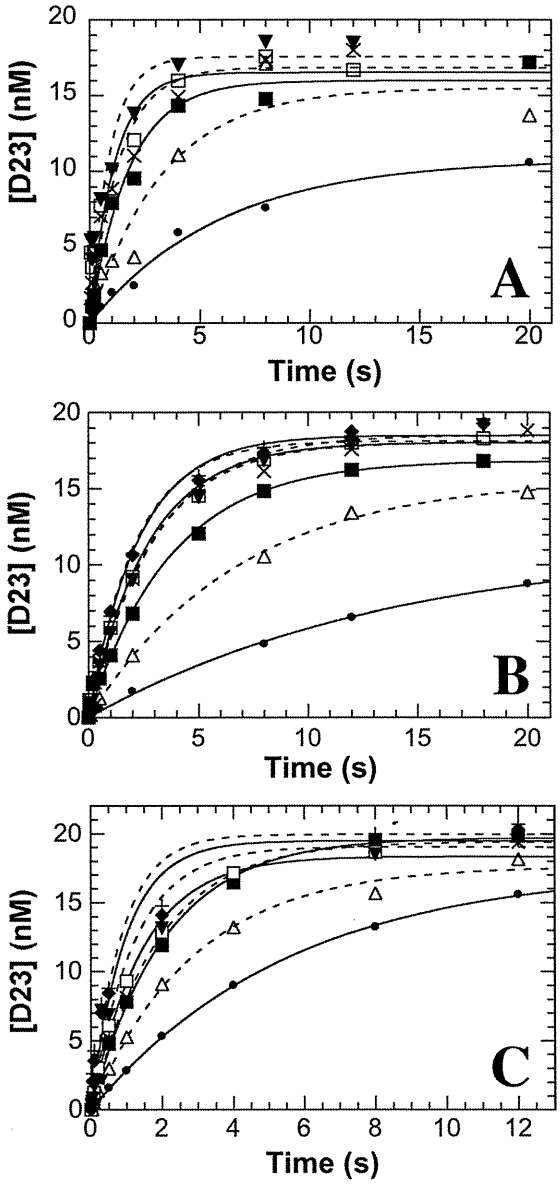


**Fig. 4.** Observed rates of incorporation by RT of dTTP or dTTP analogs opposite dA. Each point in the plot represents a single observation, and single exponential equations were used to fit the kinetic traces at varying concentrations of dTTP or dTTP analog. These six to eight experiments all contributed to the $k_{pol}$ and $K_d$ rate constant values. A, incorporation of dTTP. Concentrations of dTTP are denoted as follows: ●, 0.50 μM; △, 1.0 μM; ■, 2.0 μM; ×, 4.0 μM; □, 10 μM; ▼, 20 μM. B, incorporation of FLT-TP. Concentrations of FLT-TP are denoted as follows: ●, 0.50 μM; △, 2.0 μM; ■, 5.0 μM; ×, 8.0 μM; □, 10 μM; ▼, 12 μM; ♦, 16 μM; +, 20 μM. C, incorporation of Ed4T-TP. Concentrations of Ed4T-TP are denoted as follows: ●, 0.20 μM; △, 0.60 μM; ■, 1.0 μM; ×, 1.4 μM; □, 1.8 μM; ▼, 2.0 μM; ♦, 8.0 μM; +, 20 μM.

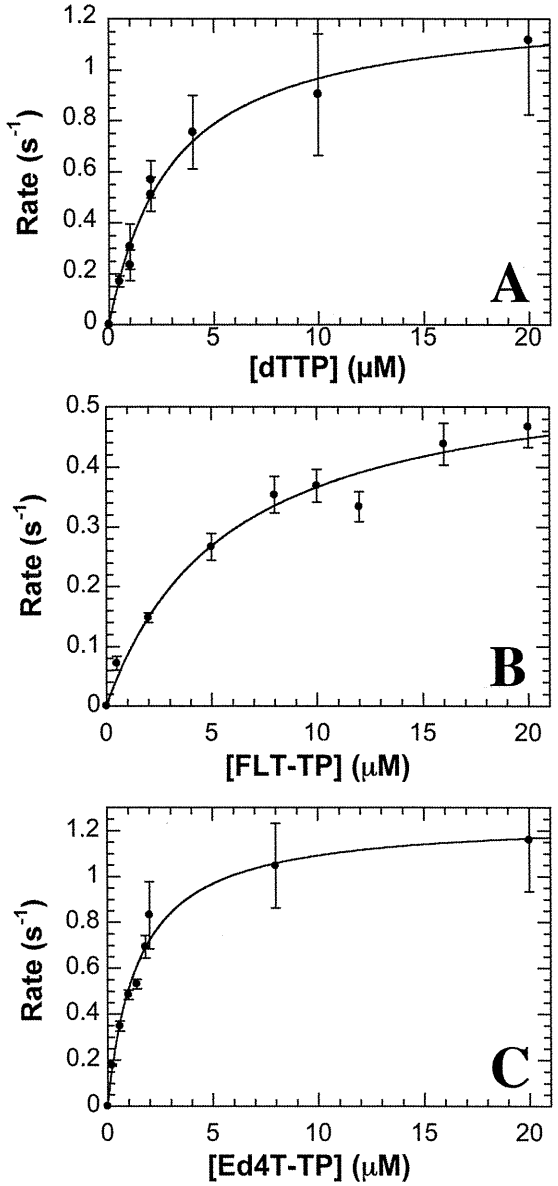


**Fig. 5.** Nucleotide concentration dependence of the observed rate of RT incorporation of dTTP analogs opposite dA. Hyperbolic equations were used to fit plots of the observed rate constants (generated from Fig. 4) versus dTTP analog concentrations to obtain $k_{pol}$ and $K_d$ values. Each point represents the observed rate generated from Fig. 4, and the S.E. is the deviance from the hyperbolic fits. Values for $k_{pol}$, $K_d$, and efficiency are presented in Table 1. A, incorporation of dTTP. B, incorporation of FLT-TP. C, incorporation of Ed4T-TP.

This supports the finding that lower doses of FLT are effective and less toxic (see above). WT pol γ very successfully discriminated against Ed4T-TP, showing 6200- and 180-fold greater discrimination, relative to values for dTTP and FLT-TP, respectively. This strikingly high level of selectivity is better than values for many currently available NRTIs (Table 2). Furthermore, in contrast to the very high level of selectivity shown by WT pol γ, RT showed very low discrimination between Ed4T-TP and dTTP. In fact, Ed4T-TP was the preferred substrate. This preference seems to be sequence-dependent, because a study using a different DNA primer/template substrate found a modest preference for dTTP over Ed4T-TP (Yang et al., 2008).

We showed that RT was poor at discriminating Ed4T and d4T from dTTP, with discrimination values of 0.51 and 0.56, respectively (Vaccaro et al., 2000) (Table 2); the preference for these two analogs over dTTP is unique among all of the NRTIs assayed to date (Table 2). The didehydro ring found in d4T and Ed4T may facilitate incorporation by RT and limit the discrimination of these analogs from the native nucleotides. Whereas d4T-TP was preferred over dTTP by RT, the corresponding selectivity by WT pol γ was 840-fold lower than for Ed4T-TP (Table 2) (Vaccaro et al., 2000; Johnson et al., 2001). Although RT preferred both analogs, there was a 13-fold difference in discrimination between RT and WT pol

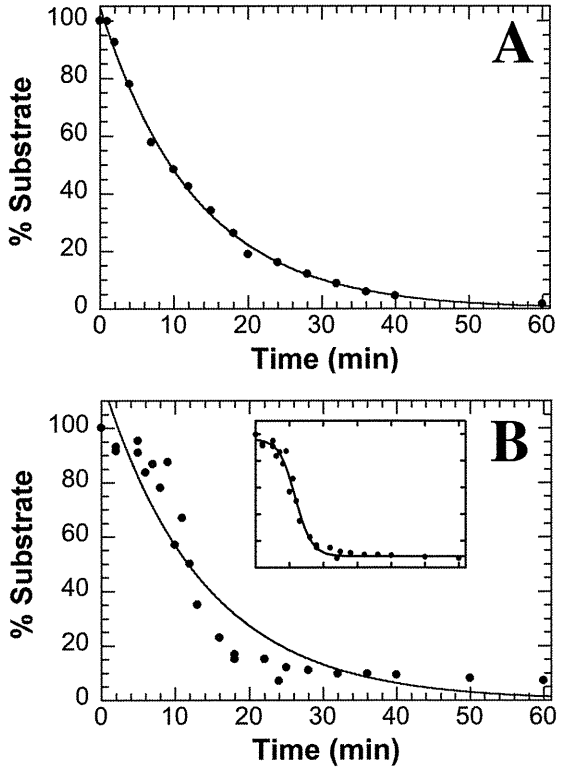


**Fig. 6.** Rates of dTTP analog excision by exo$^+$ pol $\gamma$. Single exponential decay equations were used to determine $k_{exo}$ values. A, excision of FLT-MP ($k_{exo}$ = 0.00130 ± 0.00005 $s^{-1}$). B, excision of Ed4T-MP ($k_{exo}$ = 0.0012 ± 0.0002 $s^{-1}$). Inset, sigmoidal fit of the data ($k_{exo}$ = 0.00018 $s^{-1}$).

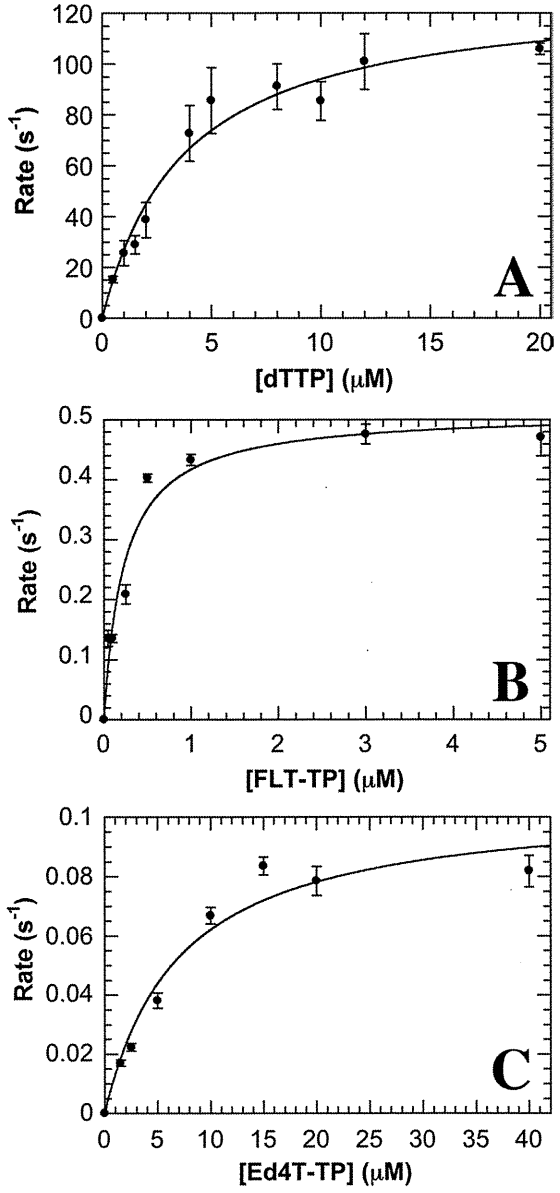


**Fig. 7.** Nucleotide concentration dependence of the observed rate of R964C pol $\gamma$ incorporation of dTTP analogs opposite dA. Hyperbolic equations were used to fit plots of the observed rate constants versus dTTP analog concentrations to obtain $k_{pol}$ and $K_d$ values. Each point represents the observed rate generated by fitting a time course with 10 different points to a single exponential expression, and the S.E. is the deviance from the hyperbolic fits. Values for $k_{pol}$, $K_d$, and efficiency are presented in Table 1. A, incorporation of dTTP. B, incorporation of FLT-TP. C, incorporation of Ed4T-TP.

$\gamma$ for d4T-TP, compared with a 12,000-fold difference in discrimination for Ed4T-TP. To our knowledge, Ed4T is the first NRTI to serve both as a preferred substrate for RT and as a nearly negligible substrate for WT pol $\gamma$.

Because the only difference between d4T and Ed4T is the presence of the 4′-ethynyl group, this functionality may serve as an enzyme selectivity moiety, in that discrimination by WT pol $\gamma$ (but importantly not RT) improves 840-fold when the ethynyl group is present (Table 2). It is likely that a similar trend would be seen with 4′-ethynyl-2-fluoro-2′-deoxyadenosine (EFdA)-TP; we found a 4300-fold preference for dATP over EFdA-TP with WT pol $\gamma$ (Sohl et al., 2012), and steady-state studies with RT yielded a discrimination value of 0.5 (i.e., EFdA-TP is preferred over dATP) (Michailidis et al., 2009). NMR spectroscopy studies probing the interaction of RT and EFdA indicated that the 4′-ethynyl group locks the sugar into a conformation favorable for incorporation by RT but not WT pol $\gamma$, which contributes to the preference of RT for the analog (Kirby et al., 2011). The 4′-ethynyl group may serve as an enzyme selectivity moiety in Ed4T, although structural studies would be required to assess this.

A more subtle characteristic of the poor discrimination shown by RT for FLT-TP and Ed4T-TP concerns the development of resistance. The rate of generation of NRTI resistance mutations in RT after d4T treatment occurs notably more slowly than with other NRTIs (Lin et al., 1994). It was proposed that this is attributable, at least in part, to poor discrimination by RT (Vaccaro et al., 2000; Ray et al., 2002b). Our findings of low levels of RT discrimination can provide an explanation for the observed slow RT mutation rates for FLT and Ed4T (Kim et al., 2001; Nitanda et al., 2005; Yang et al., 2009). We also note that important future work includes testing the efficiency of incorporation of FLT-TP and Ed4T-TP with RT containing NRTI-resistant mutations to predict effectiveness among NRTI-experienced patients.

Both FLT-MP and Ed4T-MP were excised somewhat efficiently from the primer/template substrate by exo$^+$ pol $\gamma$ (Fig. 6). Although a single exponential equation fit well the data measured for FLT-MP excision (Fig. 6A), a very slight sigmoidal characteristic of Ed4T-MP excision (Fig. 6B) resulted in a minor deviation from a similar fit. This initial slow phase caused a slower rate of excision when a sigmoidal fit was used (Fig. 6B, inset). It is possible that excision of Ed4T-MP requires a more complicated reaction scheme (i.e.,

TABLE 2

Comparison of efficiency of WT pol γ and RT incorporation of nucleoside analogs, FDA-approved NRTIs, and NRTIs under development

Discrimination (efficiency$_{dNTP}$/efficiency$_{analog}$) is shown, rather than incorporation efficiency, to minimize the rate constant variations associated with different primer/template substrates.

| dNTP Analog | Discrimination | | Reference |
|---|---|---|---|
| | WT pol γ | RT | |
| ddC-TP | 2.9 | 10 | Feng and Anderson, 1999; Feng et al., 2001; Ray et al., 2003 |
| ddA-TP | 4.0 | 5 | Johnson et al., 2001 |
| d4T-TP | 7.4 | 0.56 | Vaccaro et al., 2000; Johnson et al., 2001 |
| KP1212-TP | 26 | 14 | Murakami et al., 2005 |
| FLT-TP | 35 | 4.2 | Current study |
| (−)-3TC-TP | 2900 | 40 | Feng and Anderson, 1999; Feng et al., 2001; Ray et al., 2003 |
| EFdA-TP | 4300 | N.D.[a] | Sohl et al., 2012 |
| Ed4T-TP | 6200 | 0.51 | Current study |
| PMPApp | 11,400 | 6.1 | Johnson et al., 2001 |
| AZT-TP | 37,000 | 2.7 | Vaccaro and Anderson, 1998; Johnson et al., 2001 |
| (−)-FTC-TP | 290,000 | 16 | Feng et al., 2004 |
| CBV-TP | 900,000 | 34 | Johnson et al., 2001 |

N.D., no data; KP1212, 5-aza-5,6-dihydro-2'-deoxycytidine; 3TC, lamivudine; PMPApp, tenofovir diphosphate; FTC, emtricitabine; CBV, carbovir.
[a] Steady-state studies indicated an efficiency of 0.5 (Michailidis et al., 2009).

more steps) than does excision of FLT-MP, including conformational changes or changes related to partitioning between the two active sites. Without structural or spectral evidence to support this, our focus is on the single exponential fit, although experimentally testing alternate reaction schemes is an interest for future study. The rate of dissociation of a DNA template is ~0.02 $s^{-1}$ (Johnson and Johnson, 2001), and it is likely that DNA dissociation upon dTTP analog incorporation occurs more often than excision.

The moderate excision rates for FLT-MP and Ed4T-MP are within the range of values for most FDA-approved NRTIs (Johnson et al., 2001) and are faster than the rates of removal of ddC (0.0003 $s^{-1}$) (Hanes and Johnson, 2008) and ddA (0.0005 $s^{-1}$) (Johnson et al., 2001). Our finding of the faster (relative to ddC and ddA) Ed4T excision rate supports work examining the link between the dissociation constant of the incoming nucleotide analog and the kinetic partitioning of the DNA in the pol γ polymerase and exonuclease active sites (Hanes and Johnson, 2008). Specifically, the low affinity of WT pol γ for Ed4T may facilitate transfer of the DNA primer/template from the polymerase active site to the exonuclease active site during excision. Affinity constants for ddC-TP (Feng et al., 2001) and ddA-TP (Johnson et al., 2001), which are excised much more slowly by exo$^+$ pol γ, indicate that they bind much more tightly than Ed4T. Likewise, the $K_d$ for FLT-TP is 1 order of magnitude greater than those of ddC-TP and ddA-TP (Johnson et al., 2001), which suggests more favorable excision active site partitioning for FLT removal, compared with ddC and ddA, to help achieve the faster rate of FLT excision.

As seen with d4T treatment (Yamanaka et al., 2007), we predict that patients with the R964C pol γ mutation may show higher rates of mitochondrial toxicity. As noted for d4T (Bailey et al., 2009), alterations in incorporation kinetics were modest but significant; R964C pol γ incorporated dTTP 1.4-fold less efficiently than WT pol γ, and FLT-TP and Ed4T-TP were incorporated 1.6- and 2.5-fold more efficiently, respectively [compared with 2.1-fold more efficiently for d4T-TP (Bailey et al., 2009)] (Table 1). This resulted in overall 2.2- and 3.4-fold losses of discrimination for dTTP over FLT-TP and Ed4T-TP, respectively (Table 1), similar to the 3.2-fold loss of discrimination for dTTP over d4T-TP determined previously (Bailey et al., 2009). Using these dTTP analogs for patients with the R964C pol γ mutation may lead to higher rates of mitochondrial toxicity because of the similar kinetic profiles, compared with d4T (Yamanaka et al., 2007), although it is possible that other toxicity mechanisms also may contribute. This mutation should be taken into consideration when FLT and Ed4T are tested in clinical settings.

In summary, we found that two relatively new RT inhibitors for treatment of HIV infection, FLT-TP and Ed4T-TP, were incorporated by WT pol γ nearly 1 and >3 orders of magnitude more slowly, respectively, than dTTP. RT readily incorporated both inhibitors, however, and actually preferred Ed4T-TP over native nucleotides. Ed4T is the first NRTI shown to be preferred by RT and yet have negligible incorporation by WT pol γ. We propose that the ethynyl group in Ed4T serves as an enzyme selectivity moiety to generate the different discrimination abilities of RT and WT pol γ, which is an important finding for future NRTI design. These unique kinetic interaction profiles for FLT-TP and Ed4T-TP provide a mechanism to explain the different susceptibilities to toxicity. Such studies are critical for understanding pol γ-mediated mechanisms of toxicity for NRTIs in preclinical and clinical trials.

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**Authorship Contributions**

*Participated in research design:* Sohl and Anderson.
*Conducted experiments:* Sohl and Kim.
*Contributed new reagents or analytic tools:* Kasiviswanathan, Pradere, Schinazi, Copeland, Mitsuya, and Baba.
*Performed data analysis:* Sohl and Kim.
*Wrote or contributed to the writing of the manuscript:* Sohl and Anderson.

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# Response of Simian Immunodeficiency Virus to the Novel Nucleoside Reverse Transcriptase Inhibitor 4′-Ethynyl-2-Fluoro-2′-Deoxyadenosine *In Vitro* and *In Vivo*

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**Nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) are essential components in first-line therapy for human immunodeficiency virus (HIV) infection. However, long-term treatment with existing NRTIs can be associated with significant toxic side effects and the emergence of drug-resistant strains. The identification of new NRTIs for the continued management of HIV-infected people therefore is paramount. In this report, we describe the response of a primary isolate of simian immunodeficiency virus (SIV) to 4′-ethynyl-2-fluoro-2′-deoxyadenosine (EFdA) both *in vitro* and *in vivo*. EFdA was 3 orders of magnitude better than tenofovir (TFV), zidovudine (AZT), and emtricitabine (FTC) in blocking replication of SIV in monkey peripheral blood mononuclear cells (PBMCs) *in vitro*, and in a preliminary study using two SIV-infected macaques with advanced AIDS, it was highly effective at treating SIV infection and AIDS symptoms *in vivo*. Both animals had 3- to 4-log decreases in plasma virus burden within 1 week of EFdA therapy (0.4 mg/kg of body weight, delivered subcutaneously twice a day) that eventually became undetectable. Clinical signs of disease (diarrhea, weight loss, and poor activity) also resolved within the first month of treatment. No detectable clinical or pathological signs of drug toxicity were observed within 6 months of continuous therapy. Virus suppression was sustained until drug treatment was discontinued, at which time virus levels rebounded. Although the rebound virus contained the M184V/I mutation in the viral reverse transcriptase, EFdA was fully effective in maintaining suppression of mutant virus throughout the drug treatment period. These results suggest that expanded studies with EFdA are warranted.**

Nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) have proven highly effective in both the treatment of chronic human immunodeficiency virus (HIV) infection (11) and, more recently, as a promising microbicidal prevention strategy for sexually transmitted virus (12). There are seven NRTIs approved for current clinical use, including the nucleoside emtricitabine (FTC) and the nucleotide tenofovir (TFV). Unfortunately, the frequent use of these drugs has resulted in the emergence of resistant virus strains (7, 8, 10, 18). New compounds with potent activity on a wide range of isolates, including NRTI-resistant strains, are critically needed.

We and others have previously described a group of 4′-substituted NRTIs that are more potent and have higher selectivity indices *in vitro* than existing NRTIs (9, 13, 14, 15, 16, 17). One of the most potent of these compounds, 4′-ethynyl-2-fluoro-2′deoxyadenosine (EFdA), inhibits HIV-1 replication in primary peripheral blood mononuclear cells (PBMC) with a 50% effective concentration ($EC_{50}$) of 50 pM, a potency 4 orders of magnitude greater than that of TFV and 400-fold greater than that of AZT (15). It is also nontoxic *in vitro* at concentrations as high as 10 μM, which results in an *in vitro* selectivity index greater than 200,000. Furthermore, EFdA retains significant potency against a broad range of clinically important drug-resistant HIV isolates (13, 17).

The striking anti-HIV properties of EFdA prompted us to examine its activity on simian immunodeficiency virus (SIV) replication *in vitro* and *in vivo*. High potency against SIV would enable detailed preclinical assessments of EFdA for potential therapeutic use, as well as for use in preexposure prophylaxis and topical microbicide applications, using the SIV-macaque model for AIDS. EFdA could also provide an important tool in experiments designed to increase our understanding of host-virus interactions during latent viral infection.

In the present report, we show that EFdA surpassed the potency of TFV, AZT, and FTC in blocking replication of a primary, virulent isolate of SIV in primary monkey PBMC *in vitro*, and in a preliminary study using two SIV-infected macaques with advanced AIDS, it was highly effective at treating SIV infection *in vivo*. Both animals showed substantial decreases in plasma virus burden within 1 week of EFdA therapy that, with the exception of a single time point in one animal, remained below 100 copies/ml plasma throughout treatment. The clinical signs of disease (diarrhea, weight loss, and poor activity) also resolved during therapy. Repeated analysis of blood chemistry values during therapy, and complete histopathological examination of tissues at necropsy, also failed to reveal clinical or histopathological signs of drug-

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induced toxicity. Together, these results demonstrate a potential role for this compound in the treatment of AIDS.

## MATERIALS AND METHODS

**Macaque studies.** Male Indian-origin rhesus macaques (*Macaca mulatta*), 4 to 6 years of age, were obtained after completion of another study examining the adjunctive effect of DNA immunization and antiretroviral therapy with (*R*)-9-(2-phosphonomethoxypropyl) adenine (PMPA; Gilead Sciences, Foster City, CA) and lopinavir-ritonavir (Kaletra; Abbot Laboratories, Abbott Park, IL) (6). They were infected by intravenous inoculation of a cryopreserved stock of SIV/DeltaB670 propagated in rhesus macaque PBMC. Beginning 41 days postinoculation (p.i.), they received daily subcutaneous (s.c.) injections of 20 mg/kg of body weight PMPA and twice-daily oral administration of 16 mg (approximately 13 mg/kg) lopinavir-ritonavir. Therapy was continued without interruption for 40 weeks, during which time each animal received 6 monthly immunizations with a DNA vaccine expressing SIV *gag*, *pol*, and *env* and *E. coli* enterotoxin. Both immunizations and antiviral therapy were discontinued after 40 weeks to monitor the effects of vaccination on viral rebound and progression to disease. Although both animals responded to PMPA plus lopinavir-ritonavir and treatment appeared to prolong survival, after therapy was discontinued it had no apparent effect on controlling virus rebound in either animal, and both progressed to AIDS.

Treatment with EFdA (0.4 mg/kg delivered s.c. twice daily) was initiated approximately 34 months into the infection (24 months after cessation of PMPA plus lopinavir-ritonavir) and continued for 4 and 6 months. Monkey R395 was sacrificed after 4 months of treatment while still on therapy. EFdA was discontinued after 6 months in monkey R393 due to depletion of the drug supply, and he was sacrificed 2 months later. Tissues from all organs from both monkeys, one during therapy (R395) and one 2 months after therapy was discontinued (R393), were subjected to detailed histopathological examination.

Monkeys were 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 American Association for the Accreditation of Laboratory Animal Care International.

**Analysis of viral loads in plasma and tissues.** Virion-associated RNA in plasma was quantified by real-time PCR in a Prism 7700 (Applied Biosystems, Inc., Foster City, CA) using primers specific for the SIV long terminal repeat (LTR) as described previously (4, 5, 6, 21). This assay is linear over an 8-log range of template copy numbers and has a sensitivity threshold of 10 copies per reaction. Control amplifications of each sample omitting reverse transcriptase were also performed to control for contaminating DNA. RNA copy numbers from the unknown plasma samples were calculated from a similarly amplified external standard and expressed as viral RNA copies/ml plasma.

Full-length cell-associated SIV transcripts were quantified in TRIzol-extracted RNA from tissues or purified mononuclear cells as described previously (6). Briefly, reverse-transcribed cellular RNA was subjected to real-time quantitative reverse transcription-PCR (qRT-PCR) using TaqMan chemistry and forward and reverse primers with probe specific for the U5 region of the 5′ long terminal repeat (LTR) and the downstream primer binding site. These primers amplify a 92-bp fragment found either in the virion itself or in transcripts prepared for packaging into virions. When cell associated, they are a reliable indicator of virus production in the tissues. The assay has an amplification efficiency of 97% and a sensitivity of 10 copies/reaction.

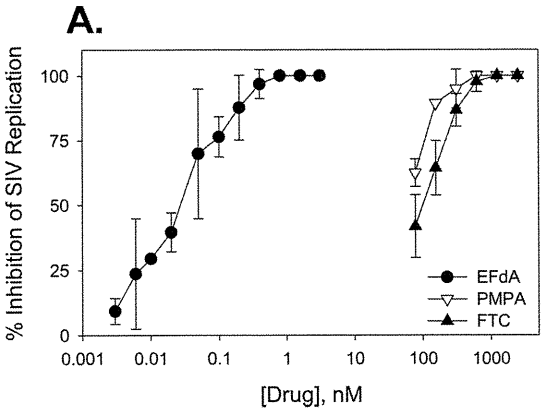
**Genotyping of plasma SIV.** cDNA obtained from purified RNA from high-speed plasma pellets was used for analysis as described previously (1). Amplicons spanning nucleotides 3353 to 3682 of the viral genome were obtained from a pool of 5 independent PCRs of each sample, cloned, and sequenced. Deduced sequences spanning amino acids 108 to 408 of the viral reverse transcriptase were aligned using SIVmac239 as the consensus sequence using software available in the Los Alamos database.

***In vitro* drug inhibition.** For the *in vitro* experiments, viably frozen rhesus macaque PBMC (rhPBMC) were thawed, washed thoroughly, and stimulated for 72 h in growth media (1× RPMI 1640 with 15% fetal bovine serum, 1% streptomycin-penicillin, and 1% L-glutamine) containing 2 μg/ml phytohemagglutinin. After thorough washing, rhPBMC were resuspended in growth media containing 10 U/ml interleukin-2 (IL-2), infected with 16.7 μl of SIV/DeltaB670 (66 50% tissue culture infectious doses [$TCID_{50}$]) per $1.0 \times 10^6$ cells, and plated in 96-well plates at $4.5 \times 10^4$ cells per well. Twofold serial dilutions of the assayed compounds were produced in IL-2 growth media and added to infected rhPBMC, generating final drug concentration ranges of 0 to 12.5 nM EFdA, 0 to 1,000 nM AZT, 0 to 10,000 nM TFV, and 0 to 10,000 nM FTC. Infected, treated rhPBMCs were cultured at 37°C in a 5% $CO_2$ incubator for 10 days. Half of the cell supernatant was harvested at days 4, 7, and 10 postinfection; after each sampling, medium was replenished with fresh IL-2 growth medium containing half the concentration of drug added at day 0. The supernatants harvested on day 10 postinfection were tested for virus replication using an SIV p27 enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's protocols (Advanced Bioscience Laboratories, Rockville, MD). The percent inhibition of SIV replication was determined by measuring the reduction of p27 in the culture supernatant at each escalating drug concentration versus untreated virus. Results are the averages from three replicates for each dilution of drug. The $EC_{50}$s and $EC_{90}$s for each drug were determined by four-parameter logistic nonlinear regression analysis.

## RESULTS

**Susceptibility of SIV to EFdA *in vitro*.** To determine whether EFdA was as effective in controlling SIV replication as it had been for HIV, we evaluated its ability to inhibit *in vitro* replication of the primary SIV isolate SIV/DeltaB670 in rhesus PBMC (rhPBMC). The inhibitory effect of two other well-studied NRTIs, TFV and FTC, were also evaluated for comparison (Fig. 1). SIV/DeltaB670 is highly virulent in macaques (19), has been propagated *in vitro* only in rhPBMC, and is comprised of a well-characterized genetic swarm (1, 2, 21, 22). A comparison of the percent inhibition and the 50 and 90% effective concentrations ($EC_{50}$ and $EC_{90}$) for each drug are shown in Fig. 1A and B, respectively. The inhibitory activity of EFdA against SIV ($EC_{50}$ of 50 pM) was identical to that observed for inhibition of HIV-1 replication in human PBMCs (15) and was 3 orders of magnitude more potent than that of either TFV or FTC. No cytotoxicity was noted upon exposure of monkey PBMCs for 10 days to EFdA concentrations up to 10 μM, providing an *in vitro* selectivity index of greater than 200,000 (data not shown).

**Susceptibility of SIV to EFdA *in vivo*.** The *in vitro* potency of EFdA against the same virulent primary stock used for our *in vivo* experiments encouraged us to treat two macaques chronically infected with SIV/DeltaB670 despite their advanced disease. The virological history of these animals is shown in Fig. 2. Both animals were originally used in a study to evaluate the immunotherapeutic potential of a DNA vaccine administered during treatment of chronic infection with TFV and lopinavir-ritonavir (6). Animals had been inoculated intravenously with the same SIV stock as that used for the *in vitro* experiments described above. Plasma virus burden was monitored by qRT-PCR (4, 5, 21). Forty-one days postinoculation (after the viral set point had been reached but while they were clinically asymptomatic), daily treatment with 20 mg/kg TFV and twice-daily administration of 16 mg/kg lopinavir-ritonavir was initiated; therapy was continued




B.

| | $EC_{50}$ (nM) | $EC_{95}$ (nM) |
|---|---|---|
| EFdA | 0.04 +/- 0.01 | 0.4 |
| PMPA | 67.5 +/- 6.5 | 240 |
| FTC | 108.6 +/- 9.2 | 580 |

FIG 1 *In vitro* response of SIV/DeltaB670 to escalating doses of EFdA, TFV, and FTC in rhesus macaque PBMC. Sixty-six $TCID_{50}$ of virus was incubated with serial dilutions of each drug, and infected cultures were monitored for p27 levels in the culture supernatant over time postinfection. (A) The percent inhibition relative to untreated control cultures for each dilution. (B) The 50 and 90% effective concentrations for each drug, determined using four-parameter logistic nonlinear regression. Values represent the means ± standard errors from one experiment with three or two replicates per dilution, respectively.

for 279 days and then discontinued. Both macaques were additionally immunized at monthly intervals during therapy with the SIV DNA vaccine.

**Initial response to TFV and lopinavir-ritonavir.** Both animals responded to the cocktail of TFV and lopinavir-ritonavir with a 5-log drop in plasma virus loads. R395 continued to respond well throughout therapy, whereas plasma virus loads in monkey R393 soon rebounded and remained at or above the threshold associated with disease progression ($>10^4$/ml plasma) (21) throughout the remainder of therapy. Treatment was discontinued at 320 days p.i.; virus loads rebounded in both animals shortly thereafter. Subsequent to viral rebound, both animals developed progressive disease characterized clinically by chronic diarrhea that was unresponsive to treatment and persistent weight loss. Thirty-four months into the infection (24 months after cessation of PMPA plus lopinavir-ritonavir), both animals had declines in $CD4^+$ T lymphocytes to less than 45% of their preinfection values, plasma virus loads greater than $10^4$ copies/ml plasma, and clinical signs of AIDS (diarrhea and weight loss greater than 20% of body weight; end-stage AIDS).

**Response to EFdA treatment.** EFdA treatment (0.4 mg/kg delivered s.c. twice daily [BID]) was initiated during this time. Despite the poor clinical conditions and high virus loads at the onset of therapy, the response to treatment with EFdA exceeded that observed with TFV plus lopinavir-ritonavir initiated during the asymptomatic stage of the disease (Fig. 2). Within 1 week, a 3- to 4-log reduction in virus burden was observed in both animals that, except for barely detectable blips in monkey R393, further declined and remained below the threshold of detection until EFdA was discontinued (Fig. 3). Four months into the therapy, monkey R395 was sacrificed and the tissues collected for analysis. EFdA therapy was continued for an additional 2 months in monkey

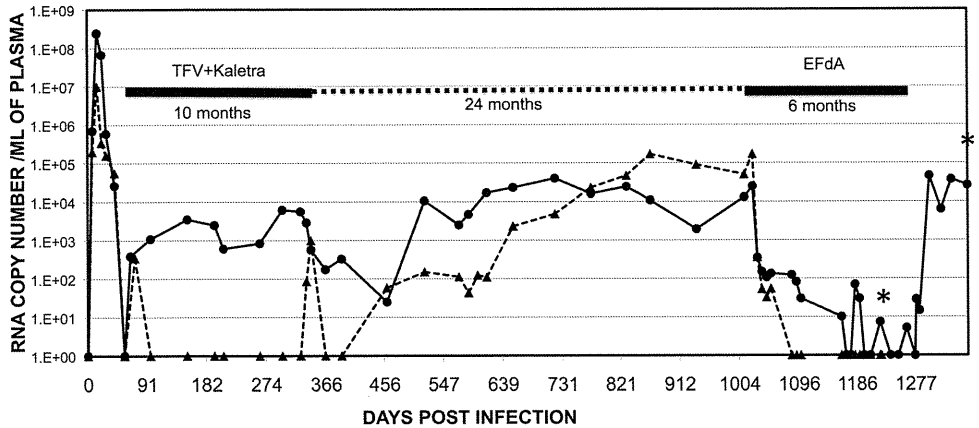



FIG 2 Virological history of SIV/DeltaB670-infected macaques treated with TFV plus lopinavir-ritonavir followed by EFdA. Monkeys were infected by intravenous inoculation and longitudinally monitored for virus burden, clinical signs of disease, and changes in $CD4^+$ T cell numbers in the blood over time during infection and treatment. Daily subcutaneous injections with TFV (20 mg/kg; once daily) and twice-daily oral administration of 13 mg/kg lopinavir-ritonavir was initiated on day 41 and discontinued on day 320 p.i. Twenty-four months later, twice-daily subcutaneous injections of 0.4 mg/kg EFdA were initiated on day 1025 and terminated on day 1224 (at sacrifice) in monkey R395 and day 1276 in monkey R393. Monkey R393 was sacrificed for tissue collection on day 1336. The black bars are indicative of the intervals of therapy. The asterisks indicate the timing of sacrifice of each animal. Plasma virus load measurements were performed over the course of the experiments as described in the text. Values fewer than 5,000 copies of SIV RNA/ml plasma is indicative of asymptomatic infection. Values of less than 100 copies were repeated to ensure accuracy. Values from monkey R395 are indicated by triangles connected by the dashed line. The circles connected by the solid line reflect values from monkey R393.