

- mutation N43D and the associated baseline polymorphism E137K on peptide sensitivity and six-helix bundle structure. *Biochemistry* 47:6662–6670.
7. Baldwin CE, Sanders RW, Deng Y, Jurriaans S, Lange JM, Lu M, Berkhout B. 2004. Emergence of a drug-dependent human immunodeficiency virus type 1 variant during therapy with the T20 fusion inhibitor. *J. Virol.* 78:12428–12437.
  8. Cabrera C, Marfil S, Garcia E, Martinez-Picado J, Bonjoch A, Bofill M, Moreno S, Ribera E, Domingo P, Clotet B, Ruiz L. 2006. Genetic evolution of gp41 reveals a highly exclusive relationship between codons 36, 38 and 43 in gp41 under long-term enfuvirtide-containing salvage regimen. *AIDS* 20:2075–2080.
  9. Champagne K, Shishido A, Root MJ. 2009. Interactions of HIV-1 inhibitory peptide T20 with the gp41 N-HR coiled coil. *J. Biol. Chem.* 284:3619–3627.
  10. Izumi K, Kodama E, Shimura K, Sakagami Y, Watanabe K, Ito S, Watabe T, Terakawa Y, Nishikawa H, Sarafianos SG, Kitaura K, Oishi S, Fujii N, Matsuoka M. 2009. Design of Peptide-based inhibitors for human immunodeficiency virus type 1 strains resistant to T-20. *J. Biol. Chem.* 284:4914–4920.
  11. Lu J, Deeks SG, Hoh R, Beatty G, Kuritzkes BA, Martin JN, Kuritzkes DR. 2006. Rapid emergence of enfuvirtide resistance in HIV-1-infected patients: results of a clonal analysis. *J. Acquir. Immune Defic. Syndr.* 43:60–64.
  12. Oliveira AC, Martins AN, Pires AF, Arruda MB, Tanuri A, Pereira HS, Brindeiro RM. 2009. Enfuvirtide (T-20) resistance-related mutations in HIV type 1 subtypes B, C, and F isolates from Brazilian patients failing HAART. *AIDS Res. Hum. Retroviruses* 25:193–198.
  13. Reeves JD, Lee FH, Miamidian JL, Jabara CB, Juntilla MM, Doms RW. 2005. Enfuvirtide resistance mutations: impact on human immunodeficiency virus envelope function, entry inhibitor sensitivity, and virus neutralization. *J. Virol.* 79:4991–4999.
  14. Watabe T, Terakawa Y, Watanabe K, Ohno H, Nakano H, Nakatsu T, Kato H, Izumi K, Kodama E, Matsuoka M, Kitaura K, Oishi S, Fujii N. 2009. X-ray crystallographic study of an HIV-1 fusion inhibitor with the gp41 S138A substitution. *J. Mol. Biol.* 392:657–665.
  15. Lalezari JP, Bellos NC, Sathasivam K, Richmond GJ, Cohen CJ, Myers RA, Jr, Henry DH, Raskino C, Melby T, Murchison H, Zhang Y, Spence R, Greenberg ML, Demasi RA, Miralles GD. 2005. T-1249 retains potent antiretroviral activity in patients who had experienced virological failure while on an enfuvirtide-containing treatment regimen. *J. Infect. Dis.* 191:1155–1163.
  16. He Y, Xiao Y, Song H, Liang Q, Ju D, Chen X, Lu H, Jing W, Jiang S, Zhang L. 2008. Design and evaluation of sifuvirtide, a novel HIV-1 fusion inhibitor. *J. Biol. Chem.* 283:11126–11134.
  17. Dwyer JJ, Wilson KL, Davison DK, Freel SA, Seedorff JE, Wring SA, Tvermoes NA, Matthews TJ, Greenberg ML, Delmedico MK. 2007. Design of helical, oligomeric HIV-1 fusion inhibitor peptides with potent activity against enfuvirtide-resistant virus. *Proc. Natl. Acad. Sci. U. S. A.* 104:12772–12777.
  18. Welch BD, VanDemark AP, Heroux A, Hill CP, Kay MS. 2007. Potent D-peptide inhibitors of HIV-1 entry. *Proc. Natl. Acad. Sci. U. S. A.* 104:16828–16833.
  19. Wang H, Qi Z, Guo A, Mao Q, Lu H, An X, Xia C, Li X, Debnath AK, Wu S, Liu S, Jiang S. 2009. ADS-J1 inhibits human immunodeficiency virus type 1 entry by interacting with the gp41 pocket region and blocking fusion-active gp41 core formation. *Antimicrob. Agents Chemother.* 53:4987–4998.
  20. Naito T, Izumi K, Kodama E, Sakagami Y, Kajiwara K, Nishikawa H, Watanabe K, Sarafianos SG, Oishi S, Fujii N, Matsuoka M. 2009. SC29EK, a peptide fusion inhibitor with enhanced alpha-helicity, inhibits replication of human immunodeficiency virus type 1 mutants resistant to enfuvirtide. *Antimicrob. Agents Chemother.* 53:1013–1018.
  21. Nishikawa H, Nakamura S, Kodama E, Ito S, Kajiwara K, Izumi K, Sakagami Y, Oishi S, Ohkubo T, Kobayashi Y, Otaka A, Fujii N, Matsuoka M. 2009. Electrostatically constrained alpha-helical peptide inhibits replication of HIV-1 resistant to enfuvirtide. *Int. J. Biochem. Cell Biol.* 41:891–899.
  22. Marqusee S, Baldwin RL. 1987. Helix stabilization by Glu-Lys<sup>+</sup> salt bridges in short peptides of de novo design. *Proc. Natl. Acad. Sci. U. S. A.* 84:8898–8902.
  23. Oishi S, Ito S, Nishikawa H, Watanabe K, Tanaka M, Ohno H, Izumi K, Sakagami Y, Kodama E, Matsuoka M, Fujii N. 2008. Design of a novel HIV-1 fusion inhibitor that displays a minimal interface for binding affinity. *J. Med. Chem.* 51:388–391.
  24. Li X, Qian H, Miyamoto F, Naito T, Kawaji K, Kajiwara K, Hattori T, Matsuoka M, Watanabe K, Oishi S, Fujii N, Kodama EN. 2012. A simple, rapid, and sensitive system for the evaluation of anti-viral drugs in rats. *Biochem. Biophys. Res. Commun.* 424:257–261.
  25. Nameki D, Kodama E, Ikeuchi M, Mabuchi N, Otaka A, Tamamura H, Ohno M, Fujii N, Matsuoka M. 2005. Mutations conferring resistance to human immunodeficiency virus type 1 fusion inhibitors are restricted by gp41 and Rev-responsive element functions. *J. Virol.* 79:764–770.
  26. Shimura K, Nameki D, Kajiwara K, Watanabe K, Sakagami Y, Oishi S, Fujii N, Matsuoka M, Sarafianos SG, Kodama EN. 2010. Resistance profiles of novel electrostatically constrained HIV-1 fusion inhibitors. *J. Biol. Chem.* 285:39471–39480.
  27. Izumi K, Nakamura S, Nakano H, Shimura K, Sakagami Y, Oishi S, Uchiyama S, Ohkubo T, Kobayashi Y, Fujii N, Matsuoka M, Kodama EN. 2010. Characterization of HIV-1 resistance to a fusion inhibitor, N36, derived from the gp41 amino-terminal heptad repeat. *Antiviral Res.* 87:179–186.
  28. Ueno M, Kodama EN, Shimura K, Sakurai Y, Kajiwara K, Sakagami Y, Oishi S, Fujii N, Matsuoka M. 2009. Synonymous mutations in stem-loop III of Rev-responsive elements enhance HIV-1 replication impaired by primary mutations for resistance to enfuvirtide. *Antiviral Res.* 82:67–72.
  29. Eggink D, Bontjer I, Langedijk JP, Berkhout B, Sanders RW. 2011. Resistance of human immunodeficiency virus type 1 to a third-generation fusion inhibitor requires multiple mutations in gp41 and is accompanied by a dramatic loss of gp41 function. *J. Virol.* 85:10785–10797.
  30. Reeves JD, Gallo SA, Ahmad N, Miamidian JL, Harvey PE, Sharron M, Pohlmann S, Sfakianos JN, Derdeyn CA, Blumenthal R, Hunter E, Doms RW. 2002. Sensitivity of HIV-1 to entry inhibitors correlates with envelope/coreceptor affinity, receptor density, and fusion kinetics. *Proc. Natl. Acad. Sci. U. S. A.* 99:16249–16254.
  31. Reeves JD, Miamidian JL, Biscione MJ, Lee FH, Ahmad N, Pierson TC, Doms RW. 2004. Impact of mutations in the coreceptor binding site on human immunodeficiency virus type 1 fusion, infection, and entry inhibitor sensitivity. *J. Virol.* 78:5476–5485.
  32. Yu X, Lu L, Cai L, Tong P, Tan S, Zou P, Meng F, Chen YH, Jiang S. 2012. Mutations of Gln64 in the HIV-1 gp41 N-terminal heptad repeat render viruses resistant to peptide HIV fusion inhibitors targeting the gp41 pocket. *J. Virol.* 86:589–593.
  33. Borrás-Blasco J, Navarro-Ruiz A, Borrás C, Castera E. 2008. Adverse cutaneous reactions associated with the newest antiretroviral drugs in patients with human immunodeficiency virus infection. *J. Antimicrob. Chemother.* 62:879–888.
  34. Liu Z, Shan M, Li L, Lu L, Meng S, Chen C, He Y, Jiang S, Zhang L. 2011. In vitro selection and characterization of HIV-1 variants with increased resistance to sifuvirtide, a novel HIV-1 fusion inhibitor. *J. Biol. Chem.* 286:3277–3287.
  35. Melby T, Demasi R, Cammack N, Miralles GD, Greenberg ML. 2007. Evolution of genotypic and phenotypic resistance during chronic treatment with the fusion inhibitor T-1249. *AIDS Res. Hum. Retroviruses* 23:1366–1373.



## RESEARCH

## Open Access

# Hypersusceptibility mechanism of Tenofovir-resistant HIV to EFdA

Eleftherios Michailidis<sup>1</sup>, Emily M Ryan<sup>1</sup>, Atsuko Hachiya<sup>2</sup>, Karen A Kirby<sup>1</sup>, Bruno Marchand<sup>1</sup>, Maxwell D Leslie<sup>1</sup>, Andrew D Huber<sup>1</sup>, Yee T Ong<sup>1</sup>, Jacob C Jackson<sup>1</sup>, Kamalendra Singh<sup>1</sup>, Eiichi N Kodama<sup>3</sup>, Hiroaki Mitsuya<sup>4,5</sup>, Michael A Parniak<sup>6</sup> and Stefan G Sarafianos<sup>1,7\*</sup>

## Abstract

**Background:** The K65R substitution in human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) is the major resistance mutation selected in patients treated with first-line antiretroviral tenofovir disoproxil fumarate (TDF). 4'-ethynyl-2'-fluoro-2'-deoxyadenosine (EFdA), is the most potent nucleoside analog RT inhibitor (NRTI) that unlike all approved NRTIs retains a 3'-hydroxyl group and has remarkable potency against wild-type (WT) and drug-resistant HIVs. EFdA acts primarily as a chain terminator by blocking translocation following its incorporation into the nascent DNA chain. EFdA is in preclinical development and its effect on clinically relevant drug resistant HIV strains is critically important for the design of optimal regimens prior to initiation of clinical trials.

**Results:** Here we report that the K65R RT mutation causes hypersusceptibility to EFdA. Specifically, in single replication cycle experiments we found that EFdA blocks WT HIV ten times more efficiently than TDF. Under the same conditions K65R HIV was inhibited over 70 times more efficiently by EFdA than TDF. We determined the molecular mechanism of this hypersensitivity using enzymatic studies with WT and K65R RT. This substitution causes minor changes in the efficiency of EFdA incorporation with respect to the natural dATP substrate and also in the efficiency of RT translocation following incorporation of the inhibitor into the nascent DNA. However, a significant decrease in the excision efficiency of EFdA-MP from the 3' primer terminus appears to be the primary cause of increased susceptibility to the inhibitor. Notably, the effects of the mutation are DNA-sequence dependent.

**Conclusion:** We have elucidated the mechanism of K65R HIV hypersusceptibility to EFdA. Our findings highlight the potential of EFdA to improve combination strategies against TDF-resistant HIV-1 strains.

**Keywords:** HIV-1, RT, EFdA, K65R

## Background

Human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) is the major target of antiretroviral drug treatments. RT inhibitors constitute the largest class of HIV-1 drugs and are grouped in two separate categories. The first category consists of the nucleos(t)ide RT inhibitors (NRTIs), which are analogs of the natural nucleosides. Most NRTIs lack a 3'-OH and act as chain terminators by blocking DNA polymerization

[1-8]. The other group includes the nonnucleoside RT inhibitors (NNRTIs), which are non-competitive RT inhibitors with respect to either dNTP or nucleic acid substrates and block DNA synthesis by binding to a hydrophobic pocket of RT [9-15]. Highly Active Antiretroviral Therapies (HAART) are based on combinations of antiretrovirals and have helped extend the lives of HIV-1 patients. However, the efficacy of combination therapies is being challenged by the selection of drug-resistant variants of HIV-1.

There are two major mechanisms of NRTI resistance [16,17]. The first is the discrimination mechanism, which is based on decreased incorporation of the nucleotide analog into the elongating DNA over the canonical dNTP substrate [16,18-21]. An example of this

\* Correspondence: [sarafianos@missouri.edu](mailto:sarafianos@missouri.edu)

<sup>1</sup>Christopher Bond Life Sciences Center, Department of Molecular Microbiology & Immunology, University of Missouri, Columbia, MO 65211, USA

<sup>7</sup>Department of Biochemistry, University of Missouri, Columbia, MO 65211, USA

Full list of author information is available at the end of the article

type of resistance is conferred by the M184V mutation, which decreases HIV susceptibility to lamivudine (3TC) and emtricitabine (FTC) [20-24]. The second mechanism is the excision mechanism, which is based on the enhanced ability of the mutant RT to remove the chain-terminating inhibitor from the DNA terminus [25-28] through a phosphorolytic reaction that uses primarily adenosine triphosphate (ATP) as a substrate. Upon removal of the inhibitor DNA synthesis resumes. The excision reaction is facilitated by Excision Enhancement Mutations (EEMs), typically M41L, D67N, K70R, T215Y/E, L210W, and K219E/Q, which are also known as Thymidine Associated Mutations (TAMs) because they were historically linked to resistance to thymidine analogs AZT and d4T [29,30].

Tenofovir disoproxil fumarate (TDF) is one of the most prescribed anti-HIV drugs, and is described as a key component of all first-line regimens in the DHHS HIV guidelines (<http://aidsinfo.nih.gov/contentfiles/lvguidelines/adultandadolescentgl.pdf>). The K65R mutation in HIV-1 RT is the signature mutation selected during tenofovir-based therapy. Viruses carrying K65R have reduced susceptibility to tenofovir and other NRTIs, but remain susceptible to zidovudine (AZT) [31-36]. This mutation has also been associated with a reduction in viral replication capacity, NRTI excision, NRTI incorporation, and dNTP incorporation [37-43]. Recent crystallographic data suggest that the K65R mutation disrupts the interaction between the side chains of 65R and 72R resulting in structural changes that lead to NRTI resistance [44].

We have previously shown that a series of NRTIs with 4'-substitutions and a 3'-OH group are very potent inhibitors of WT and multi-drug resistant HIV-1. The most effective of these compounds is the adenosine analog 4'-ethynyl-2-fluoro-2'-deoxyadenosine (EFdA) [45,46]. We have demonstrated that EFdA acts in a DNA-sequence specific manner, primarily inhibiting DNA synthesis as an immediate chain terminator, but less often, at some DNA sequences can also act as a delayed chain terminator [46]. Compounds that exhibit this novel mechanism of inhibition have been dubbed Translocation Defective Reverse Transcriptase Inhibitors (TDRTIs) [46].

In an effort to investigate the effect of EFdA against drug-resistant strains of HIV-1 we found that RT mutation K65R confers hypersusceptibility to EFdA. We carried out a series of biochemical experiments to elucidate the mechanism of this phenomenon and we propose here that K65R increases the susceptibility to EFdA mainly by suppressing the ATP- or PPi-dependent repair of EFdA-MP-terminated DNA. Understanding the molecular basis of K65R hypersusceptibility to EFdA may lead to new and more effective combination therapies.

## Results

### The K65R RT mutation enhances susceptibility of HIV to EFdA

In order to determine the susceptibility of HIV-1 to EFdA we performed single infectivity viral replication assays according to the experimental procedures described in Methods section. We used as a positive control of resistance to K65R HIV-1 the nucleotide analog TDF. Table 1 shows that K65R-containing viruses are at least 2.5-fold more susceptible to EFdA than WT viruses. In contrast, there was a 3-fold resistance to tenofovir caused by K65R RT mutation.

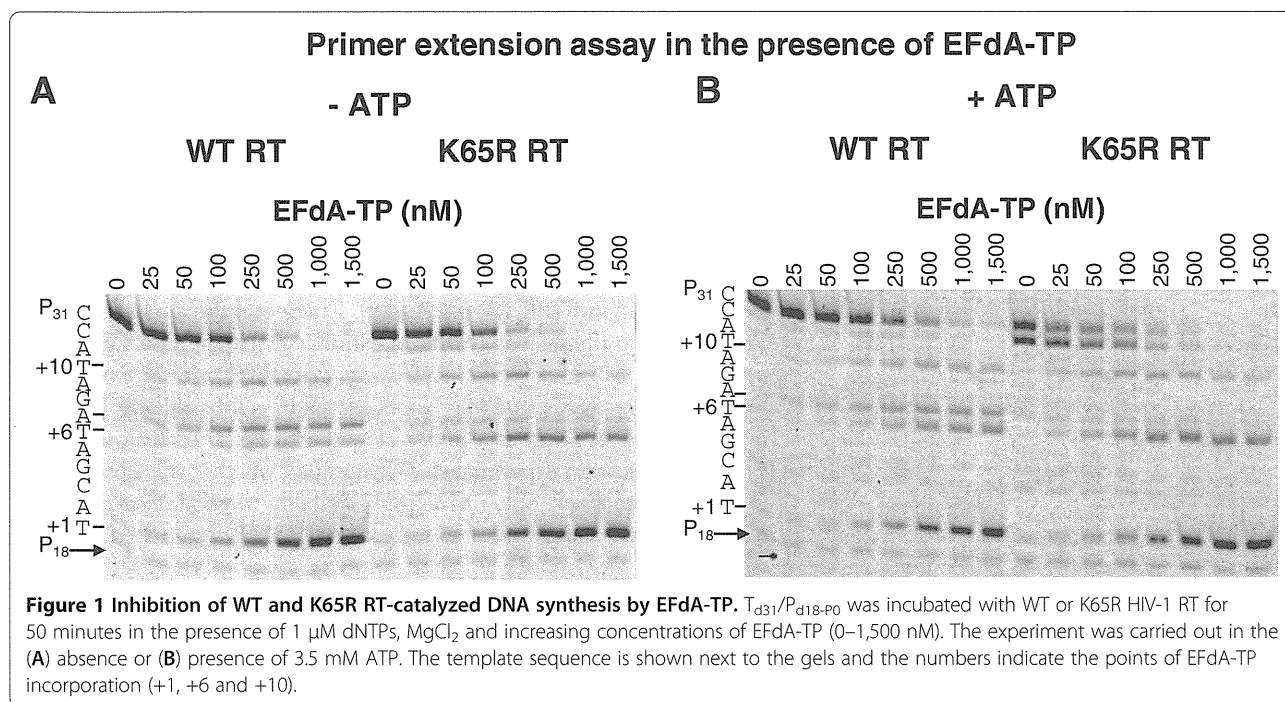
### The K65R mutation enhances susceptibility of RT to EFdA-TP

In order to recapitulate the HIV hypersusceptibility to EFdA observed in cell-based assays and determine the biochemical mechanism of this phenomenon we carried out a series of biochemical experiments. We used a primer extension assay to compare the effect of EFdA-TP on DNA-dependent DNA polymerization by WT and K65R RTs. In order to assess the effect of ATP-based excision on the susceptibility of RT to EFdA-TP we performed the reactions in the absence (Figure 1A) and in the presence of ATP (Figure 1B). In the absence of ATP, any changes in the susceptibility to the inhibitor would be caused by the "decreased incorporation" mechanism as there are no NRTI excision events under these conditions. However, changes in inhibitor susceptibility in the presence of ATP could be caused either by "changes in inhibitor incorporation" or "changes in inhibitor excision", or both. Figure 1 and Table 2 show that RT mutation K65R causes hypersusceptibility to EFdA-TP. In the presence of ATP the K65R mutation caused a 2.5-fold increase in susceptibility to EFdA-TP (Table 2). These data are consistent with the results from the cell-based assays shown in Table 1. The ~2-fold effect of ATP-based excision on the hypersusceptibility to EFdA suggests that excision is the major mechanism of this phenomenon and is further characterized in the subsequent experiments.

**Table 1 EC<sub>50</sub> determination of EFdA and TDF in single cycle cell-based assays**

Virus	EC <sub>50</sub> ± SD (nM) (Fold change)	
	EFdA	TDF
WT	3.2 ± 0.7 (1)	32 ± 6 (1)
K65R	1.3 ± 0.4 (0.4)	96 ± 3 (3)

The data show "mean value ± standard deviation" obtained from the results of at least three independent experiments ( $P < 0.013$ ). The relative increase in EC<sub>50</sub> values against K65R virus compared with WT virus is given in parentheses.



The  $T_{d31}/P_{d18-P0}$  DNA substrate (Table 3) allows incorporation of dATP analogs opposite template dTs at positions “1”, “6” and “10” (Figure 1). The stopping pattern of the gels showed that EFdA-TP caused major pauses at all possible points of incorporation (positions 1, 6 and 10), suggesting that EFdA-TP inhibits RT mainly as an immediate chain terminator at the point of incorporation. Interestingly, EFdA-TP caused an additional strong stop of WT RT at position “+7”, which is one nucleotide after its incorporation (Figure 1), thereby acting as delayed chain terminator at this site. Hence, this appears to be a sequence-dependent phenomenon, as we did not observe delayed chain extension at positions +2 and +11 and there was no pause at this site in the absence of inhibitor (Figure 1).

#### The K65R RT mutation does not enhance susceptibility to EFdA by significantly affecting incorporation of the inhibitor

To determine the biochemical mechanism of increased K65R HIV inhibition by EFdA we examined several

possible mechanisms. The first hypothesis was that the K65R RT mutation selectively enhances incorporation of the EFdA-TP inhibitor into DNA because of changes in kinetic parameters such as binding or turnover rate of inhibitor incorporation. To evaluate this hypothesis we performed single nucleotide incorporation assays under steady state conditions. In order to eliminate a sequence-dependent bias we used three different template/primers (T/P) ( $T_{d26}/P_{d18-P5}$ ,  $T_{d31}/P_{d18-P0}$ , and  $T_{d31A}/P_{d21}$  [Table 3]). Our results showed that under these conditions the ratio of the incorporation efficiency ( $k_{cat}/K_m$ ) of EFdA-TP and the incorporation efficiency of dATP by K65R RT was between 0.8 and 1. These results suggest that the K65R mutation does not have a significant effect on the binding and incorporation of EFdA-TP (Table 4).

#### The K65R RT mutation does not enhance susceptibility to EFdA by significantly affecting enzyme translocation on EFdA-MP-terminated template/primers

We have previously shown that the inability of RT to form a stable ternary complex with  $T/P_{EFdA-MP}$  and the next

**Table 2** Enhancement of hypersusceptibility to EFdA-TP under ATP-based excision conditions

Enzyme	IC <sub>50</sub> (nM) of EFdA-TP $\pm$ SD (Fold change)		Hypersusceptibility enhancement in the presence of ATP
	Without ATP	With 3.5 mM ATP	
WT RT	186 $\pm$ 40 <sup>a</sup> (1) <sup>b</sup>	318 $\pm$ 99 <sup>a</sup> (1) <sup>b</sup>	1 <sup>c</sup>
K65R RT	125 $\pm$ 28 <sup>a</sup> (0.7) <sup>b</sup>	131 $\pm$ 28 <sup>a</sup> (0.4) <sup>b</sup>	1.8 <sup>c</sup>

<sup>a</sup>The data show “mean value  $\pm$  standard deviation” obtained from the results of at least four independent experiments ( $P < 0.011$ ).

<sup>b</sup>The relative increase in IC<sub>50</sub> value in K65R RT compared with WT RT without, or with ATP is given in parentheses.

<sup>c</sup>The effect of ATP-based excision on hypersusceptibility is calculated by the relative change in IC<sub>50</sub> for K65R compared to WT RT without ATP/the relative change in IC<sub>50</sub> for K65R compared to WT RT with ATP.

**Table 3 Sequences of oligonucleotides used in this study**

Polymerization assays	
T <sub>d31</sub>	5'CCA TAG ATA GCA TTG GTG CTC GAA CAG TGA C
P <sub>d18-P0</sub>	5'Cy3 GTC ACT GTT CGA GCA CCA
T <sub>d26</sub>	5'CCA TAG ATA GCA TTG GTG CTC GAA CA
P <sub>d18-P5</sub>	5'Cy3 TGT TCG AGC ACC AAT GCT
T <sub>d31A</sub>	5'AAA AAA AAA TGG ATA CAT ATG GTT AAA GTA T
P <sub>d21</sub>	5'Cy3 ATA CTT TAA CCA TAT GTA TCC
Footprinting assays	
T <sub>d43</sub>	5'Cy3 CCA TAG ATA GCA T TG GTG CTC GAA CAG TGA CAA TCA GTG TAG A
P <sub>d30</sub>	5'TCT ACA CTG ATT GTC ACT GTT CGA GCA CCA

complementary dNTP was due to the inability of the 3'-terminal EFdA-MP primer to efficiently translocate from the nucleotide binding site (N site, which is also the pre-translocation site) to the post-translocation primer site (P site or the post-translocation site) [46]. Hence, another possible mechanism by which the K65R mutation could enhance susceptibility to EFdA is by further suppressing translocation of RT on the EFdA-MP-terminated T/P. To evaluate this hypothesis we used the site-specific Fe<sup>2+</sup> footprinting assay [46,47] to assess the translocation state of WT and K65R RT·T/P<sub>EFdA-MP</sub> complexes in the absence, and in the presence of varying concentrations of the next incoming dNTP. Figure 2 shows that EFdA-TP blocked translocation and acted as a strong TDRTI against both WT and K65R RTs. At physiological dNTP concentrations

(1-25 μM) we observed a 1.5-fold decrease in the translocation efficiency of K65R compared to WT RT under these conditions. To determine whether the lower amount of observed translocated K65R RT·T/P<sub>EFdA-MP</sub> complex (Figure 2) is due to a decreased affinity of K65R RT for EFdA-MP-terminated T/P we studied the effect of K65R on the formation of RT·T/P<sub>EFdA-MP</sub> binary complex using gel-shift assays. Data in Additional file 1: Figure S1 show that WT RT binds EFdA-MP-terminated T/P only slightly stronger than K65R RT (~1.3-fold). Therefore, the small differences in the selectivity, translocation activity, and DNA binding of WT and K65R RTs were not sufficient to explain the hypersusceptibility we observed in cell-based and RT assays with EFdA and EFdA-TP respectively. Therefore, in the following experiments we examined whether the K65R substitution could enhance susceptibility to EFdA by suppressing the ability of RT to unblock EFdA-MP-terminated primers.

#### ATP- and PPI-dependent Excision/Rescue of EFdA-MP

We have previously demonstrated that using simple pyrophosphorolysis reactions (in the absence of concurrent DNA polymerization) is not an effective way to monitor unblocking of EFdA-MP-terminated primers. This is because the net phosphorolysis is limited from the apparently facile reincorporation of the newly excised EFdA-TP [46]. Hence, to better study the potential role of the excision mechanism in EFdA resistance we employed rescue assays, where in addition to the ATP or PPI which are used as unblocking reagents, we also include dNTPs that compete with and prevent reincorporation of EFdA-TP, and also allow further DNA synthesis. For these experiments we used as a substrate

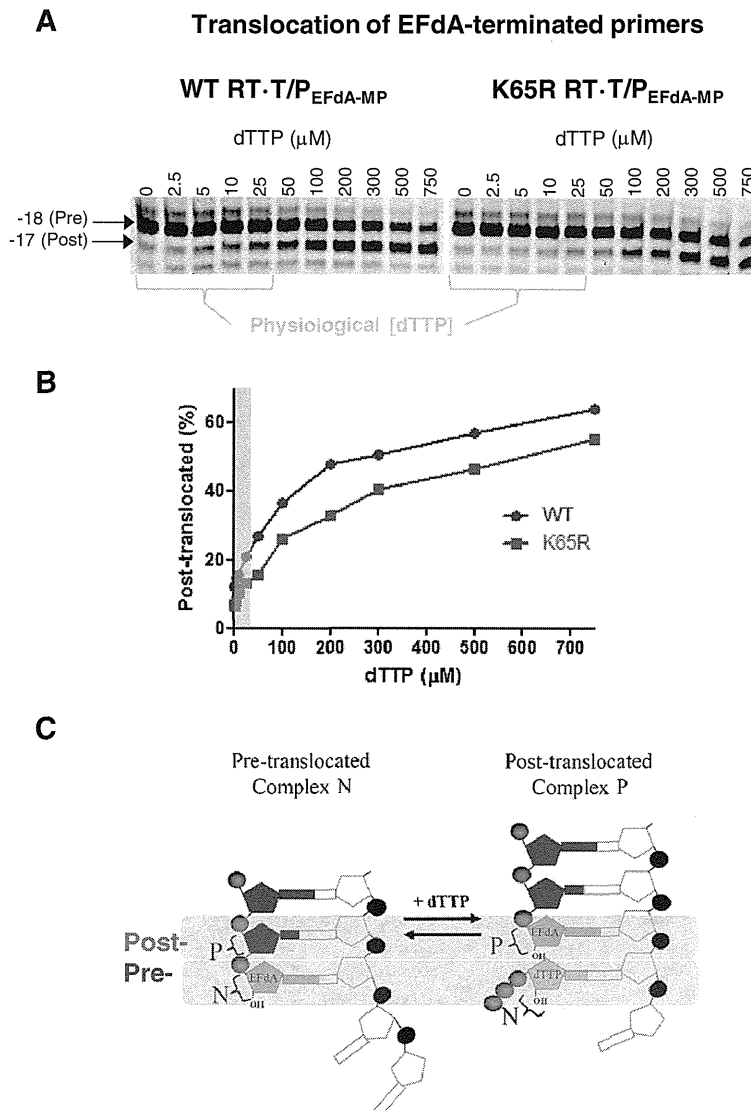
**Table 4 Steady state kinetic parameters for EFdA-TP and dATP incorporation by WT and K65R HIV-1 RTs**

Enzyme	dNTP	K <sub>m</sub> (μM)	k <sub>cat</sub> (min <sup>-1</sup> )	k <sub>cat</sub> / K <sub>m</sub> (min <sup>-1</sup> · μM <sup>-1</sup> )	Selectivity <sup>a</sup>	Fold change <sup>b</sup>
<b>T<sub>d26</sub>/P<sub>d18-P5</sub></b>						
WT RT	dATP	2.38 ± 0.27	1.58 ± 0.12	0.66	1	1
	EFdA-TP	0.66 ± 0.04	3.26 ± 0.42	4.94	7.5	
K65R RT	dATP	5.99 ± 0.81	0.92 ± 0.14	0.15	1	0.8
	EFdA-TP	1.83 ± 0.34	1.74 ± 0.42	0.95	6.3	
<b>T<sub>d31</sub>/P<sub>d18-P0</sub></b>						
WT RT	dATP	0.33 ± 0.07	6.32 ± 0.14	19.15	1	1
	EFdA-TP	0.23 ± 0.01	4.73 ± 0.33	20.57	1.1	
K65R RT	dATP	0.42 ± 0.01	3.62 ± 0.71	8.62	1	1
	EFdA-TP	0.31 ± 0.02	3.07 ± 0.15	9.90	1.1	
<b>T<sub>d31A</sub>/P<sub>d21</sub></b>						
WT RT	dATP	0.37 ± 0.08	3.64 ± 0.57	9.84	1	1
	EFdA-TP	0.19 ± 0.06	3.54 ± 0.44	18.63	1.9	
K65R RT	dATP	1.06 ± 0.11	3.68 ± 0.28	3.47	1	0.9
	EFdA-TP	0.53 ± 0.05	3.38 ± 0.29	6.38	1.8	

Values are mean ± S.D. of two to four independent experiments and were determined from Michaelis-Menten equation using GraphPad Prism 4.

<sup>a</sup>Selectivity is the ratio of the incorporation efficiency (k<sub>cat</sub>/K<sub>m</sub>) of EFdA-TP over that of dATP ([k<sub>cat</sub>/K<sub>m</sub>]<sub>EFdA-TP</sub> / [k<sub>cat</sub>/K<sub>m</sub>]<sub>dATP</sub>).

<sup>b</sup>Fold Change is the ratio of the selectivity in K65R over the selectivity in WT RT.



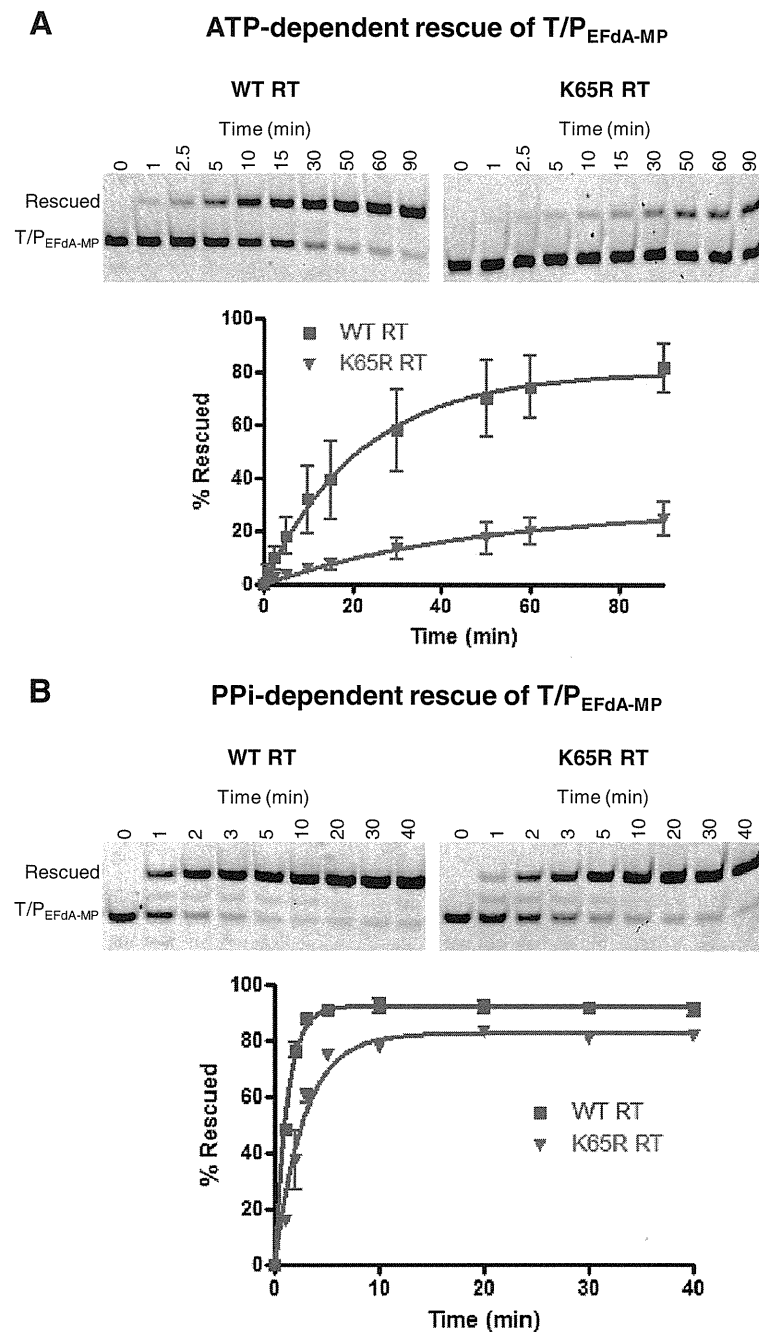
**Figure 2** Effect of K65R mutation on the translocation state of RT bound to T/P<sub>EFdA-MP</sub>. **(A)** The translocation state of HIV-1 RT after EFdA-MP incorporation was determined using site-specific Fe<sup>2+</sup> footprinting. T<sub>d43</sub>/P<sub>d30-EFdA-MP</sub> (100 nM) with 5'-Cy3-label on the DNA template was incubated with WT or K65R HIV-1 RT (600 nM) and various concentrations of the next incoming nucleotide (dTTP). The complexes were treated for 5 minutes with ammonium iron sulphate (1 mM) and resolved on a polyacrylamide 7 M urea gel. An excision at position -18 indicates a pre-translocation complex, while the one at position -17 represents a post-translocation complex. **(B)** The post-translocated complexes were determined from the gels and plotted using GraphPad Prism. Light blue indicates the physiological dNTP concentrations. **(C)** Schematic representation of the position of EFdA-MP-terminated primers at the pre- and post-translocated sites.

nucleic acid having at the 3'-primer terminus EFdA-MP (T<sub>d31</sub>/P<sub>d18-PO-EFdA-MP</sub>). Using ATP as the pyrophosphate donor, we found that the initial rates of the rescue reactions were 2.8-fold slower by K65R than by WT RT (Figure 3A). A 6.5-fold decrease was also observed in PPi-based rescue (Figure 3B). As previously reported [48] the PPi-rescue was faster than the ATP-based rescue assay. Whereas the PPi-based hydrolysis is exactly the opposite of DNA synthesis in reverse, ATP-based hydrolysis has some differences, as we have also structurally demonstrated in the crystal structure of RT in complex with DNA and tetraphosphate excision

product [49]. The above experiments provide strong evidence that K65R mutation confers hypersusceptibility to EFdA mainly through decreased excision.

## Discussion

Tenofovir is a major component of current antiviral therapies (<http://aidsinfo.nih.gov/contentfiles/lvguidelines/adultandadolescentgl.pdf>) and new HIV drugs are likely to be used in patients that have failed tenofovir-based treatment. Hence, the ability of novel HIV inhibitors to efficiently block tenofovir-resistant viruses is critical for their potential utility



**Figure 3 ATP- and PPI-dependent rescue of EFdA-MP terminated primers by WT and K65R RTs.** (A) ATP-dependent rescue of  $T_{d31}/P_{d18-PO-EFdA-MP}$ . Purified  $T_{d31}/P_{d18-PO-EFdA-MP}$  was incubated with WT or K65R RT in the presence of 10 mM  $MgCl_2$ , 3.5 mM ATP, 100  $\mu$ M dATP, 0.5  $\mu$ M dTTP, and 10  $\mu$ M ddGTP at 37°C. Aliquots of the reaction were stopped at the indicated time points (0–90 min). The results of at four independent experiments were plotted using one site hyperbola in Graphpad Prism 4. (B) PPI-dependent rescue of  $T_{d31}/P_{d18-PO-EFdA-MP}$ . Purified  $T_{d31}/P_{d18-PO-EFdA-MP}$  was incubated with WT or K65R RT in the presence of 6 mM  $MgCl_2$ , 150  $\mu$ M PPI, 100  $\mu$ M dATP, 0.5  $\mu$ M dTTP, and 10  $\mu$ M ddGTP at 37°C. Aliquots of the reaction were stopped at the indicated time points (0–40 min). The results of two independent experiments were plotted using one site hyperbola in Graphpad Prism 4.

as HIV therapeutics. The clinical cut-off for tenofovir resistance is defined as a 2.1-fold reduction in virological response. It is associated with the presence of the tenofovir-resistance signature mutation K65R in the reverse transcriptase gene

[50]. We report here that EFdA is highly potent against tenofovir-resistant K65R HIV, and inhibits this mutant 2.5-fold *more* efficiently than WT HIV. Given the fact that clinical resistance to tenofovir is considered a 2.1-fold decrease

in susceptibility, we consider a 2-fold increase in susceptibility as significant hypersusceptibility. Understanding the mechanism by which HIV becomes resistant or more susceptible to EFdA could allow us to overcome drug resistance challenges and improve the current combination therapies. We have previously demonstrated that EFdA is highly efficient in suppressing viral replication of clinical isolates harboring signature mutations to other NRTIs and NNRTIs, including isolates containing 3TC/FTC resistance mutation M184V; TAMs or Q151M complex mutations that confer resistance to AZT, d4T, and abacavir; and nevirapine and efavirenz resistance mutations K103N and Y181C [45]. In addition, we have recently shown that EFdA is 3 logs more potent in SIV inhibition than tenofovir, AZT, and 3TC, and EFdA treatment decreases viral load in SIV-infected macaques by 3–4 logs within 1 week of SIV therapy and eventually to non-detectable levels [51]. The present study demonstrates that the K65R tenofovir-resistance RT mutation confers HIV hypersensitivity to EFdA compared to WT HIV. Other studies have shown that NRTI resistance mutations can confer enhanced susceptibility to other NRTIs. Specifically, the K65R and to a lesser extent the L74V RT mutations have been reported to suppress AZT resistance [43,52–55]. In addition, we have previously reported that K65R and L74V HIVs can be hypersusceptible to NRTIs with 4'-ethynyl substitutions [45,56]. The NNRTI-resistance mutation Y181C also increases susceptibility to AZT [57,58]. Moreover, the 3TC/FTC-resistance mutation M184V also increases HIV sensitivity to AZT by decreasing the excision efficiency of AZT-MP [22,53,59–61]. Finally, we have recently shown that the 172K polymorphism can enhance susceptibility to both NRTIs and NNRTIs [62].

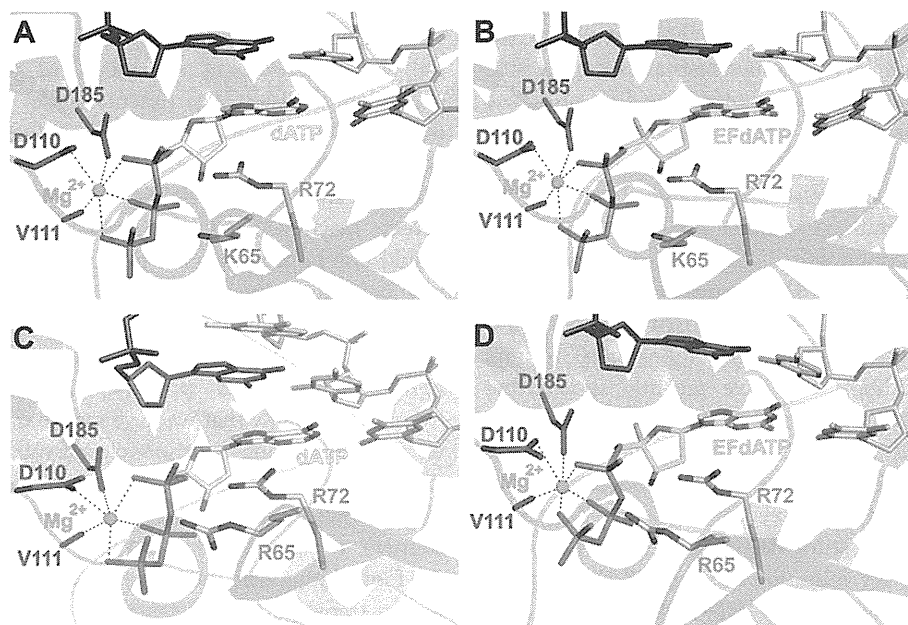
To determine whether the K65R RT mutation has the same effect at the enzyme level as well, we also carried out inhibitor susceptibility experiments with WT and K65R recombinant RT enzymes. Indeed, our enzymatic assays clearly showed that K65R RT is more susceptible to inhibition by EFdA-TP than WT RT. We thus focused on the biochemical mechanism of the enhanced EFdA susceptibility. We previously reported that EFdA is a TDRTI and inhibits primarily by blocking translocation after its incorporation at the 3'-end of the primer [45,46]. Hence, we investigated the effect of the K65R mutation on translocation using the site-specific  $\text{Fe}^{2+}$  footprinting assay. We found that K65R mutation has only a small effect on the translocation state of the EFdA-MP-terminated DNA-RT complex suggesting that the EFdA-MP-terminated primers stay at the nucleotide binding site (N site) of K65R RT as much as they do at the N site of WT RT. Since the EFdA resistance was not the result of changes in translocation efficiency, we hypothesized that K65R affects either the incorporation of the inhibitor itself, or its excision from EFdA-terminated

primers. The effect on incorporation efficiency was assessed with single nucleotide incorporation experiments, whereas the effect on excision was measured in PPI- and ATP-dependent excision experiments under steady state conditions. Our results showed that the K65R mutation decreased the incorporation efficiencies of EFdA-TP and dATP to the same extent. Since pyrophosphorolysis is the reverse reaction of polymerization we hypothesized that it would also be slower in the presence of this mutation. This was confirmed by a PPI-based excision assay where we measured unblocking of EFdA-MP from the 3'-end of the primer. We found that K65R reduced excision and kept EFdA-MP-terminated primers blocked, explaining the hypersusceptibility that we have reported. In addition, when we used conditions that more closely mimic cell-based conditions, with ATP as the unblocking reagent and also all dNTPs present in the reaction to extend the unblocked primers, we also found that K65R reduced excision. Since the footprinting data did not show any significant difference in the translocation efficiency we can therefore conclude that the excision is not decreased because the EFdA-MP-terminated primers reside less at the excisable site. A decreased unblocking of EFdA-MP-terminated primers is not due to their inability to bind at the excisable N site of K65R RT. Instead, the molecular models in Figure 4 suggest that residues R65 and K65 interact differently with R72 and the phosphate moieties of EFdA-TP or dNTP, and thus may differentially affect the recognition of the pyrophosphate donor (ATP or PPI) and its nucleophilic attack on EFdA-terminated primers. Future crystallographic studies should provide more details on the molecular basis of excision-based EFdA resistance.

## Conclusion

We have provided virological and biochemical data demonstrating that the K65R RT mutation confers enhanced sensitivity to EFdA. We reported here that the mechanism of hypersensitivity is mainly through reduced excision of the chain terminating EFdA-MP. Our findings demonstrated that EFdA is a very potent NRTI and it could be used not only against WT HIV but also against tenofovir-resistant HIVs. The primary resistance mutation for EFdA is M184V and combination with tenofovir could be similar to the pair of mutations for 3TC/AZT combination. Unlike AZT and 3TC which are analogs of different deoxynucleosides, EFdA and tenofovir are both deoxyadenosine analogs and would theoretically compete to each other. However, they are activated/phosphorylated by different pathways [45]. Therefore, combination of EFdA with tenofovir could help suppress K65R resistance. This conclusion has significant potential therapeutic implications. Moreover, EFdA would be a good candidate in salvage therapies for patients that fail tenofovir-treatment due to K65R resistance.





**Figure 4** Molecular models of dATP and EFdA-TP in the active sites of WT and K65R HIV RT. dATP (yellow sticks, **A** and **C**) and EFdA-TP (cyan sticks, **B** and **D**) are shown at the active sites of WT HIV RT, (**A** and **B**) or K65R HIV RT (**C** and **D**). The fingers and palm subdomains are shown in blue and red cartoon, respectively. The primer and template strands are shown in dark gray and light gray sticks, respectively. Figures were made using PyMOL (The PyMOL Molecular Graphics System, Version 1.3 Schrödinger, LLC).

## Methods

### Cells and viruses

TZM-bl cells (CCR5 transduced HeLa-CD4/LTR- $\beta$ -gal and luciferase cells) were obtained from the AIDS Research and Reference Reagent Program, the National Institutes of Health (NIH). 293T and TZM-bl cells were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin, and used for transfection and antiviral assays, respectively.

K65R RT mutation was introduced by site-directed mutagenesis as described previously [63,64]. Briefly, the desired mutations were introduced into the *Xma*I - *Nhe*I region (759 bp) of pTZNX1, which encodes nucleotides Gly-15 to Ala-267 of HIV-1 RT. After mutagenesis, the *Xma*I - *Nhe*I cassettes were inserted back into pNL101 and confirmed by sequencing. Viral stocks were obtained by transfection of each molecular clone into 293T cells using Fugene 6 (Roche, Mannheim, Germany), harvested and stored at  $-80^{\circ}\text{C}$  until use.

### Cell-based drug susceptibility assays

Single-replication-cycle drug susceptibility assays were performed in triplicates using TZM-bl cells. TZM-bl cells were infected with diluted virus stock at 400,000 relative light units (RLU) in the presence of increasing concentrations of RTIs and cultured for 48 h. The luciferase marker gene expressions were measured using the Bright-

Glo (Promega, WI). Susceptibility to NRTIs was calculated as the concentration that reduces RLU (infection) by 50% (50% effective concentration [ $\text{EC}_{50}$ ]). The data were obtained from the results of at least three independent experiments and the P values were determined using *t*-test statistical analysis.

### Enzymes and nucleic acids

HIV-1 RTs were expressed in JM-109 (Invitrogen) bacteria and purified by nickel affinity chromatography and monoQ anion exchange chromatography as previously described [46,65-69]. Oligonucleotides used in this study were chemically synthesized and purchased from Integrated DNA Technologies (Coralville, IA). Sequences of the DNA substrates are shown in Table 3. Deoxynucleotide triphosphates and dideoxynucleotide triphosphates were purchased from Fermentas (Glen Burnie, MD). EFdA was synthesized by Yamasa Corporation (Chiba, Japan) as described before [70]. Using EFdA as starting material the triphosphate form EFdA-TP was synthesized by TriLink BioTechnologies (San Diego, CA). Concentrations of nucleotides and EFdA-TP were calculated spectrophotometrically on the basis of absorption at 260 nm and their extinction coefficients. All nucleotides were treated with inorganic pyrophosphatase (Roche Diagnostics) as described previously [26] to remove traces of PPi contamination that might interfere with the rescue assay.

### Enzymatic drug susceptibility assays

#### *Inhibition of HIV-1 RT-catalyzed DNA Synthesis by EFdA-TP*

DNA template was annealed to 5'-Cy3 labeled DNA primer (3:1 molar ratio) ( $T_{d31}/P_{d18-P0}$ ). To monitor primer extension, the DNA/DNA hybrid (20 nM) was incubated at 37°C with WT or K65R HIV-1 RT (20 nM) in a buffer containing 50 mM Tris (pH 7.8) and 50 mM NaCl (RT buffer). Subsequently, varying amounts of EFdA-TP were added and the reactions were initiated by the addition of 6 or 10 mM  $MgCl_2$  in a final volume of 20  $\mu$ l. All dNTPs were present at a final concentration of 1  $\mu$ M in the presence or absence of 3.5 mM ATP. The reactions were terminated after 50 minutes by adding equal volume of 100% formamide containing traces of bromophenol blue. The products were resolved on 15% polyacrylamide 7 M urea gels. In this and in subsequent assays the gels were scanned with a Typhoon FLA 9000 PhosphorImager (GE Healthcare, NJ). The bands corresponding to fully-extended product were quantified using Multi Gauge software. The results of at least four independent experiments were plotted as percent full extension using one site-competition nonlinear regression in GraphPad Prism 4 to determine the mean and standard deviation of the  $IC_{50}$  for EFdA-TP.

### Steady-state Kinetics

#### *Single nucleotide incorporation of dATP and EFdA-TP by WT and K65R RTs*

Steady-state kinetic parameters  $K_m$  and  $k_{cat}$  for incorporation of EFdA-TP or dATP were determined using single nucleotide incorporation in gel-based assays under saturating concentrations of T/P (10-fold excess over RT). Reactions were carried out in RT buffer, 6 mM  $MgCl_2$ , 100 nM  $T_{d26}/P_{d18-P5}$  or  $T_{d31}/P_{d18-P0}$  or  $T_{d31A}/P_{d21}$  (Table 3) and 10 nM WT or K65R HIV-1 RT in a final volume of 20  $\mu$ l and stopped at indicated reaction times. The products were resolved and quantified as described above.  $K_m$  and  $k_{cat}$  were determined graphically using the Michaelis-Menten equation. Reactions were carried out in two to four independent experiments to determine the mean and standard deviation values.

#### *Site-specific $Fe^{2+}$ footprinting assay*

Site-specific  $Fe^{2+}$  footprints were monitored on 5'-Cy3-labeled DNA templates. 100 nM of 5'-Cy3- $T_{d43}/P_{d30}$  was incubated with 600 nM WT or K65R HIV-1 RT in a buffer containing 120 mM sodium cacodylate (pH 7), 20 mM NaCl, 6 mM  $MgCl_2$ , and 1  $\mu$ M EFdA-TP, to allow quantitative chain-termination. Prior to the treatment with  $Fe^{2+}$ , complexes were pre-incubated for 7 min with increasing concentrations of the next incoming nucleotide (dTTP). The complexes were treated with ammonium iron sulfate (1 mM) as previously

described [46,47]. This reaction relies on autoxidation of  $Fe^{2+}$  [71] to create a local concentration of hydroxyl radicals, which cleave the DNA at the nucleotide closest to the  $Fe^{2+}$  specifically bound to the RNase H active site. These experiments were performed at least twice.

#### *ATP- and PPI-dependent excision and rescue of T/P<sub>EFdA-MP</sub>*

##### *ATP-dependent rescue of T/P<sub>EFdA-MP</sub>*

Template/primer with EFdA-MP at the 3' primer terminus ( $T/P_{EFdA-MP}$ ) was prepared by incubating 500 nM  $T_{d31}/P_{d18-P0}$  with 1  $\mu$ M HIV-1 RT in RT buffer and 6 mM  $MgCl_2$ . EFdA-TP was added into the reaction and the mixture was incubated at 37°C for 1 h. After incorporation of EFdA-TP, the  $T/P_{EFdA-MP}$  was purified using the QIAquick nucleotide removal kit (Qiagen, Valencia, CA). Under these conditions, the extension of T/P to  $T/P_{EFdA-MP}$  was complete. 20 nM of purified  $T_{d31}/P_{d18-P0-EFdA-MP}$  was incubated with 60 nM WT or K65R HIV-1 RT in the presence of 3.5 mM ATP, 100  $\mu$ M dATP, 0.5  $\mu$ M dTTP, and 10  $\mu$ M ddGTP in RT buffer and 10 mM  $MgCl_2$ . Aliquots of the reaction were stopped at different time points (0–90 min). The data from at least four independent experiments were analyzed using GraphPad Prism 4.

##### *PPI-dependent rescue of T/P<sub>EFdA-MP</sub>*

20 nM of purified  $T_{d31}/P_{d18-P0-EFdA-MP}$  was incubated at 37°C with 60 nM WT or K65R HIV-1 RT in the presence of 150  $\mu$ M PPI, 100  $\mu$ M dATP, 0.5  $\mu$ M dTTP, and 10  $\mu$ M ddGTP in RT buffer and 6 mM  $MgCl_2$ . Aliquots of the reaction were stopped at different times (0–40 min). The data from at least two independent experiments were plotted using GraphPad Prism 4.

### Molecular modeling

Molecular models of dATP and EFdA-TP in the active site of WT HIV RT were made using PDB ID 1 T05 [72] as a starting model (WT HIV RT in complex with tenofovir diphosphate). A molecular model of EFdA-TP in the active site of K65R HIV RT was made using PDB ID 3JYT [44] as a starting model (K65R HIV RT in complex with dATP). The sketch module of SYBYL (Version 7.3.5, Tripos International, St. Louis, MO) was used to make dATP and EFdA-TP molecules. dATP and EFdA-TP were each superposed to tenofovir diphosphate in the WT complex, after which the tenofovir diphosphate was removed. Gasteiger-Huckel charges were calculated and molecular minimization of the WT-dATP and WT-EFdA-TP were performed in SYBYL using the Powell method. SYBYL was also used to add the 2-fluoro and 4'-ethynyl groups to dATP in the K65R complex. Gasteiger-Huckel charges were then calculated and molecular minimization was performed as for the WT complexes.

## Additional file

**Additional file 1: Figure S1.** Effect of K65R mutation on the formation of RT:T/P<sub>EFdA-MP</sub> complex. Purified T/P<sub>EFdA-MP</sub> (25 nM) was incubated at room temperature for 10 min with different concentrations of WT or K65R RTs in RT buffer and 6 mM MgCl<sub>2</sub>. RT was used at different concentrations to obtain RT:DNA ratios that ranged from 0 to 10. Four  $\mu$ l of 20% sucrose was added to each mixture in a final volume of 24  $\mu$ l. The complexes were subsequently resolved on a native 6% polyacrylamide Tris borate gel and visualized as described in Methods.

### Abbreviations

HIV: Human immunodeficiency virus; RT: Reverse transcriptase; NRTI: Nucleoside reverse transcriptase inhibitor; TDRIT: Translocation-defective RT inhibitor; EFdA: 4'-ethynyl-2-fluoro-2'-deoxyadenosine; MP: Monophosphate; TP: Triphosphate; TDF: Tenofovir disoproxil fumarate; T/P: Template/primer; T/P<sub>EFdA-MP</sub>: Template/primer possessing EFdA-MP at the 3'-primer terminus (or T/P chain terminated by EFdA-MP).

### Competing interests

Hiroaki Mitsuya and Eiichi Kodama are inventors of EFdA.

### Authors' contributions

EM designed the biochemical experiments. EM and EMR carried out the biochemical experiments. MDL, ADH, and KS assisted in some of the biochemical experiments. AH designed and carried out the cell-based assays. KAK and SGS performed the molecular modeling studies. YTO and JCY participated in the initial biochemical studies. BM participated in the design of biochemical experiments and interpretation of data. ENK, HM, and MAP helped with preliminary virological data. MAP helped in the data interpretation. EM drafted the manuscript. SGS conceived and coordinated the study and drafted the manuscript. All authors read and approved the final manuscript.

### Acknowledgements

Dr. Tatiana Ilna's contribution in the preparation of the RT plasmids is acknowledged. This work was supported, in whole or in part, by National Institutes of Health grants AI076119, AI074389, AI076119-S1, AI076119-02S1, AI100890, AI099284, and GM103368 (S. G. S.) and AI079801 (M. A. P.).

### Author details

<sup>1</sup>Christopher Bond Life Sciences Center, Department of Molecular Microbiology & Immunology, University of Missouri, Columbia, MO 65211, USA. <sup>2</sup>Clinical Research Center, Department of Infectious Diseases and Immunology, National Hospital Organization Nagoya Medical Center, Nagoya 4600001, Japan. <sup>3</sup>Division of Emerging Infectious Diseases, Tohoku University, Sendai 980-8575, Japan. <sup>4</sup>Department of Internal Medicine, Kumamoto University, Kumamoto 860-8556, Japan. <sup>5</sup>Experimental Retrovirology Section, HIV/AIDS Malignancy Branch, NIH, Bethesda, MD 20892, USA. <sup>6</sup>Department of Microbiology and Molecular Genetics, University of Pittsburgh, Pittsburgh, PA 15219, USA. <sup>7</sup>Department of Biochemistry, University of Missouri, Columbia, MO 65211, USA.

Received: 22 April 2013 Accepted: 13 June 2013

Published: 24 June 2013

### References

- Parniak MA, Sluis-Cremer N: Inhibitors of HIV-1 reverse transcriptase. *Advances in pharmacology (San Diego, Calif)* 2000, **49**:67-109.
- Sharma PL, Nurpeisov V, Hernandez-Santiago B, Beltran T, Schinazi RF: Nucleoside inhibitors of human immunodeficiency virus type 1 reverse transcriptase. *Curr Top Med Chem* 2004, **4**:895-919.
- Hammer SM, Saag MS, Schechter M, Montaner JS, Schooley RT, Jacobsen DM, Thompson MA, Carpenter CC, Fischl MA, Gazzard BG, *et al*: Treatment for adult HIV infection: 2006 recommendations of the International AIDS Society-USA panel. *Top HIV Med* 2006, **14**:827-843.
- Schinazi RF, Hernandez-Santiago B, Hurwitz SJ: Pharmacology of current and promising nucleosides for the treatment of human immunodeficiency viruses. *Antiviral Res* 2006, **71**:322-334.
- De Clercq E: Anti-HIV drugs. *Verh K Acad Geneesk Belg* 2007, **69**:81-104.
- Sluis-Cremer N, Tachedjian G: Mechanisms of inhibition of HIV replication by non-nucleoside reverse transcriptase inhibitors. *Virus Res* 2008, **134**:147-156.
- Deval J: Antimicrobial strategies: inhibition of viral polymerases by 3'-hydroxyl nucleosides. *Drugs* 2009, **69**:151-166.
- Sarafianos SG, Marchand B, Das K, Himmel DM, Parniak MA, Hughes SH, Arnold E: Structure and Function of HIV-1 Reverse Transcriptase: Molecular Mechanisms of Polymerization and Inhibition. *J Mol Biol* 2009, **385**:693-713.
- Ren J, Esnouf R, Garman E, Somers D, Ross C, Kirby I, Keeling J, Darby G, Jones Y, Stuart D, *et al*: High resolution structures of HIV-1 RT from four RT-inhibitor complexes. *Nat Struct Biol* 1995, **2**:293-302.
- Ding J, Das K, Hsiou Y, Sarafianos SG, Clark AD Jr, Jacobo-Molina A, Tantillo C, Hughes SH, Arnold E: Structure and functional implications of the polymerase active site region in a complex of HIV-1 RT with a double-stranded DNA template-primer and an antibody Fab fragment at 2.8 Å resolution. *J Mol Biol* 1998, **284**:1095-1111.
- Kohlstaedt LA, Wang J, Friedman JM, Rice PA, Steitz TA: Crystal structure at 3.5 Å resolution of HIV-1 reverse transcriptase complexed with an inhibitor. *Science* 1992, **256**:1783-1790.
- Rodgers DW, Gamblin SJ, Harris BA, Ray S, Culp JS, Hellmig B, Woolf DJ, Debouck C, Harrison SC: The structure of unliganded reverse transcriptase from the human immunodeficiency virus type 1. *Proc Natl Acad Sci USA* 1995, **92**:1222-1226.
- Hsiou Y, Ding J, Das K, Clark AD Jr, Hughes SH, Arnold E: Structure of unliganded HIV-1 reverse transcriptase at 2.7 Å resolution: implications of conformational changes for polymerization and inhibition mechanisms. *Structure* 1996, **4**:853-860.
- Spence RA, Kati WM, Anderson KS, Johnson KA: Mechanism of inhibition of HIV-1 reverse transcriptase by nonnucleoside inhibitors. *Science* 1995, **267**:988-993.
- Rittinger K, Divita G, Goody RS: Human immunodeficiency virus reverse transcriptase substrate-induced conformational changes and the mechanism of inhibition by nonnucleoside inhibitors. *Proc Natl Acad Sci USA* 1995, **92**:8046-8049.
- Sluis-Cremer N, Arion D, Parniak MA: Molecular mechanisms of HIV-1 resistance to nucleoside reverse transcriptase inhibitors (NRTIs). *Cell Mol Life Sci* 2000, **57**:1408-1422.
- Menendez-Arias L: Mechanisms of resistance to nucleoside analogue inhibitors of HIV-1 reverse transcriptase. *Virus Res* 2008, **134**:124-146.
- Martin-Hernandez AM, Domingo E, Menendez-Arias L: Human immunodeficiency virus type 1 reverse transcriptase: role of Tyr115 in deoxynucleotide binding and misinsertion fidelity of DNA synthesis. *EMBO J* 1996, **15**:4434-4442.
- Gao G, Orlova M, Georgiadis MM, Hendrickson WA, Goff SP: Conferring RNA polymerase activity to a DNA polymerase: a single residue in reverse transcriptase controls substrate selection. *Proc Natl Acad Sci USA* 1997, **94**:407-411.
- Gao HQ, Boyer PL, Sarafianos SG, Arnold E, Hughes SH: The role of steric hindrance in 3TC resistance of human immunodeficiency virus type-1 reverse transcriptase. *J Mol Biol* 2000, **300**:403-418.
- Sarafianos SG, Das K, Clark AD Jr, Ding J, Boyer PL, Hughes SH, Arnold E: Lamivudine (3TC) resistance in HIV-1 reverse transcriptase involves steric hindrance with beta-branched amino acids. *Proc Natl Acad Sci USA* 1999, **96**:10027-10032.
- Tisdale M, Kemp SD, Parry NR, Larder BA: Rapid in vitro selection of human immunodeficiency virus type 1 resistant to 3'-thiacytidine inhibitors due to a mutation in the YMDD region of reverse transcriptase. *Proc Natl Acad Sci USA* 1993, **90**:5653-5656.
- Feng JY, Anderson KS: Mechanistic studies examining the efficiency and fidelity of DNA synthesis by the 3TC-resistant mutant (184V) of HIV-1 reverse transcriptase. *Biochemistry* 1999, **38**:9440-9448.
- Back NK, Nijhuis M, Keulen W, Boucher CA, Oude Essink BO, van Kuilenburg AB, van Gennip AH, Berkhout B: Reduced replication of 3TC-resistant HIV-1 variants in primary cells due to a processivity defect of the reverse transcriptase enzyme. *EMBO J* 1996, **15**:4040-4049.
- Arion D, Kaushik N, McCormick S, Borkow G, Parniak MA: Phenotypic mechanism of HIV-1 resistance to 3'-azido-3'-deoxythymidine (AZT): increased polymerization processivity and enhanced sensitivity to pyrophosphate of the mutant viral reverse transcriptase. *Biochemistry* 1998, **37**:15908-15917.
- Meyer PR, Matsuura SE, So AG, Scott WA: Unblocking of chain-terminated primer by HIV-1 reverse transcriptase through a nucleotide-dependent mechanism. *Proc Natl Acad Sci USA* 1998, **95**:13471-13476.

27. Meyer PR, Matsuura SE, Mian AM, So AG, Scott WA: A mechanism of AZT resistance: an increase in nucleotide-dependent primer unblocking by mutant HIV-1 reverse transcriptase. *Mol Cell* 1999, **4**:35–43.
28. Meyer PR, Matsuura SE, Tolun AA, Pfeifer I, So AG, Mellors JW, Scott WA: Effects of specific zidovudine resistance mutations and substrate structure on nucleotide-dependent primer unblocking by human immunodeficiency virus type 1 reverse transcriptase. *Antimicrob Agents Chemother* 2002, **46**:1540–1545.
29. Boyer PL, Sarafianos SG, Arnold E, Hughes SH: Selective excision of AZTMP by drug-resistant human immunodeficiency virus reverse transcriptase. *J Virol* 2001, **75**:4832–4842.
30. Larder BA, Kemp SD: Multiple mutations in HIV-1 reverse transcriptase confer high-level resistance to zidovudine (AZT). *Science* 1989, **246**:1155–1158.
31. Winters MA, Shafer RW, Jellinger RA, Mamtora G, Gingeras T, Merigan TC: Human immunodeficiency virus type 1 reverse transcriptase genotype and drug susceptibility changes in infected individuals receiving dideoxynucleoside monotherapy for 1 to 2 years. *Antimicrob Agents Chemother* 1997, **41**:757–762.
32. Harrigan PR, Stone C, Griffin P, Najera I, Bloor S, Kemp S, Tisdale M, Larder B: Resistance profile of the human immunodeficiency virus type 1 reverse transcriptase inhibitor abacavir (1592U89) after monotherapy and combination therapy. CNA2001 Investigative Group. *J Infect Dis* 2000, **181**:912–920.
33. Margot NA, Lu B, Cheng A, Miller MD, Study T: Resistance development over 144 weeks in treatment-naïve patients receiving tenofovir disoproxil fumarate or stavudine with lamivudine and efavirenz in Study 903. *HIV Med* 2006, **7**:442–450.
34. Wainberg MA, Miller MD, Quan Y, Salomon H, Mulato AS, Lamy PD, Margot NA, Anton KE, Cherrington JM: In vitro selection and characterization of HIV-1 with reduced susceptibility to PMPA. *Antivir Ther* 1999, **4**:87–94.
35. Margot NA, Isaacson E, McGowan I, Cheng AK, Schooley RT, Miller MD: Genotypic and phenotypic analyses of HIV-1 in antiretroviral-experienced patients treated with tenofovir DF. *AIDS* 2002, **16**:1227–1235.
36. Feng JY, Myrick FT, Margot NA, Mulamba GB, Rimsky L, Borroto-Esoda K, Selmi B, Canard B: Virologic and enzymatic studies revealing the mechanism of K65R- and Q151M-associated HIV-1 drug resistance towards emtricitabine and lamivudine. *Nucleosides Nucleotides Nucleic Acids* 2006, **25**:89–107.
37. Shah FS, Curr KA, Hamburg ME, Parniak M, Mitsuya H, Arnez JG, Prasad VR: Differential influence of nucleoside analog-resistance mutations K65R and L74V on the overall mutation rate and error specificity of human immunodeficiency virus type 1 reverse transcriptase. *J Biol Chem* 2000, **275**:27037–27044.
38. Frankel FA, Invernizzi CF, Oliveira M, Wainberg MA: Diminished efficiency of HIV-1 reverse transcriptase containing the K65R and M184V drug resistance mutations. *AIDS* 2007, **21**:665–675.
39. Parikh UM, Zelina S, Sluis-Cremer N, Mellors JW: Molecular mechanisms of bidirectional antagonism between K65R and thymidine analog mutations in HIV-1 reverse transcriptase. *AIDS* 2007, **21**:1405–1414.
40. White KL, Margot NA, Ly JK, Chen JM, Ray AS, Pavelko M, Wang R, McDermott M, Swaminathan S, Miller MD: A combination of decreased NRTI incorporation and decreased excision determines the resistance profile of HIV-1 K65R RT. *AIDS* 2005, **19**:1751–1760.
41. Deval J, Navarro JM, Selmi B, Courcambecq J, Boretto J, Halfon P, Garrido-Urbani S, Sire J, Canard B: A loss of viral replicative capacity correlates with altered DNA polymerization kinetics by the human immunodeficiency virus reverse transcriptase bearing the K65R and L74V dideoxynucleoside resistance substitutions. *J Biol Chem* 2004, **279**:25489–25496.
42. Deval J, White KL, Miller MD, Parkin NT, Courcambecq J, Halfon P, Selmi B, Boretto J, Canard B: Mechanistic basis for reduced viral and enzymatic fitness of HIV-1 reverse transcriptase containing both K65R and M184V mutations. *J Biol Chem* 2004, **279**:509–516.
43. White KL, Chen JM, Feng JY, Margot NA, Ly JK, Ray AS, Macarthur HL, McDermott MJ, Swaminathan S, Miller MD: The K65R reverse transcriptase mutation in HIV-1 reverses the excision phenotype of zidovudine resistance mutations. *Antivir Ther* 2006, **11**:155–163.
44. Das K, Bandwar RP, White KL, Feng JY, Sarafianos SG, Tuske S, Tu X, Clark AD Jr, Boyer PL, Hou X, *et al*: Structural basis for the role of the K65R mutation in HIV-1 reverse transcriptase polymerization, excision antagonism, and tenofovir resistance. *J Biol Chem* 2009, **284**:35092–35100.
45. Kawamoto A, Kodama E, Sarafianos SG, Sakagami Y, Kohgo S, Kitano K, Ashida N, Iwai Y, Hayakawa H, Nakata H, *et al*: 2'-deoxy-4'-C-ethynyl-2-halo-adenosines active against drug-resistant human immunodeficiency virus type 1 variants. *Int J Biochem Cell Biol* 2008, **40**:2410–2420.
46. Michailidis E, Marchand B, Kodama EN, Singh K, Matsuoka M, Kirby KA, Ryan EM, Sawani AM, Nagy E, Ashida N, *et al*: Mechanism of inhibition of HIV-1 reverse transcriptase by 4'-Ethynyl-2-fluoro-2'-deoxyadenosine triphosphate, a translocation-defective reverse transcriptase inhibitor. *J Biol Chem* 2009, **284**:35681–35691.
47. Marchand B, Gotte M: Site-specific footprinting reveals differences in the translocation status of HIV-1 reverse transcriptase. Implications for polymerase translocation and drug resistance. *J Biol Chem* 2003, **278**:35362–35372.
48. Ray AS, Murakami E, Basavapathruni A, Vaccaro JA, Ulrich D, Chu CK, Schinazi RF, Anderson KS: Probing the molecular mechanisms of AZT drug resistance mediated by HIV-1 reverse transcriptase using a transient kinetic analysis. *Biochemistry* 2003, **42**:8831–8841.
49. Tu X, Das K, Han Q, Bauman JD, Clark AD Jr, Hou X, Frenkel YV, Gaffney BL, Jones RA, Boyer PL, *et al*: Structural basis of HIV-1 resistance to AZT by excision. *Nat Struct Mol Biol* 2010, **17**:1202–1209.
50. Winters B, Montaner J, Harrigan PR, Gazzard B, Pozniak A, Miller MD, Emery S, van Leth F, Robinson P, Baxter JD, *et al*: Determination of clinically relevant cutoffs for HIV-1 phenotypic resistance estimates through a combined analysis of clinical trial and cohort data. *J Acquir Immune Defic Syndr* 2008, **48**:26–34.
51. Murphey-Corb M, Rajakumar P, Michael H, Nyaundi J, Didier PJ, Reeve AB, Mitsuya H, Sarafianos SG, Parniak MA: Response of simian immunodeficiency virus to the novel nucleoside reverse transcriptase inhibitor 4'-ethynyl-2-fluoro-2'-deoxyadenosine in vitro and in vivo. *Antimicrob Agents Chemother* 2012, **56**:4707–4712.
52. St Clair MH, Martin JL, Tudor-Williams G, Bach MC, Vavro CL, King DM, Kellam P, Kemp SD, Larder BA: Resistance to ddI and sensitivity to AZT induced by a mutation in HIV-1 reverse transcriptase. *Science* 1991, **253**:1557–1559.
53. Quan Y, Gu Z, Li X, Liang C, Parniak MA, Wainberg MA: Endogenous reverse transcriptase assays reveal synergy between combinations of the M184V and other drug resistance-conferring mutations in interactions with nucleoside analog triphosphates. *J Mol Biol* 1998, **277**:237–247.
54. Bazmi HZ, Hammond JL, Cavalcanti SC, Chu CK, Schinazi RF, Mellors JW: In vitro selection of mutations in the human immunodeficiency virus type 1 reverse transcriptase that decrease susceptibility to (–)-beta-D-dioxolane-guanosine and suppress resistance to 3'-azido-3'-deoxythymidine. *Antimicrob Agents Chemother* 2000, **44**:1783–1788.
55. Parikh UM, Bachelier L, Koontz D, Mellors JW: The K65R mutation in human immunodeficiency virus type 1 reverse transcriptase exhibits bidirectional phenotypic antagonism with thymidine analog mutations. *J Virol* 2006, **80**:4971–4977.
56. Kodama EI, Kohgo S, Kitano K, Machida H, Gatanaga H, Shigeta S, Matsuoka M, Ohru H, Mitsuya H: 4'-Ethynyl nucleoside analogs: potent inhibitors of multidrug-resistant human immunodeficiency virus variants in vitro. *Antimicrob Agents Chemother* 2001, **45**:1539–1546.
57. Larder BA: 3'-Azido-3'-deoxythymidine resistance suppressed by a mutation conferring human immunodeficiency virus type 1 resistance to nonnucleoside reverse transcriptase inhibitors. *Antimicrob Agents Chemother* 1992, **36**:2664–2669.
58. Selmi B, Deval J, Alvarez K, Boretto J, Sarfati S, Guerreiro C, Canard B: The Y181C substitution in 3'-azido-3'-deoxythymidine-resistant human immunodeficiency virus, type 1, reverse transcriptase suppresses the ATP-mediated repair of the 3'-azido-3'-deoxythymidine 5'-monophosphate-terminated primer. *J Biol Chem* 2003, **278**:40464–40472.
59. Boucher CA, Cammack N, Schipper P, Schuurman R, Rouse P, Wainberg MA, Cameron JM: High-level resistance to (–) enantiomeric 2'-deoxy-3'-thiacytidine in vitro is due to one amino acid substitution in the catalytic site of human immunodeficiency virus type 1 reverse transcriptase. *Antimicrob Agents Chemother* 1993, **37**:2231–2234.
60. Boyer PL, Sarafianos SG, Arnold E, Hughes SH: The M184V mutation reduces the selective excision of zidovudine 5'-monophosphate (AZTMP) by the reverse transcriptase of human immunodeficiency virus type 1. *J Virol* 2002, **76**:3248–3256.
61. Gotte M, Arion D, Parniak MA, Wainberg MA: The M184V mutation in the reverse transcriptase of human immunodeficiency virus type 1 impairs rescue of chain-terminated DNA synthesis. *J Virol* 2000, **74**:3579–3585.

62. Hachiya A, Marchand B, Kirby KA, Michailidis E, Tu X, Palczewski K, Ong YT, Li Z, Griffin DT, Schuckmann MM, *et al*: HIV-1 reverse transcriptase (RT) polymorphism 172K suppresses the effect of clinically relevant drug resistance mutations to both nucleoside and non-nucleoside RT inhibitors. *J Biol Chem* 2012, **287**:29988–29999.
63. Hachiya A, Gatanaga H, Kodama E, Ikeuchi M, Matsuoka M, Harada S, Mitsuya H, Kimura S, Oka S: Novel patterns of nevirapine resistance-associated mutations of human immunodeficiency virus type 1 in treatment-naive patients. *Virology* 2004, **327**:215–224.
64. Hachiya A, Kodama EN, Sarafianos SG, Schuckmann MM, Sakagami Y, Matsuoka M, Takiguchi M, Gatanaga H, Oka S: Amino acid mutation N348I in the connection subdomain of human immunodeficiency virus type 1 reverse transcriptase confers multiclass resistance to nucleoside and nonnucleoside reverse transcriptase inhibitors. *J Virol* 2008, **82**:3261–3270.
65. Bauman JD, Das K, Ho WC, Baweja M, Himmel DM, Clark AD Jr, Oren DA, Boyer PL, Hughes SH, Shatkin AJ, Arnold E: Crystal engineering of HIV-1 reverse transcriptase for structure-based drug design. *Nucleic Acids Res* 2008, **36**:5083–5092.
66. Schuckmann MM, Marchand B, Hachiya A, Kodama EN, Kirby KA, Singh K, Sarafianos SG: The N348I mutation at the connection subdomain of HIV-1 reverse transcriptase decreases binding to nevirapine. *J Biol Chem* 2010, **285**:38700–38709.
67. Ndongwe TP, Adedeji AO, Michailidis E, Ong YT, Hachiya A, Marchand B, Ryan EM, Rai DK, Kirby KA, Whatley AS, *et al*: Biochemical, inhibition and inhibitor resistance studies of xenotropic murine leukemia virus-related virus reverse transcriptase. *Nucleic Acids Res* 2012, **40**:345–359.
68. Kirby KA, Marchand B, Ong YT, Ndongwe TP, Hachiya A, Michailidis E, Leslie MD, Sietsema DV, Fetterly TL, Dorst CA, *et al*: Structural and Inhibition Studies of the RNase H Function of Xenotropic Murine Leukemia Virus-Related Virus Reverse Transcriptase. *Antimicrob Agents Chemother* 2012, **56**(4):2048–61.
69. Sarafianos SG, Clark AD Jr, Tuske S, Squire CJ, Das K, Sheng D, Ilankumaran P, Ramesha AR, Kroth H, Sayer JM, *et al*: Trapping HIV-1 reverse transcriptase before and after translocation on DNA. *J Biol Chem* 2003, **278**:16280–16288.
70. Ohru H, Kohgo S, Hayakawa H, Kodama E, Matsuoka M, Nakata T, Mitsuya H: 2'-deoxy-4'-C-ethynyl-2-fluoroadenosine: a nucleoside reverse transcriptase inhibitor with highly potent activity against wide spectrum of HIV-1 strains, favorable toxic profiles, and stability in plasma. *Nucleosides Nucleotides Nucleic Acids* 2007, **26**(10-12):1543–6.
71. Biaglow JE, Kachur AV: The generation of hydroxyl radicals in the reaction of molecular oxygen with polyphosphate complexes of ferrous ion. *Radiat Res* 1997, **148**:181–187.
72. Tuske S, Sarafianos SG, Clark AD Jr, Ding J, Naeger LK, White KL, Miller MD, Gibbs CS, Boyer PL, Clark P, *et al*: Structures of HIV-1 RT-DNA complexes before and after incorporation of the anti-AIDS drug tenofovir. *Nat Struct Mol Biol* 2004, **11**:469–474.

doi:10.1186/1742-4690-10-65

Cite this article as: Michailidis *et al*: Hypersusceptibility mechanism of Tenofovir-resistant HIV to EFD. *Retrovirology* 2013 **10**:65.

Submit your next manuscript to BioMed Central  
and take full advantage of:

- Convenient online submission
- Thorough peer review
- No space constraints or color figure charges
- Immediate publication on acceptance
- Inclusion in PubMed, CAS, Scopus and Google Scholar
- Research which is freely available for redistribution

Submit your manuscript at  
[www.biomedcentral.com/submit](http://www.biomedcentral.com/submit)



## Evaluation of Combinations of 4'-Ethynyl-2-Fluoro-2'-Deoxyadenosine with Clinically Used Antiretroviral Drugs

Atsuko Hachiya,<sup>a,b\*</sup> Aaron B. Reeve,<sup>c</sup> Bruno Marchand,<sup>a</sup> Eleftherios Michailidis,<sup>a</sup> Yee Tsuey Ong,<sup>a</sup> Karen A. Kirby,<sup>a</sup> Maxwell D. Leslie,<sup>a</sup> Shinichi Oka,<sup>b</sup> Eiichi N. Kodama,<sup>d</sup> Lisa C. Rohan,<sup>e,f</sup> Hiroaki Mitsuya,<sup>g,h</sup> Michael A. Parniak,<sup>c</sup> Stefan G. Sarafianos<sup>a,i</sup>

Christopher Bond Life Sciences Center, Department of Molecular Microbiology and Immunology, University of Missouri School of Medicine, Columbia, Missouri, USA<sup>a</sup>; AIDS Clinical Center, National Center for Global Health and Medicine, Tokyo, Japan<sup>b</sup>; Department of Microbiology and Molecular Genetics, University of Pittsburgh, Pittsburgh, Pennsylvania, USA<sup>c</sup>; Division of Emerging Infectious Diseases, Tohoku University, Tohoku Medical Megabank Organization, Miyagi, Japan<sup>d</sup>; Magee-Women's Research Institute<sup>e</sup> and Department of Pharmaceutical Sciences, School of Pharmacy,<sup>f</sup> University of Pittsburgh, Pittsburgh, Pennsylvania, USA; Department of Hematology and Infectious Diseases, Kumamoto University, Kumamoto, Japan<sup>g</sup>; Experimental Retrovirology Section, HIV/AIDS Malignancy Branch, National Institutes of Health, Bethesda, Maryland, USA<sup>h</sup>; Department of Biochemistry, University of Missouri, Columbia, Missouri, USA<sup>i</sup>

**Drug combination studies of 4'-ethynyl-2-fluoro-2'-deoxyadenosine (EFdA) with FDA-approved drugs were evaluated by two different methods, MacSynergy II and CalcuSyn. Most of the combinations, including the combination of the two adenosine analogs EFdA and tenofovir, were essentially additive, without substantial antagonism or synergism. The combination of EFdA and rilpivirine showed apparent synergism. These studies provide information that may be useful for the design of EFdA combination regimens for initial and salvage therapy assessment.**

Combination antiretroviral therapies provide durable viral suppression and constitute the standard of care for HIV infection (<http://www.aidsinfo.nih.gov/guidelines/>) (1). For example, the combination of tenofovir disoproxil fumarate (TDF) and emtricitabine (FTC) (Truvada) is one of the preferred regimens for treatment of HIV-1 infection (2, 3). *In vitro* studies have shown that tenofovir (TFV) and FTC have synergistic antiretroviral activity (4, 5).

The investigational nucleoside reverse transcriptase inhibitor (NRTI), 4'-ethynyl-2-fluoro-2'-deoxyadenosine (EFdA) is presently under preclinical evaluation. Unlike other NRTIs used in the treatment of HIV infection, EFdA retains the 3'-hydroxyl moiety. It also contains a 2-fluoro group on the adenine base and a 4'-ethynyl group on the deoxyribose ring. Although EFdA is an adenosine analog, its activation is initiated by phosphorylation by 2'-deoxycytidine kinase (dCK), and the drug is highly resistant to degradation by adenosine deaminase (ADA) (6). EFdA shows exceptional antiretroviral activity *in vitro* (6–8) and *in vivo* (9, 10) as well as a favorable cross-resistance profile with current reverse transcriptase inhibitors (RTIs) used in the clinic.

Studies on potential interactions between EFdA and other antiretroviral drugs can provide information that could be useful in the development of combinatorial therapeutic strategies. The present study evaluates anti-HIV efficacy in combinations of EFdA with representative FDA-approved RTIs *in vitro*.

We first determined the antiviral potencies of five NRTIs (zidovudine [AZT], lamivudine [3TC], FTC, TDF, and EFdA) and three nonnucleoside RTIs (NNRTIs; efavirenz [EFV], etravirine [ETR], and rilpivirine [RPV]) against HIV-1<sub>NL4-3</sub> in order to obtain an optimal range of drug concentrations for use in combination assay analyses. As previously demonstrated (6, 7), EFdA inhibited HIV-1 replication several orders of magnitude more efficiently than other currently approved NRTIs (Table 1). In the same cell-based assays, we evaluated antiretroviral activity of EFdA in combination with the FDA-approved RTIs. To obtain more comprehensive evaluations of drug combinations and to reduce analysis bias, we use two algorithms provided by the software packages MacSynergy II (version 1.0; Ann Arbor, MI) and

TABLE 1 Antiviral activity of HIV-1 inhibitors

Compound class and name	EC <sub>50</sub> (nM) for anti-HIV-1 activity <sup>a</sup>
NRTI	
AZT	180 ± 60
3TC	1,210 ± 240
FTC	370 ± 70
TDF	14 ± 2
EFdA	3 ± 1
NNRTI	
EFV	1.6 ± 0.4
ETR	1 ± 0.1
RPV	0.4 ± 0.1

<sup>a</sup> EC<sub>50</sub>, 50% effective concentration. Values were determined using the TZM-bl cell line obtained from the NIH AIDS Research and Reference Reagent Program. All assays were conducted in triplicates. The data shown are mean values ± standard deviations obtained from the results of at least three independent experiments.

CalcuSyn (Biosoft, Ferguson, MO), which are based on the Bliss independence model (11, 12) and the median effect principle (13), respectively. Quantitative differences in data analyses by the two algorithms used by the MacSynergy II and CalcuSyn programs are not uncommon (14). In the present work, drug interactions were considered significant only if detected by both computational approaches.

Received 25 February 2013 Returned for modification 6 April 2013

Accepted 15 June 2013

Published ahead of print 24 June 2013

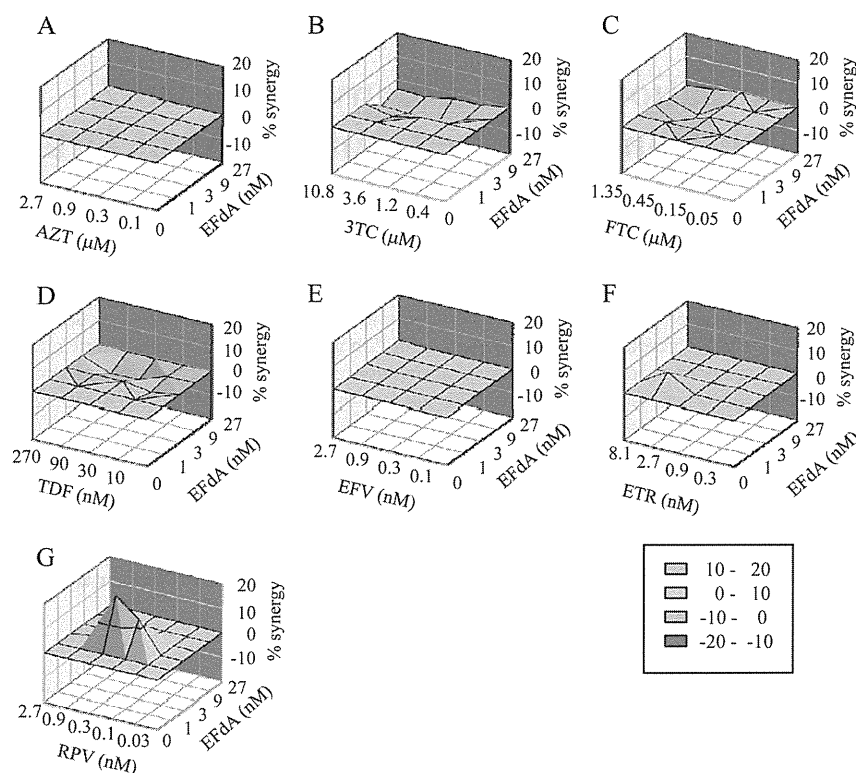
Address correspondence to Stefan G. Sarafianos, [sarafianos@missouri.edu](mailto:sarafianos@missouri.edu).

\* Present address: Atsuko Hachiya, Clinical Research Center, Department of Infectious Diseases and Immunology, National Hospital Organization Nagoya Medical Center, Nagoya, Japan.

A.H. and A.B.R. contributed equally to this article.

Copyright © 2013, American Society for Microbiology. All Rights Reserved.

doi:10.1128/AAC.00283-13



**FIG 1** Effects of EFdA in combination with other anti-HIV-1 agents. The calculated additive surface, which represents the predicted additive interactions, is then subtracted from the experimental surface to reveal regions of greater-than-expected interactions (synergy). A resulting surface appearing as a horizontal plane at 0% inhibition above the calculated additive surface suggests that the interactions are merely additive. Peaks of statistically significant synergy (positive value) or antagonism (negative value) that deviate significantly from the expected additive drug interactions derived from 95% confidence interval data are shown in the different plots of the interaction between EFdA and other anti-HIV-1 agents in the cell-based assay, as follows: AZT (A), 3TC (B), FTC (C), TDF (D), EFV (E), ETR (F), and RPV (G). Units of  $\mu\text{M}^2\%$  are analogous to the units for area under a dose-response curve in the two-dimensional situation.

Most drugs tested, including the adenosine analog TDF, showed little or no drug interactions in combination with EFdA (Fig. 1 and Table 2). Data analysis with CalcuSyn suggested that the combinations of both EFdA and 3TC (EFdA-3TC) and EFdA-FTC are moderately antagonistic (Fig. 1 and Table 2). Using

MacSynergy, the EFdA-3TC combination was assessed as minor antagonism, whereas the EFdA-FTC combination was considered additive; however, its value ( $-23.4 \mu\text{M}^2\%$ ) was very close to minor antagonism ( $-25 \mu\text{M}^2\%$ ). The observed borderline antagonism may arise from competition at the first and rate-limiting

**TABLE 2** Interactions of drug combinations for inhibition of HIV-1 virus or RT enzyme

Drug class and EFdA combination	Target	MacSynergy analysis		CalcuSyn analysis		
		Synergy/antagonism ( $\mu\text{M}^2\%$ ) <sup>a</sup>	Predicted interaction	CI <sup>b</sup>	Predicted interaction	Proposed interaction <sup>c</sup>
<b>NRTI</b>						
AZT	Virus	0/0	Additive	1.18	Additive	Neutral
TDF	Virus	0/-12.8	Additive	1.36	Moderate antagonism	Neutral
3TC	Virus	0/-39.5	Minor antagonism	1.23	Moderate antagonism	Possible antagonism
FTC	Virus	0/-23.4	Additive	1.25	Moderate antagonism	Neutral
<b>NNRTI</b>						
EFV	Virus	1.9/0	Additive	0.9	Additive	Neutral
ETR	Virus	7.8/0	Additive	1.05	Additive	Neutral
RPV	Virus	41.0/-0.04	Minor synergy	0.64	Synergy	Synergy
RPV	Enzyme	34.1/0	Minor synergy	0.56	Synergy	Synergy

<sup>a</sup> The volume of the peaks in the difference plots at the 95% confidence level, which corresponds to the area under a dose-response curve and is considered to provide a quantitative measure of possible drug interactions. MacSynergy II defines  $\mu\text{M}^2\%$  values as follows: 25 to 50, minor synergy; 50 to 100, moderate synergy; >100, strong synergy. The corresponding negative value ranges reflect minor, moderate, and strong antagonism, respectively. Values between 25 and  $-25 \mu\text{M}^2\%$  are considered insignificant.

<sup>b</sup> CI, combination Index. CalcuSyn defines CI values as follows: 0.1 to 0.3, strong synergy; 0.3 to 0.7, synergy; 0.7 to 0.85, moderate synergy; 0.85 to 1.2, additive; 1.2 to 1.45, moderate antagonism; 1.45 to 3.3, antagonism; 3.3 to 10, strong antagonism.

<sup>c</sup> Proposed interaction is assessed on the basis of the predictions by the two computational methods. Neutral, a lack of drug interaction in the combination (additivity).

**TABLE 3** Antiviral and prophylactic activity of EFdA, RPV, and EFdA-RPV combinations

Activity type (pretreatment) and virus	Drug treatment <sup>a</sup>	EC <sub>50</sub> (nM) <sup>b</sup>	CI <sub>50</sub> <sup>c</sup>					
Antiviral activity	Wild type	EFdA	0.9 ± 0.2	0.55				
		RPV	0.7 ± 0.1					
		EFdA-RPV	0.4 ± 0.2					
	M184V	EFdA	15 ± 3		0.5			
		RPV	0.6 ± 0.2					
		EFdA-RPV	0.7 ± 0.1					
	L100I/K103N	EFdA	1 ± 0.5			0.45		
		RPV	10 ± 2					
		EFdA-RPV	9 ± 2					
Prophylaxis (2-h preincubation)	Wild type	EFdA	11 ± 5	0.75				
		RPV	18 ± 4					
		EFdA-RPV	5 ± 2					
	M184V	EFdA	97 ± 22		1			
		RPV	8 ± 2					
		EFdA-RPV	10 ± 3					
	L100I/K103N	EFdA	12 ± 3			0.6		
		RPV	175 ± 42					
		EFdA-RPV	9 ± 2					
	Prophylaxis (18-h preincubation)	Wild type	EFdA				3 ± 1	0.9
			RPV				21 ± 4	
			EFdA-RPV				4 ± 1	
M184V		EFdA	53 ± 13	0.6				
		RPV	14 ± 4					
		EFdA-RPV	13 ± 40					
L100I/K103N		EFdA	6 ± 1		0.75			
		RPV	250 ± 25					
		EFdA-RPV	5 ± 2					

<sup>a</sup> EFdA and RPV were combined at a 1:1 ratio.

<sup>b</sup> EC<sub>50</sub>, 50% effective concentration. The data shown are mean values ± standard deviations obtained from the results of at least three independent experiments using P4R5-MAGI cells (32).

<sup>c</sup> CI<sub>50</sub> is the calculated combination index at a 50% inhibitory concentration.

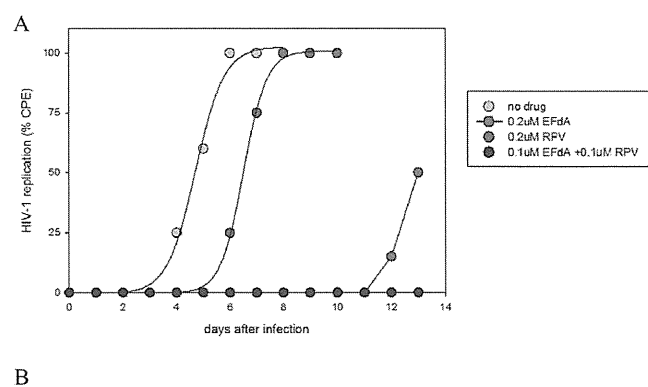
phosphorylation step as EFdA, 3TC, and FTC are all initially activated by 2'-deoxycytidine kinase (6, 15, 16). Small differences in the effect of 3TC versus FTC may arise from the longer half-life of FTC. In contrast, the combination of EFdA-RPV demonstrated apparently significant synergy, as assessed by the two different methods (41 μM<sup>2</sup>% in MacSynergy and 0.64 combination index [CI] in CalcuSyn). To confirm the synergy of HIV-1 inhibition by EFdA-RPV, we further evaluated this combination in the enzymatic assay for reverse transcriptase. Primer extension assays (7, 17) were performed with Quant-iT PicoGreen reagent (Invitrogen, Carlsbad, CA) (18). As shown in Table 2, the combination of EFdA with RPV provided synergistic effects on inhibition of reverse transcription.

We further compared the antiretroviral activity of various concentrations of EFdA alone, RPV alone, and a 1:1 molar combination of EFdA and RPV against wild-type virus and two HIV-1 mutants with reverse transcriptase (RT) mutations M184V and L100I/K103N. To evaluate the ability of the drugs to establish a barrier to subsequent infection in the absence of exogenous drug, cells were pretreated with various concentrations of each drug alone or in combination, followed by removal of exogenous drug and inoculation with HIV-1. These conditions assess intracellular persistence of drug following exogenous drug clearance, which is dependent on the intracellular half-life of the test drugs. The EFdA-RPV combination provided additive to synergistic inhibition of wild-type HIV-1 and both mutant strains (Table 3). The protective effect established by EFdA pretreatment is likely the result of EFdA resistance to degradation by adenosine deaminase

(ADA) (6), consistent with a longer intracellular half-life (19). Hence, our data suggest that EFdA could be a strong candidate for use in preexposure prophylaxis, an approach in which TDF has shown efficacy in clinical studies (20, 21).

Finally, we evaluated the ability of EFdA-RPV pretreatment to protect MT-2 lymphoblastoid cells from infection by a mixed virus population consisting of six HIV-1 strains: the wild type; the mutants K65R, Y181C, M184V, and D67N/K70R/T215F/K219Q (thymidine analogue mutations [TAMs]); and L100I/K103N. Cells were exposed to appropriate drugs for 16 h, and then exogenous drug was removed (Fig. 2). The breakthrough virus population harvested from cells not exposed to drug contained all six input viruses, plus some recombinant strains (Fig. 2B). Rapid virus breakthrough was evident in cells pretreated with RPV alone, and the only virus detected in the RPV-breakthrough population was the NNRTI-resistant L100I/K103N (100%). In contrast, breakthrough in cells pretreated with EFdA alone was significantly delayed. The EFdA-breakthrough population contained a mixture of the NRTI-resistant M184V mutant (54%) and TAMs (32%), plus some recombinant strains consisting of M184V plus one or more TAMs. No breakthrough was noted in cells pretreated with the EFdA-RPV combination, suggesting an enhanced protective effect of the drug combination compared to either drug alone.

Synergistic interactions between NRTIs and NNRTIs have been previously reported in both viral (5, 22–24) and enzymatic assays (5, 25–31). NNRTIs may act synergistically with NRTIs by



**FIG 2** HIV-1 breakthrough in cells pretreated with EFdA, RPV, or a combination of EFdA and RPV. (A) MT-2 cells were incubated with the indicated concentrations of drug for 18 h and then extensively washed to remove exogenous drug. The washed cells were infected with a mixture of six virus strains (the wild type and five drug-resistant variants). Cells were examined daily for HIV-induced cytopathic effects. (B) Genotypic composition of breakthrough virus is shown. At least 20 clones from each breakthrough virus were sequenced. EFdA (0.1 μM) and RPV (0.1 μM) were used in combination. No virus breakthrough was noted in cells pretreated with the EFdA-RPV combination. TAMs (thymidine analog resistance mutations) included the D67N, K70R, T210F, and T219Q RT mutations. Recombinant virus possessed mutations derived from at least two of the input virus strains.



suppressing the phosphorolytic unblocking of NRTI-terminated primers, possibly by stabilizing the primer terminus at a posttranslocation position, where it cannot undergo phosphorolytic removal (5, 26, 27, 29). The mechanism for the apparent synergistic activity of the EFdA-RPV combination is under investigation.

In conclusion, EFdA in combination with RPV may provide a beneficial interaction against replication of drug-sensitive and certain RTI-resistant HIV-1 strains. The results of the present study indicate that EFdA may act as promising component of future antiretroviral therapies.

## ACKNOWLEDGMENTS

This work was supported by a grant for the Bilateral International Collaborative R&D Program from the Korean Food and Drug Administration and the Ministry of Knowledge and Economy (S.G.S.), by National Institutes of Health (NIH) research grants AI076119-S1, AI076119-02S1, AI100890, AI099284, AI094715, AI076119, AI074389, and GM103368 to S.G.S. and AI079801 to M.A.P., and by a Grant-in-Aid for the research on HIV/AIDS (H22-AIDS-001) from the Ministry of Health, Labor, and Welfare of Japan (S.O.). B.M. was the recipient of an amfAR Mathilde Krim Fellowship and a Canadian Institutes of Health Research (CIHR) Fellowship. We acknowledge the Yamasa Corporation for providing EFdA for this study.

H.M. and E.N.K. are coinventors of EFdA.

A.H. carried out the cell-based drug combination assays and wrote the manuscript. A.B.R. carried out the viral breakthrough experiments and studies with drug-resistant HIV and wrote portions of the manuscript. B.M., E.M., Y.T.O., K.A.K., and M.D.L. carried out the biochemical assays. S.G.S., M.A.P., S.O., and H.M. contributed to the design of the study, and S.G.S., L.C.R., E.N.K., and M.A.P. edited the manuscript. All authors read this paper and approved the final manuscript.

## REFERENCES

- Thompson MA, Aberg JA, Hoy JF, Telenti A, Benson C, Cahn P, Eron JJ, Gunthard HF, Hammer SM, Reiss P, Richman DD, Rizzardini G, Thomas DL, Jacobsen DM, Volberding PA. 2012. Antiretroviral treatment of adult HIV infection: 2012 recommendations of the International Antiviral Society-USA panel. *JAMA* 308:387–402.
- James JS. 2004. FDA approves two combination pills, Epzicom and Truvada; comment on commercial race to once-a-day nucleosides. *AIDS Treat News* 403:6. <http://www.aidsnews.org/2004/08/epzicom-truvada.html>.
- Killingly B, Pozniak A. 2007. The first once-daily single-tablet regimen for the treatment of HIV-infected patients. *Drugs Today* 43:427–442.
- Borroto-Esoda K, Vela JE, Myrick F, Ray AS, Miller MD. 2006. In vitro evaluation of the anti-HIV activity and metabolic interactions of tenofovir and emtricitabine. *Antivir. Ther.* 11:377–384.
- Feng JY, Ly JK, Myrick F, Goodman D, White KL, Svarovskaia ES, Borroto-Esoda K, Miller MD. 2009. The triple combination of tenofovir, emtricitabine and efavirenz shows synergistic anti-HIV-1 activity in vitro: a mechanism of action study. *Retrovirology* 6:44. doi:10.1186/1742-4690-6-44.
- Kawamoto A, Kodama E, Sarafianos SG, Sakagami Y, Kohgo S, Kitano K, Ashida N, Iwai Y, Hayakawa H, Nakata H, Mitsuya H, Arnold E, Matsuoka M. 2008. 2'-Deoxy-4'-C-ethynyl-2-halo-adenosines active against drug-resistant human immunodeficiency virus type 1 variants. *Int. J. Biochem. Cell Biol.* 40:2410–2420.
- Michailidis E, Marchand B, Kodama EN, Singh K, Matsuoka M, Kirby KA, Ryan EM, Sawani AM, Nagy E, Ashida N, Mitsuya H, Parniak MA, Sarafianos SG. 2009. Mechanism of inhibition of HIV-1 reverse transcriptase by 4'-ethynyl-2-fluoro-2'-deoxyadenosine triphosphate, a translocation-defective reverse transcriptase inhibitor. *J. Biol. Chem.* 284:35681–35691.
- Kirby KA, Singh K, Michailidis E, Marchand B, Kodama EN, Ashida N, Mitsuya H, Parniak MA, Sarafianos SG. 2011. The sugar ring conformation of 4'-ethynyl-2-fluoro-2'-deoxyadenosine and its recognition by the polymerase active site of HIV reverse transcriptase. *Cell Mol. Biol.* 57:40–46.
- Murphey-Corb M, Rajakumar P, Michael H, Nyaundi J, Didier PJ, Reeve AB, Mitsuya H, Sarafianos SG, Parniak MA. 2012. Response of simian immunodeficiency virus to the novel nucleoside reverse transcriptase inhibitor 4'-ethynyl-2-fluoro-2'-deoxyadenosine in vitro and in vivo. *Antimicrob. Agents Chemother.* 56:4707–4712.
- Hattori S, Ide K, Nakata H, Harada H, Suzu S, Ashida N, Kohgo S, Hayakawa H, Mitsuya H, Okada S. 2009. Potent activity of a nucleoside reverse transcriptase inhibitor, 4'-ethynyl-2-fluoro-2'-deoxyadenosine, against human immunodeficiency virus type 1 infection in a model using human peripheral blood mononuclear cell-transplanted NOD/SCID Janus kinase 3 knockout mice. *Antimicrob. Agents Chemother.* 53:3887–3893.
- Prichard MN, Prichard LE, Shipman C, Jr. 1993. Strategic design and three-dimensional analysis of antiviral drug combinations. *Antimicrob. Agents Chemother.* 37:540–545.
- Prichard MN, Shipman C, Jr. 1990. A three-dimensional model to analyze drug-drug interactions. *Antiviral Res.* 14:181–205.
- Chou TC, Talalay P. 1984. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv. Enzyme Regul.* 22:27–55.
- Pirrone V, Thakkar N, Jacobson JM, Wigdahl B, Krebs FC. 2011. Combinatorial approaches to the prevention and treatment of HIV-1 infection. *Antimicrob. Agents Chemother.* 55:1831–1842.
- Chang CN, Skalski V, Zhou JH, Cheng YC. 1992. Biochemical pharmacology of (+)- and (–)-2',3'-dideoxy-3'-thiacytidine as anti-hepatitis B virus agents. *J. Biol. Chem.* 267:22414–22420.
- Sabini E, Hazra S, Konrad M, Lavie A. 2007. Nonenantioselectivity property of human deoxycytidine kinase explained by structures of the enzyme in complex with L- and D-nucleosides. *J. Med. Chem.* 50:3004–3014.
- Hachiya A, Kodama EN, Schuckmann MM, Kirby KA, Michailidis E, Sakagami Y, Oka S, Singh K, Sarafianos SG. 2011. K70Q adds high-level tenofovir resistance to “Q151M complex” HIV reverse transcriptase through the enhanced discrimination mechanism. *PLoS One* 6:e16242. doi:10.1371/journal.pone.0016242.
- Kirby KA, Marchand B, Ong YT, Ndongwe TP, Hachiya A, Michailidis E, Leslie MD, Sietsema DV, Fetterly TL, Dorst CA, Singh K, Wang Z, Parniak MA, Sarafianos SG. 2012. Structural and inhibition studies of the RNase H function of xenotropic murine leukemia virus-related virus reverse transcriptase. *Antimicrob. Agents Chemother.* 56:2048–2061.
- Nakata H, Amano M, Koh Y, Kodama E, Yang G, Bailey CM, Kohgo S, Hayakawa H, Matsuoka M, Anderson KS, Cheng YC, Mitsuya H. 2007. Activity against human immunodeficiency virus type 1, intracellular metabolism, and effects on human DNA polymerases of 4'-ethynyl-2-fluoro-2'-deoxyadenosine. *Antimicrob. Agents Chemother.* 51:2701–2708.
- Abdool Karim Q, Abdool Karim SS, Frohlich JA, Grobler AC, Baxter C, Mansoor LE, Kharsany AB, Sibeko S, Mlisana KP, Omar Z, Gengiah TN, Maarschalk S, Arulappan N, Mlotshwa M, Morris L, Taylor D. 2010. Effectiveness and safety of tenofovir gel, an antiretroviral microbicide, for the prevention of HIV infection in women. *Science* 329:1168–1174.
- Grant RM, Lama JR, Anderson PL, McMahan V, Liu AY, Vargas L, Goicochea P, Casapia M, Guanira-Carranza JV, Ramirez-Cardich ME, Montoya-Herrera O, Fernandez T, Veloso VG, Buchbinder SP, Charney S, Schechter M, Bekker LG, Mayer KH, Kallas EG, Amico KR, Mulligan K, Bushman LR, Hance RJ, Ganoza C, Defechereux P, Postle B, Wang F, McConnell JJ, Zheng JH, Lee J, Rooney JF, Jaffe HS, Martinez AI, Burns DN, Glidden DV. 2010. Preexposure chemoprophylaxis for HIV prevention in men who have sex with men. *N. Engl. J. Med.* 363:2587–2599.
- Richman D, Rosenthal AS, Skoog M, Eckner RJ, Chou TC, Sabo JP, Merluzzi VJ. 1991. BI-RG-587 is active against zidovudine-resistant human immunodeficiency virus type 1 and synergistic with zidovudine. *Antimicrob. Agents Chemother.* 35:305–308.
- Brennan TM, Taylor DL, Bridges CG, Leyda JP, Tyms AS. 1995. The inhibition of human immunodeficiency virus type 1 in vitro by a non-nucleoside reverse transcriptase inhibitor MKC-442, alone and in combination with other anti-HIV compounds. *Antiviral Res.* 26:173–187.
- King RW, Klabe RM, Reid CD, Erickson-Viitanen SK. 2002. Potency of nonnucleoside reverse transcriptase inhibitors (NNRTIs) used in

- combination with other human immunodeficiency virus NNRTIs, NRTIs, or protease inhibitors. *Antimicrob. Agents Chemother.* 46:1640–1646.
25. Maga G, Hubscher U, Pregnotato M, Ubiali D, Gosselin G, Spadari S. 2001. Potentiation of inhibition of wild-type and mutant human immunodeficiency virus type 1 reverse transcriptases by combinations of non-nucleoside inhibitors and D- and L-(beta)-dideoxynucleoside triphosphate analogs. *Antimicrob. Agents Chemother.* 45:1192–1200.
  26. Borkow G, Arion D, Wainberg MA, Parniak MA. 1999. The thiocarboxanilide nonnucleoside inhibitor UC781 restores antiviral activity of 3'-azido-3'-deoxythymidine (AZT) against AZT-resistant human immunodeficiency virus type 1. *Antimicrob. Agents Chemother.* 43:259–263.
  27. Odriozola L, Cruchaga C, Andreola M, Dolle V, Nguyen CH, Tarrago-Litvak L, Perez-Mediavilla A, Martinez-Irujo JJ. 2003. Non-nucleoside inhibitors of HIV-1 reverse transcriptase inhibit phosphorolysis and resensitize the 3'-azido-3'-deoxythymidine (AZT)-resistant polymerase to AZT-5'-triphosphate. *J. Biol. Chem.* 278:42710–42716.
  28. Cruchaga C, Odriozola L, Andreola M, Tarrago-Litvak L, Martinez-Irujo JJ. 2005. Inhibition of phosphorolysis catalyzed by HIV-1 reverse transcriptase is responsible for the synergy found in combinations of 3'-azido-3'-deoxythymidine with nonnucleoside inhibitors. *Biochemistry* 44:3535–3546.
  29. Basavapathruni A, Bailey CM, Anderson KS. 2004. Defining a molecular mechanism of synergy between nucleoside and nonnucleoside AIDS drugs. *J. Biol. Chem.* 279:6221–6224.
  30. Radzio J, Sluis-Cremer N. 2008. Efavirenz accelerates HIV-1 reverse transcriptase ribonuclease H cleavage, leading to diminished zidovudine excision. *Mol. Pharmacol.* 73:601–606.
  31. Shaw-Reid CA, Feuston B, Munshi V, Getty K, Krueger J, Hazuda DJ, Parniak MA, Miller MD, Lewis D. 2005. Dissecting the effects of DNA polymerase and ribonuclease H inhibitor combinations on HIV-1 reverse-transcriptase activities. *Biochemistry* 44:1595–1606.
  32. Parikh UM, Koontz DL, Chu CK, Schinazi RF, Mellors JW. 2005. In vitro activity of structurally diverse nucleoside analogs against human immunodeficiency virus type 1 with the K65R mutation in reverse transcriptase. *Antimicrob. Agents Chemother.* 49:1139–1144.

厚生労働科学研究費補助金エイズ対策研究事業

「安全かつ効果的な抗HIV療法開発のための研究」班

平成25年度 総括・分担研究報告書

---

発行日 2014年3月31日

発行者 研究代表者 湯永 博之

発行所 研究班事務局

(独)国立国際医療研究センターエイズ治療・研究開発センター

〒162-8655 東京都新宿区戸山1-21-1

---

