Antiviral Research 101 (2014) 62–67

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Molecular mechanism of HIV-1 resistance to 3′-azido-2′, 3′-dideoxyguanosine

Jeffrey D. Meteer [a], Raymond F. Schinazi [b,c], John W. Mellors [a], Nicolas Sluis-Cremer [a,*]

[a] Division of Infectious Diseases, Department of Medicine, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA
[b] Center for AIDS Research, Department of Pediatrics, Emory University School of Medicine, Atlanta, GA, USA
[c] Veterans Affairs Medical Center, Decatur, GA, USA

ARTICLE INFO

Article history:
Received 7 October 2013
Revised 28 October 2013
Accepted 30 October 2013
Available online 7 November 2013

Keywords:
HIV-1
Reverse transcriptase
3′-Azido-2′,3′-dideoxyguanosine
Resistance
Excision
Discrimination

ABSTRACT

We reported that 3′-azido-2′,3′-dideoxyguanosine (3′-azido-ddG) selected for the L74V, F77L, and L214F mutations in the polymerase domain and K476N and V518I mutations in the RNase H domain of HIV-1 reverse transcriptase (RT). In this study, we have defined the molecular mechanisms of 3′-azido-ddG resistance by performing in-depth biochemical analyses of HIV-1 RT containing mutations L74V, F77L, V106I, L214F, R277K, and K476N (SGS3). The SGS3 HIV-1 RT was from a single-genome-derived full-length RT sequence obtained from 3′-azido-ddG resistant HIV-1 selected *in vitro*. We also analyzed two additional constructs that either lacked the L74V mutation (SGS3-L74V) or the K476N mutation (SGS3-K476N). Pre-steady-state kinetic experiments revealed that the L74V mutation allows RT to effectively discriminate between the natural nucleotide (dGTP) and 3′-azido-ddG-triphosphate (3′-azido-ddGTP). 3′-azido-ddGTP discrimination was primarily driven by a decrease in 3′-azido-ddGTP binding affinity (K_d) and not by a decreased rate of incorporation (k_{pol}). The L74V mutation was found to severely impair RT's ability to excise the chain-terminating 3′-azido-ddG-monophosphate (3′-azido-ddGMP) moiety. However, the K476N mutation partially restored the enzyme's ability to excise 3′-azido-ddGMP on an RNA/DNA, but not on a DNA/DNA, template/primer by selectively decreasing the frequency of secondary RNase H cleavage events. Collectively, these data provide strong additional evidence that the nucleoside base structure is major determinant of HIV-1 resistance to the 3′-azido-2′,3′-dideoxynucleosides.

1. Introduction

Since zidovudine (AZT) was first approved by the Food and Drug Administration in 1987, nucleoside reverse transcriptase (RT) inhibitors (NRTIs) have become the cornerstone of effective combination antiretroviral therapies (cART) for both antiretroviral-naïve and -experienced HIV-infected individuals. However, their effectiveness can be limited by the emergence of NRTI-resistant virus (Tang and Shafer, 2012). NRTI-associated resistance mutations can be broadly categorized into two groups depending on their phenotypic mechanism of resistance. The mutations K65R, K70E, L74V, Q151M (in complex with A62V, V75I, F77L, and F116Y) and M184V increase the selectivity of RT for incorporation of natural dNTP substrate *versus* the NRTI-triphosphate (TP) (diphosphate (DP) for tenofovir) (Sluis-Cremer et al., 2000, 2007; Deval et al., 2004, 2002; Parikh et al., 2007; Ly et al., 2007; Feng and Anderson, 1999). This resistance mechanism has been termed NRTI-DP/TP discrimination. In comparison, the mutations M41L, D67N, K70R, L210W, T215F/Y, and K219Q/E are typically referred to as thymidine analog mutations (TAMs). These mutations augment the ability of HIV-1 RT to excise a chain-terminating NRTI-monophosphate (NRTI-MP) from a prematurely terminated DNA chain (Meyer et al., 1998, 1999). This resistance mechanism has been termed NRTI-MP excision.

AZT typically selects for TAMs in HIV-1 (Larder et al., 1989; Larder and Kemp, 1989). AZT-MP is also more readily excised by HIV-1 RT containing TAMs than are other sugar modified NRTI-MP analogs (Meyer et al., 2000). Initially, Boyer et al. proposed that this was due to the 3′-azido group, which anchored the chain-terminating AZT-MP in the excision-competent nucleotide-binding site (N-site) and prevented its translocation to the excision-incompetent primer-binding site (P-site) (Boyer et al., 2001). However, we reported that the 3′-azido-2′,3′-dideoxypurines retained

Abbreviations: RT, reverse transcriptase; RNase H, ribonuclease H; WT, wild-type; NRTI, nucleoside RT inhibitor; TP, triphosphate; DP, diphosphate; MP, monophosphate; T/P, template/primer; NRTIs, nucleoside reverse transcriptase inhibitors; 3′-Azido-ddG, 3′-azido-2′,3′-dideoxyguanosine; 3′-Azido-ddGMP, 3′-azido-ddG-monophosphate; IC_{50}, 50% inhibitory concentration; 3′-Azido-ddGTP, 3′-azido-ddG-triphosphate; AZT, zidovudine.

* Corresponding author. Tel.: +1 412 648 8457; fax: +1 412 648 8521.
E-mail addresses: jdm69@pitt.edu (J.D. Meteer), rschina@emory.edu (R.F. Schinazi), jwm1@pitt.edu (J.W. Mellors), nps2@pitt.edu (N. Sluis-Cremer).

0166-3542/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.antiviral.2013.10.017

activity against HIV-1 variants that contained multiple TAMs (Sluis-Cremer et al., 2005, 2009). This finding suggested that the 3′-azido-2′,3′-dideoxynucleoside base was a major determinant of HIV-1 resistance. To further explore this hypothesis, we conducted *in vitro* selection experiments by serial passage of HIV-1_{LAI} in MT-2 cells in the presence of increasing concentrations of 3′-azido-ddG (Meteer et al., 2011). 3′-Azido-ddG selected for virus that was 5.3-fold resistant to the nucleoside compared to wildtype (WT) HIV-1_{LAI} passaged in the absence of drug. Population sequencing of the entire reverse transcriptase (RT) coding region identified L74V, F77L, and L214F mutations in the polymerase domain and K476N and V518I mutations in the RNase H domain. Under similar conditions, AZT selected for highly resistant virus (>16,200-fold over WT) that contained the TAMs D67N, K70R, T215F, A371V, and Q509L in RT (Brehm et al., 2007, 2008). The selection of divergent mutations indicates that the phenotypic mechanisms responsible for resistance between 3′-azido-ddG and AZT are different.

We therefore investigated the molecular mechanisms of resistance to 3′-azido-ddG by performing in-depth biochemical analyses of wild-type and mutants HIV-1 RTs containing L74V, L74V/F77L/V106I/L214F/R277K/K476N (SGS3), F77L/V106I/L214F/R277K/K476N (SGS3-L74V), and L74V/F77L/V106I/L214F/R277K (SGS3-K476N). We report that the L74V mutation allows HIV-1 RT to effectively discriminate between the natural nucleotide (dGTP) and 3′-azido-ddG-triphosphate (3′-azido-ddGTP). We also show that the K476N mutation partially restores the enzyme's ability to excise 3′-azido-ddGMP on an RNA/DNA, but not DNA/DNA, template/primer by selectively decreasing the frequency of secondary RNase H cleavage events.

2. Materials and methods

2.1. Materials

AZT-TP and 3′-azido-ddGTP were purchased from Trilink Biotechnologies (San Diego, CA). ATP, deoxyribonucleotide triphosphates (dNTPs) and dideoxynucleoside triphosphates were purchased from GE Healthcare (Piscataway, New Jersey, USA), and [γ-^{32}P]ATP was acquired from PerkinElmer Life Sciences (Boston, Massachusetts, USA). RNA and DNA oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, Iowa, USA).

2.2. Cloning, site-directed mutagenesis and purification of HIV-1 RT

We previously reported that HIV-1 containing the L74V mutation, or L74V in combination with F77L, L214F, K476N, and/or V518I, exhibited only a ~2.0-fold decrease in susceptibility to 3′-azido-ddG (Meteer et al., 2011). However, if we generated HIV-1 clones containing single-genome-derived full-length RT sequences from the 3′-azido-ddG resistant virus population selected *in vitro* (Meteer et al., 2011), the recombinant virus yielded higher levels of 3′-azido-ddG resistance (range 3.2–4.0-fold). Therefore, in this study we cloned into the p6HRT-PROT prokaryotic expression vector (Le Grice and Grüninger-Leitch, 1990) one of these single-genome-derived full-length RT sequences (SGS3) that contained L74V/F77L/V106I/L214F/R277K/K476N mutations. The contributions of the L74V and K476N mutations were studied in the context of SGS3 HIV-1 RT by reverting out the mutations by site-directed mutagenesis (QuikChange Lightning site-directed mutagenesis kit; Stratagene, La Jolla, CA) to generate the F77L/V106I/L214F/R277K/ K476N (SGS3-L74V) and L74V/F77L/V106I/L214F/R277K (SGS3-K476N) enzymes. We also introduced the L74V mutation into WT HIV-1_{LAI} RT by site-directed mutagenesis. Full-length sequencing of mutant RTs was performed to confirm the presence of the desired mutations and to exclude adventitious mutations introduced during mutagenesis. WT and mutant recombinant HIV-1 RTs were over-expressed and purified to homogeneity as described previously (Le Grice and Grüninger-Leitch, 1990). RT concentration was determined spectrophotometrically at 280 nM using an extinction co-efficient (ε_{280}) of 260 450 M^{-1} cm^{-1}. All experiments described in this study were carried out with at least 2 different preparations of enzyme.

2.3. Assays of DNA polymerization by WT and mutant HIV-1 RT

A 19 nucleotide DNA primer (P19; 5′-TTGTAGCACCATCCAAAGG-3′) annealed to a 36 nucleotide DNA template (T36; 5′-AGAGCCCCCGAGACCTTTGGATGGTGCTACAAG CT-3′) was used in these experiments. P19 was 5′-radiolabeled with [γ-^{32}P]-ATP and T4 polynucleotide kinase, as described previously (Sluis-Cremer et al., 2000). 5′-^{32}P-labeled P19 was then annealed to T36 by adding a 1:1.5 molar ratio of primer to template at 90 °C and allowing the mixture to slowly cool to ambient room temperature. DNA polymerization was assessed by incubating 200 nM WT or mutant HIV-1 RT with 20 nM template/primer (T/P; T36/P19) in 50 mM Tris–HCl (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$. The reaction was initiated by the addition of 0.1 or 1 μM mixed dNTPs. After defined incubation periods, aliquots were removed and processed as described previously (Parikh et al., 2007). In some experiments, an excess (100 nM) of unlabeled T/P was added to the reaction to trap unbound RT.

2.4. Assays of 3′-azido-ddGTP incorporation and 3′-azido-ddGMP excision by WT and mutant HIV-1 RT

In these assays, we assessed the ability of WT or mutant HIV-1 RT to synthesize full-length DNA product on the T36/P19 T/P in the presence of 5 μM 3′-azido-ddGTP and 3 mM ATP. Briefly, 200 nM WT or mutant HIV-1 RT was pre-incubated with 20 nM 5′-^{32}P-end-labeled T/P in 50 mM Tris–HCl (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$. Reactions were initiated by the addition of 0.5 μM mixed dNTPs, 5 μM 3'-azido-ddGTP, and 3 mM ATP. After defined incubation periods, aliquots were removed and processed as described above.

2.5. Pre-steady-state assays of dGTP or 3′-azido-ddGTP incorporation by WT or mutant HIV-1 RT

A 5′-^{32}P-labeled 20 nucleotide DNA primer (P20; 5′-TCGGGCGCCACTGCTAGAGA-3′) annealed to a 57 nucleotide DNA template (T57; 5′-CTCAGACCCTTTTAGTCAGAATG GAAACTCTCTAG CAGTGGCGCCCGAACAGGGACA-3′) was used in these experiments. A Kintek RQF-3 instrument (Kintek Corporation, Clarence, PA) was used for pre-steady state experiments with reaction times ranging from 5 ms to 3 min. The typical experiment was performed at 37 °C in 50 mM Tris–HCl (pH 7.5) containing 50 mM KCl, 10 mM $MgCl_2$, and varying concentrations of dGTP or 3′-azido-ddGTP (0.5–10 μM). All concentrations reported refer to the final concentrations after mixing. The active site concentrations of WT and mutant RTs were calculated from pre-steady-state burst experiments, as described previously (Sluis-Cremer et al., 2005). Burst amplitudes of 57%, 48%, and 51% were calculated for the WT, SGS3, and L74V RT enzymes, respectively. All experiments described below were performed using corrected RT active site concentrations. WT or mutant HIV-1 RT (200 nM) was pre-incubated with 20 nM T/P, prior to rapid mixing with nucleotide and divalent metal ions to initiate the reaction that was quenched with 50 mM EDTA. Prod-

ucts were resolved and analyzed, as described previously (Sluis-Cremer et al., 2007). Data were fitted by nonlinear regression with Sigma Plot software (Systat Software, Inc., San Jose, CA) using the appropriate equations (Johnson, 1995). The apparent burst rate constant (k_{obs}) for each particular concentration of dGTP or 3′-azido-ddGTP was determined by fitting the time courses for the formation of product using the following equation: product = $A[1 - \exp(-k_{obs}t)]$, where A represents the burst amplitude. The turnover number (k_{pol}) and apparent dissociation constant for the nucleotide analog (K_d) were then obtained by plotting the apparent catalytic rates (k_{obs}) against nucleotide analog concentrations and fitting the data with the following hyperbolic equation: $k_{obs} = (k_{pol}[dNTP])/([dNTP] + K_d)$. Catalytic efficiency was calculated as the ratio of turnover number over dissociation constant (k_{pol}/K_d). Selectivity for natural dGTP *versus* 3′-azido-ddGTP was calculated as the ratio of catalytic efficiency of dGTP over that of the analog $(k_{pol}/K_d)^{dGTP}/(k_{pol}/K_d)^{3'\text{-azido-ddGTP}}$.

2.6. Assays of 3′-azido-ddGMP excision by WT or mutant HIV-1 RT

A 23 nucleotide primer (5′-TTGTAGCACCATCCAAAGGTCTC-3′) was 5′-end labeled with [γ-^{32}P]-ATP, chain-terminated with 3′-azido-ddGMP and annealed to a DNA (T36) or RNA ($T36^{RNA}$; 5′-rCrArGrArGrCrCrCrCrGrArGrArCrCrUrUrUrGrGrArUrGrGrUrGrCrUrArCrArArGrCrU-3′) template, as described previously (Brehm et al., 2008). 200 nM WT or mutant HIV-1 RT was pre-incubated with 20 nM T/P in 50 mM Tris–HCl (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$. Reactions were initiated by the addition of 2 μM dGTP, 40 μM ddCTP, and 3 mM ATP. After defined incubation periods, aliquots were removed and processed as described above.

2.7. Assay for RT RNase H activity

WT and mutant RT RNase H activity was evaluated using the same 3′-azido-ddGMP chain-terminated RNA/DNA T/P substrate described above, except the 5′-end of the RNA was ^{32}P-end-labeled. Assays were carried out using 20 nM $T_{RNA}/P_{3'\text{-azido-ddG}}$, 3 mM ATP and 10 mM $MgCl_2$ in a buffer containing 50 mM Tris–HCl (pH 7.5) and 50 mM KCl. Reactions were initiated by the addition of 200 nM WT or mutant HIV-1 RT. Aliquots were removed, quenched at varying times, and analyzed as described above.

3. Results

3.1. DNA polymerase activity of WT and mutant HIV-1 RT

As described in the materials and methods, we cloned a single-genome-derived full-length RT sequence (SGS3) derived from a 3′-azido-ddG resistant virus population selected *in vitro* (Meteer et al., 2011) that contained the L74V/F77L/V106I/L214F/R277K/K476N mutations into the p6HRT-PROT prokaryotic expression vector. We also generated 2 additional constructs that lacked either the L74V mutation (SGS3-L74V) or the K476N mutation (SGS3-K476N). Each of the mutant enzymes was purified to homogeneity and analyzed for DNA-dependent DNA polymerase activity using either 0.1 μM or 1.0 μM dNTP. The DNA polymerization activity of all 3 mutant RTs was compromised compared to the WT enzyme (Fig. 1A). Specifically, the mutant RTs generated less full-length DNA product and there was an increase in the accumulation of shorter DNA products compared to the WT enzyme. We also assessed DNA polymerization by SGS3 and WT HIV-1 RT under processive conditions. The results in Fig. 1B show that SGS3 RT is less processive than the WT enzyme at low (1.0 μM) and high (10.0 μM) concentrations of dNTP.

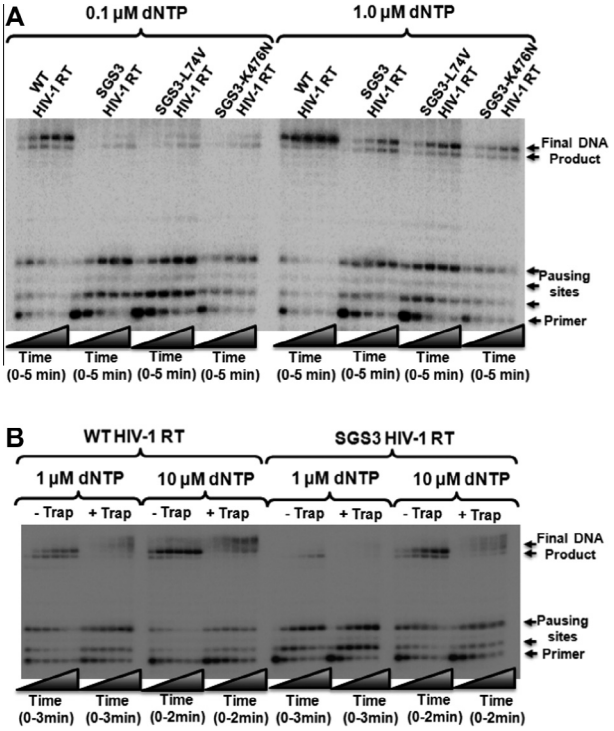

Fig. 1. DNA-dependent DNA polymerase activity of WT, SGS3, SGS3-L74V, and SGS3-K476N HIV-1 RT. (A) Representative autoradiogram of the DNA-dependent DNA polymerase activity of WT, SGS3, SGS3-L74V, and SGS3-K476N HIV-1 RT. Reactions were carried out at 0.1 and 1.0 μM dNTP. Reactions times were 0, 1, 2, 3, 4, and 5 min. (B) Representative autoradiogram of the DNA-dependent DNA polymerase activity of WT, SGS3, SGS3-L74V, and SGS3-K476N HIV-1 RT. Reactions were carried out at 1.0 and 10 μM dNTP. Unlabeled T36/P19 T/P was used as a trap. Reaction times were 0, 30, 60, 90, 120, and 180 s for 1.0 μM dNTP or 0, 15, 30, 60, 90, and 120 s for 10 μM dNTP.

3.2. 3′-Azido-ddGTP incorporation and 3′-azido-ddGMP excision activity of WT and mutant HIV-1 RT

During HIV-1 replication there are multiple opportunities for RT to incorporate and excise nucleotide analogs. As such, we initially assessed the ability of WT and mutant HIV-1 RT to synthesize full-length DNA product in the presence of 3′-azido-ddGTP and ATP. Fig. 2 shows that in the presence of 5 μM 3′-azido-ddGTP and 3 mM ATP, the SGS3, and SGS3-K476N RTs synthesized significantly greater amounts of full-length DNA than did the WT or

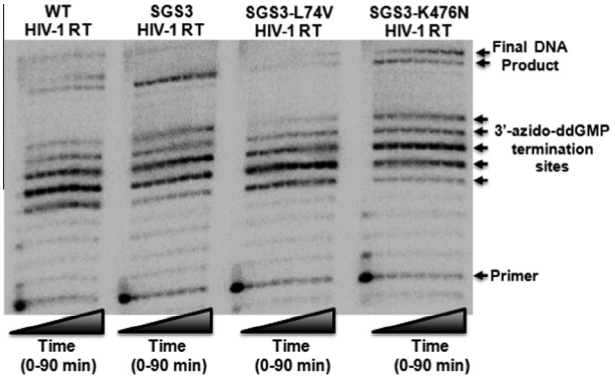

Fig. 2. 3′-azido-ddGTP incorporation and 3′-azido-ddGMP excision activity of WT and mutant HIV-1 RT. Representative autoradiogram of the DNA-dependent DNA polymerase activity of WT, SGS3, SGS3-L74V, and SGS3-K476N HIV-1 RT in the presence of 5 μM 3′-azido-ddGTP and 3 mM ATP. Reaction times were 0, 5, 10, 15, 30, 45, 60, and 90 min.

SGS3-L74V enzymes. This increase in DNA product formation by these RTs appeared to be driven by a decrease in the frequency of 3′-azido-ddGMP chain-termination. Of note, this assay was carried-out over a long time period (5–90 min) to allow for the enzyme to excise the chain-terminating 3′-azido-ddGMP moiety. In this regard, there was no evidence of a decrease in chain-termination through excision at any of the sites at which 3′-azido-ddGTP had been incorporated. Taken together, this data suggested that 3′-azido-ddG resistance was driven by a discrimination phenotype mediated by the L74V mutation.

3.3. Pre-steady-state incorporation of dGTP and 3′-azido-ddGTP by WT and mutant HIV-1 RT

Pre-steady state kinetic analyses were carried out to elucidate the interactions of dGTP and 3′-azido-ddGTP with the polymerase active sites of WT and SGS3 HIV-1 RT (Table 1). These experiments defined the maximum rates of nucleotide incorporation (k_{pol}), the nucleotide dissociation constants (K_d), and the catalytic efficiencies of incorporation (k_{pol}/K_d). The k_{pol}/K_d values for the incorporation of dGTP by WT or SGS3 HIV-1 RT were essentially identical, suggesting that the L74V/F77L/V106I/L214F/R277K/K476N mutations do not adversely affect single nucleotide turn-over events. The selectivity of RT, which is defined as $(k_{pol}/K_d)^{dGTP}/(k_{pol}/K_d)^{3'\text{-azido-ddGTP}}$, is an indication of the ability of the WT or SGS RT to discriminate between dGTP and 3′-azido-ddGTP. As reported previously, the WT enzyme cannot discriminate between dGTP and 3′-azido-ddGTP (selectivity < 1) (Sluis-Cremer et al., 2005). By contrast, the mutations in SGS3 RT independently increased the selectivity of the enzyme for the natural substrate *versus* 3′-azido-ddGTP (Table 1). The observed 3′-azido-ddGTP resistance of SGS3 RT could primarily be attributed to an increase in K_d and not a decrease in k_{pol}. Unfortunately, we were unable to purify sufficient quantities of the SGS3-L74V and SGS3-K476N RTs to perform pre-steady-state kinetic assays. Therefore, we also carried out analyses to elucidate the interactions of dGTP and 3′-azido-ddGTP with the polymerase active site of L74V HIV-1 RT (Table 1). Similar to SGS3 RT, the L74V enzyme could effectively discriminate between dGTP and 3′-azido-ddGTP by decreasing the affinity of the nucleotide analog for DNA polymerase active site. Of note, the calculated 3′-azido-ddGTP fold-resistance (Fold-R) values for SGS3 and L74V RT were similar.

3.4. Excision of 3′-azido-ddGMP by WT and mutant HIV-1 RT

Prior studies have shown that the L74V mutation significantly attenuates RTs ability to excise the chain-terminating NRTI-MP on DNA/DNA (Miranda et al., 2005; Frankel et al., 2005) and RNA/DNA T/P (Radzio et al., 2010) substrates. Moreover, we recently demonstrated that the N348I mutation in the connection domain of RT could augment the excision activity of the L74V enzyme on an RNA/DNA T/P by selectively decreasing the frequency of secondary RNase H cleavages that reduce the overall efficiency of the excision reaction (Radzio et al., 2010). Therefore, we next examined the ability of WT, SGS3, SGS3-L74V, and SGS3-K476N HIV-1 RT to excise 3′-azido-ddGMP and rescue DNA synthesis from chain-terminated DNA/DNA and RNA/DNA T/Ps (Fig. 3). An excision-competent RT containing the TAMs D67N/K70R/T215F/K219Q (AZT^R) was also included as a control in these experiments. On the DNA/DNA T/P substrate, the SGS3 and SGS3-K476N RTs were significantly less efficient in excising 3′-azido-ddGMP than was the WT enzyme. The ATP-mediated excision activity of the SGS3-L74V RT was similar to that of the WT enzyme. These observations are consistent with the L74V mutation significantly reducing RTs ability to excise chain-terminating nucleotide analogs. In contrast, on the RNA/DNA T/P substrate, the ATP-mediated excision activity of SGS3 RT was comparable to that of the WT enzyme. Reversion of L74V to the WT codon (i.e. SGS3-L74V) significantly increased the enzyme's ability to excise 3′-azido-ddGMP suggesting that the F77L/V106I/L214F/R277K/K476N mutations contribute to an excision phenotype on an RNA/DNA, but not DNA/DNA, T/P. Reversion of K476N to the WT codon (i.e. SGS3-K476N RT) almost completely abolished RTs ability to excise 3′-azido-ddGMP indicating that the K476N mutation counteracts the negative effect of L74 on excision.

We previously delineated the relationship between AZT-MP excision efficiency and RNase H activity on a RNA/DNA T/P substrate that was essentially identical to the one used in these experiments (Radzio and Sluis-Cremer, 2008). These studies showed that the primary polymerase-dependent RNase H cleavages do not impact the enzyme's excision efficiency, but polymerase-independent RNase H cleavages that reduce the RNA/DNA duplex length to less than 12 nucleotides abolish the excision activity. In light of this, we next evaluated the RNase H activity of the WT and mutant RTs that occurred during the ATP-mediated excision reactions. The data in Fig. 4 shows that the SGS3 and SGS3-L74V RTs carryout less secondary RNase H cleavages. As a result, there is prolonged preservation of T/P substrates with duplex lengths of 15–18 nucleotides. As described above, RT can efficiently excise a chain-terminating NRTI-MP from these substrates (Radzio and Sluis-Cremer, 2008). In contrast, these T/P substrates (with duplex lengths of 15–18 nucleotides) are not preserved in reactions carried out by the WT and SGS3-K476N RTs.

4. Discussion

In this study we show that the L74V/F77L/V106I/L214F/R277K/K476N mutations in HIV-1 RT confer 3′-azido-ddG resistance primarily through discrimination with some contribution from excision. The NRTI-DP/TP discrimination resistance

Table 1
Pre-steady-state kinetic values for incorporation of dGTP and 3′-azido-ddGTP by WT and mutant HIV-1 RT.

Nucleotide	k_{pol} (s^{-1})	K_d (μM)	k_{pol}/k_d ($\mu M^{-1}\ s^{-1}$)	Selectivity[a]	Fold-resistance[b]
WT RT					
dGTP	18.1 ± 6.7[c]	1.3 ± 1.2	13.98	–	
3′-azido-ddGTP	18.2 ± 7.6	0.7 ± 0.1	26.47	0.53	1
SGS3 RT					
dGTP	20.1 ± 1.8	1.8 ± 0.9	11.2	–	
3′-azido-ddGTP	20.0 ± 4.6	3.6 ± 1.7	5.6	1.99	3.75
L74V RT					
dGTP	21.5 ± 11.6	0.6 ± 0.3	38.49	–	
3′-azido-ddGTP	28.2 ± 11.3	1.2 ± 0.6	23.57	1.63	3.1

[a] Selectivity is $(k_{pol}/K_d)^{dGTP}/(k_{pol}/K_d)^{3'\text{-azido-ddGTP}}$.
[b] Resistance (*n*-fold) is $\text{selectivity}^{mutant}/\text{selectivity}^{WT}$.
[c] Data are the mean ± S.D. determined from at least three independent experiments.

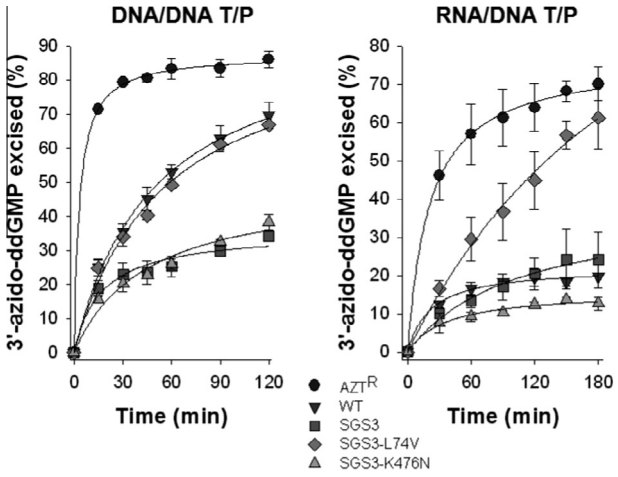

Fig. 3. ATP-mediated excision of 3′-azido-ddGMP and rescue of DNA synthesis by WT and mutant HIV-1 RT on chain-terminated DNA/DNA and RNA/DNA T/P substrates. Data are the mean ± standard deviation determined from at least 3 independent experiments.

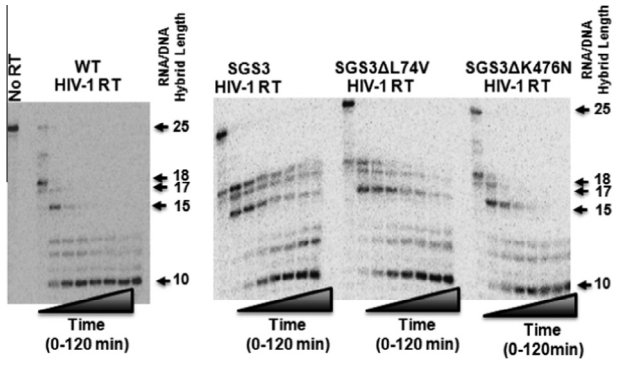

Fig. 4. Representative autoradiogram of the RNase H cleavage activity of WT, SGS3, SGS3-L74V, and SGS3-K476N HIV-1 RT that occurs during the 3′-azido-ddGMP excision reaction. The reaction times were 0, 5 15, 30, 45, 60, 90, and 120 min.

phenotype was mediated by the L74V mutation in HIV-1 RT, whereas the NRTI-MP excision phenotype was primarily mediated by the K476N mutation. We were unable to clarify the relative contributions of the F77L, V106I, L214F, and R277K in HIV-1 RT to 3′-azido-ddG resistance. F77L is typically co-selected with Q151M (Ueno et al., 1995), and in combination with the A62V, V75I, and F116Y mutations may partially increase the ability of HIV-1 RT containing Q151M to effectively discriminate against NRTI-DP/TP (Deval et al., 2002). V106I is a nonnucleoside RT inhibitor resistance mutation and has not been associated with NRTI resistance. L214F and R277K are polymorphisms that exist in both treatment-naïve and -experienced individuals, but both mutations have been associated with NRTI resistance (Puertas et al., 2009; Garriga et al., 2009; Betancor et al., 2010). However, in antiviral drug-susceptibility assays the F77L and L214F mutations did not significantly appear to increase 3′-azido-ddG resistance in viruses containing L74V (Meteer et al., 2011).

Our kinetic data shows that the L74V mutation allows HIV-1 RT to effectively discriminate between dGTP and 3′-azido-ddGTP by selectively decreasing the nucleotide analog's binding affinity (i.e. K_d). L74V is selected by other purine analogs including didanosine and abacavir (Miller et al., 2000; Martin et al., 1993), and a prior pre-steady-state kinetic study reported that the L74V mutation allows RT to discriminate between dATP and ddATP (Deval et al., 2004). Interestingly, L74V in HIV-1 RT confers ~3-fold resistance to 3′-azido-ddGTP (Table 1), but HIV-1 containing the L74V mutation remains sensitive to inhibition by 3′-azido-ddG (Sluis-Cremer et al., 2009). In this regard, it has been shown that the K65R mutation in HIV-1 RT allows the enzyme to discriminate between TTP and AZT-TP (Parikh et al., 2007; Ly et al., 2007). Like L74V, K65R significantly decreases the ATP-mediated excision activity of the enzyme, and it was proposed that the combination of these opposing mechanisms results in the increased susceptibility of HIV-1 containing the K65R mutation to AZT (White et al., 2005). In this study, we show that HIV-1 has compensated for the decreased excision activity of L74V RT through the gain of additional mutations, particularly K476N, that augments the enzyme's ability unblock the chain-terminating 3′-azido-ddGMP moiety on RNA/DNA T/P substrates. Indeed, we found that K476N restored the enzyme's ability to excise 3′-azido-ddGMP on an RNA/DNA, but not DNA/DNA, T/P by selectively decreasing the frequency of secondary RNase H cleavage events that preserved excision-competent RNA/DNA T/P substrates with duplex lengths ranging from 15 to 18 nucleotides. Residue K476 forms part of the RNase H primer grip, a region that is important for the proper binding and positioning of the T/P to RT (Sarafianos et al., 2001). Consistent with our data, prior studies have shown that point mutations in the HIV-1 RNase H primer grip (including at residue T476) can reduce RNase H activity, alter RNase H cleavage specificity or impact the NRTI-MP excision phenotype of HIV-1 RT (Rausch et al., 2002; Julias et al., 2003; Delviks-Frankenberry et al., 2007). Taken together, these findings suggest that K476N, like the A360V, N348I, and Q509L mutations, impacts the efficiency of the excision reaction by an RNase H-dependent mechanism (Brehm et al., 2007, 2012; Ehteshami et al., 2008; Yap et al., 2007). Of note, Betancor et al. (2010) reported that thumb subdomain polymorphisms-including R277K-can impact the ATP-mediated excision activity of HIV-1 RT on an RNA/DNA T/P substrate by altering the apparent dissociation equilibrium constant (K_d) between enzyme and substrate. In this regard, we found no evidence of a defect in the ability of our RT constructs to bind the T/P substrate as assessed by gel-shift analysis (data not shown).

In Fig. 1A, we show that SGS3 RT has reduced DNA polymerase activity compared to the WT enzyme. However, the pre-steady-state kinetic analyses demonstrated that the catalytic efficiency ratios (i.e. k_{pol}/K_d) for SGS3 and WT HIV-1 RT were comparable. These data suggest that the L74V/F77L/V106I/L214F/R277K/K476N mutations do not directly impact the DNA polymerase active site of HIV-1 RT. Instead, we show that the decrease in DNA polymerase activity of SGS3 RT is likely due to decreased processivity (Fig. 1B). A decrease in the *in vitro* processivity of L74V HIV-1 RT has been documented previously (Deval et al., 2004; Sharma et al., 2009).

The level of resistance that HIV-1 achieves to 3′-azido-ddG after *in vitro* selection is modest (3–4 fold) and requires multiple resistance mutations, including L74V that markedly reduce nucleotide excision. This L74V-mediated reduction in excision was only partially reversed by K476N in the RNase H domain, suggesting that there are molecular constraints on HIV-1 RT such that it does not readily evolve high level resistance to 3′-azido-ddG through discrimination and excision mechanisms, either alone or in combination. Therefore, the data presented in this report provide genetic and biochemical insights into the favorable activity profile of 3′-azido-ddG against NRTI-resistant virus.

In conclusion, our analyses reveal that HIV-1 resistance to 3′-azido-ddG is mediated by both the NRTI-DP/TP discrimination and NRTI-MP excision phenotypes. By comparison, AZT selects for TAMs in HIV-1 that confer resistance exclusively via the NRTI-MP excision phenotype. As such, these data strongly reinforce the thesis that the nucleoside base structure is major determinant of HIV-1 resistance to the 3′-azido-2′,3′-dideoxynucleosides and that further optimization of base structure is possible

to enhance the activity and resistance profile of 3′-azido-2′,3′-dideoxynucleosides.

Acknowledgments

This study was supported by Grants AI071846 (to J.W.M. and R.F.S.) and AI081571 (to N.S.-C.) from the National Institutes of Health, and by the Department of Veterans Affairs (to R.F.S.).

References

Betancor, G., Puertas, M.C., Nevot, M., Garriga, C., Martínez, M.A., Martinez-Picado, J., Menéndez-Arias, L., 2010. Mechanisms involved in the selection of HIV-1 reverse transcriptase thumb subdomain polymorphisms associated with nucleoside analogue therapy failure. Antimicrob. Agents Chemother. 54, 4799–47811.

Boyer, P.L., Sarafianos, S.G., Arnold, E., Hughes, S.H., 2001. Selective excision of AZTMP by drug-resistant human immunodeficiency virus reverse transcriptase. J. Virol. 75, 4832–4842.

Brehm, J.H., Koontz, D., Meteer, J.D., Pathak, V., Sluis-Cremer, N., Mellors, J.W., 2007. Selection of mutations in the connection and RNase H domains of human immunodeficiency virus type 1 reverse transcriptase that increase resistance to 3′-azido-3′-dideoxythymidine. J. Virol. 81, 7852–7859.

Brehm, J.H., Mellors, J.W., Sluis-Cremer, N., 2008. Mechanism by which a glutamine to leucine substitution at residue 509 in the ribonuclease H domain of HIV-1 reverse transcriptase confers zidovudine resistance. Biochemistry 47, 14020–14027.

Brehm, J.H., Scott, Y., Koontz, D.L., Perry, S., Hammer, S., Katzenstein, D., Mellors, J.W., Sluis-Cremer, N., 2012. Zidovudine (AZT) monotherapy selects for the A360V mutation in the connection domain of HIV-1 reverse transcriptase. PLoS One 7, e31558, For the AIDS Clinical Trials Group Study 175 Protocol Team.

Delviks-Frankenberry, K.A., Nikolenko, G.N., Barr, R., Pathak, V.K., 2007. Mutations in human immunodeficiency virus type 1 RNase H primer grip enhance 3′-azido-3′-deoxythymidine resistance. J. Virol. 81, 6837–6845.

Deval, J., Selmi, B., Boretto, J., Egloff, M.P., Guerreiro, C., Sarfati, S., Canard, B., 2002. The molecular mechanism of multidrug resistance by the Q151M human immunodeficiency virus type 1 reverse transcriptase and its suppression using alpha-boranophosphate nucleotide analogues. J. Biol. Chem. 277, 42097–42104.

Deval, J., Navarro, J.M., Selmi, B., Courcambeck, J., Boretto, J., Halfon, P., Garrido-Urbani, S., Sire, J., Canard, B., 2004. 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. 279, 25489–25496.

Ehteshami, M., Beilhartz, G.L., Scarth, B.J., Tchesnokov, E.P., McCormick, S., Wynhoven, B., Harrigan, P.R., Götte, M., 2008. Connection domain mutations N348I and A360V in HIV-1 reverse transcriptase enhance resistance to 3′-azido-3′-deoxythymidine through both RNase H-dependent and -independent mechanisms. J. Biol. Chem. 283, 22222–22232.

Feng, J.Y., Anderson, K.S., 1999. Mechanistic studies examining the efficiency and fidelity of DNA synthesis by the 3TC-resistant mutant (184V) of HIV-1 reverse transcriptase. Biochemistry 38, 9440–9448.

Frankel, F.A., Marchand, B., Turner, D., Götte, M., Wainberg, W.A., 2005. Impaired rescue of chain-terminated DNA synthesis associated with the L74V mutation in human immunodeficiency virus type 1 reverse transcriptase. Antimicrob. Agents Chemother. 49, 2657–2664.

Garriga, C., Pérez-Elías, M.J., Delgado, R., Ruiz, L., Pérez-Alvarez, L., Pumarola, T., López-Lirola, A., González-García, J., Menéndez-Arias, L., 2009. HIV-1 reverse transcriptase thumb subdomain polymorphisms associated with virological failure to nucleoside drug combinations. J. Antimicrob. Chemother. 64, 251–258 (Spanish Group for the Study of Antiretroviral Drug Resistance).

Johnson, K.A., 1995. Rapid quench kinetic analysis of polymerases, adenosinetriphosphatases, and enzyme intermediates. Methods Enzymol. 249, 38–61.

Julias, J.G., McWilliams, M.J., Sarafianos, S.G., Alvord, W.G., Arnold, E., Hughes, S.H., 2003. Mutation of amino acids in the connection domain of human immunodeficiency virus type 1 reverse transcriptase that contact the template-primer affects RNase H activity. J. Virol. 77, 8548–8554.

Larder, B.A., Kemp, S.D., 1989. Multiple mutations in HIV-1 reverse transcriptase confer high-level resistance to zidovudine (AZT). Science 246, 1155–1158.

Larder, B.A., Darby, G., Richman, D.D., 1989. HIV with reduced sensitivity to zidovudine (AZT) isolated during prolonged therapy. Science 243, 1731–1734.

Le Grice, S.F., Grüninger-Leitch, F., 1990. Rapid purification of homodimer and heterodimer HIV-1 reverse transcriptase by metal chelate affinity chromatography. Eur. J. Biochem. 187, 307–314.

Ly, J.K., Margot, N.A., MacArthur, H.L., Hung, M., Miller, M.D., White, K.L., 2007. The balance between NRTI discrimination and excision drives the susceptibility of HIV-1 RT mutants K65R, M184V and K65R+M184V. Antivir. Chem. Chemother. 18, 307–316.

Martin, J.L., Wilson, J.E., Haynes, R.L., Furman, P.A., 1993. Mechanism of resistance of human immunodeficiency virus type 1 to 2′,3′-dideoxyinosine. Proc. Natl. Acad. Sci. U.S.A. 90, 6135–6139.

Meteer, J.D., Koontz, D., Asif, G., Zhang, H.W., Detorio, M., Solomon, S., Coats, S.J., Sluis-Cremer, N., Schinazi, R.F., Mellors, J.W., 2011. The base component of 3′-azido-2′,3′-dideoxynucleosides influences resistance mutations selected in HIV-1 reverse transcriptase. Antimicrob. Agents Chemother. 55, 3758–3764.

Meyer, P.R., Matsuura, S.E., So, A.G., Scott, W.A., 1998. Unblocking of chain-terminated primer by HIV-1 reverse transcriptase through a nucleotide-dependent mechanism. Proc. Natl. Acad. Sci. U.S.A. 95, 13471–13476.

Meyer, P.R., Matsuura, S.E., Mian, A.M., So, A.G., Scott, W.A., 1999. A mechanism of AZT resistance. an increase in nucleotide-dependent primer unblocking by mutant HIV-1 reverse transcriptase. Mol. Cell 4, 35–43.

Meyer, P.R., Matsuura, S.E., Schinazi, R.F., So, A.G., Scott, W.A., 2000. Differential removal of thymidine nucleotide analogues from blocked DNA chains by human immunodeficiency virus reverse transcriptase in the presence of physiological concentrations of 2′-deoxynucleoside triphosphates. Antimicrob. Agents Chemother. 44, 3465–3472.

Miller, V., Ait-Khaled, M., Stone, C., Griffin, P., Mesogiti, D., Cutrell, A., Harrigan, R., Staszewski, S., Katlama, C., Pearce, G., Tisdale, M., 2000. HIV-1 reverse transcriptase (RT) genotype and susceptibility to RT inhibitors during abacavir monotherapy and combination therapy. AIDS 14, 163–171.

Miranda, L.R., Götte, M., Liang, F., Kuritzkes, D.R., 2005. The L74V mutation in human immunodeficiency virus type 1 reverse transcriptase counteracts enhanced excision of zidovudine monophosphate associated with thymidine analog resistance mutations. Antimicrob. Agents Chemother. 49, 2648–2656.

Parikh, U.M., Zelina, S., Sluis-Cremer, N., Mellors, J.W., 2007. Molecular mechanisms of bidirectional antagonism between K65R and thymidine analog mutations in HIV-1 reverse transcriptase. AIDS 21, 1405–1414.

Puertas, M.C., Buzón, M.J., Artese, A., Alcaro, S., Menendez-Arias, L., Perno, C.F., Clotet, B., Ceccherini-Silberstein, F., Martinez-Picado, J., 2009. Effect of the human immunodeficiency virus type 1 reverse transcriptase polymorphism Leu-214 on replication capacity and drug susceptibility. J. Virol. 83, 7434–7439.

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.

Radzio, J., Yap, S.H., Tachedjian, G., Sluis-Cremer, N., 2010. N348I in reverse transcriptase provides a genetic pathway for HIV-1 to select thymidine analogue mutations and mutations antagonistic to thymidine analogue mutations. AIDS 24, 659–667.

Rausch, J.W., Lener, D., Miller, J.T., Julias, J.G., Hughes, S.H., Le Grice, S.F., 2002. Altering the RNase H primer grip of human immunodeficiency virus reverse transcriptase modifies cleavage specificity. Biochemistry 41, 4856–4865.

Sarafianos, S.G., Das, K., Tantillo, C., Clark Jr., A.D., Ding, J., Whitcomb, J.M., Boyer, P.L., Hughes, S.H., Arnold, E., 2001. Crystal structure of HIV-1 reverse transcriptase in complex with a polypurine tract RNA:DNA. EMBO J. 20, 1449–1461.

Sharma, P.L., Nettles, J.H., Feldman, A., Rapp, K., Schinazi, R.F., 2009. Comparative analysis of in vitro processivity of HIV-1 reverse transcriptases containing mutations 65R, 74V, 184V and 65R+74V. Antiviral Res. 83, 317–323.

Sluis-Cremer, N., Arion, D., Kaushik, N., Lim, H., Parniak, M.A., 2000. Mutational analysis of Lys65 of HIV-1 reverse transcriptase. Biochem. J. 348, 77–82.

Sluis-Cremer, N., Arion, D., Parikh, U., Koontz, D., Schinazi, R.F., Mellors, J.W., Parniak, M.A., 2005. The 3′-azido group is not the primary determinant of 3′-azido-3′-deoxythymidine (AZT) responsible for the excision phenotype of AZT-resistant HIV-1. J. Biol. Chem. 280, 29047–29052.

Sluis-Cremer, N., Sheen, C.W., Zelina, S., Torres, P.S., Parikh, U.M., Mellors, J.W., 2007. Molecular mechanism by which the K70E mutation in human immunodeficiency virus type 1 reverse transcriptase confers resistance to nucleoside reverse transcriptase inhibitors. Antimicrob. Agents Chemother. 51, 48–53.

Sluis-Cremer, N., Koontz, D., Bassit, L., Hernandez-Santiago, B.I., Detorio, M., Rapp, K.L., Amblard, F., Bondada, L., Grier, J., Coats, S.J., Schinazi, R.F., Mellors, J.W., 2009. Anti-human immunodeficiency virus activity, cross-resistance, cytotoxicity, and intracellular pharmacology of the 3′-azido-2′,3′-dideoxypurine nucleosides. Antimicrob. Agents Chemother. 53, 3715–3719.

Tang, M.W., Shafer, R.W., 2012. HIV-1 antiretroviral resistance. scientific principles and clinical applications. Drugs 72, e1–25.

Ueno, T., Shirasaka, T., Mitsuya, H., 1995. Enzymatic characterization of human immunodeficiency virus type 1 reverse transcriptase resistant to multiple 2′,3′-dideoxynucleoside 5′-triphosphates. J. Biol. Chem. 270, 23605–23611.

White, K.L., Margot, N.A., Ly, J.K., Chen, J.M., Ray, A.S., Pavelko, M., Wang, R., McDermott, M., Swaminathan, S., Miller, M.D., 2005. A combination of decreased NRTI incorporation and decreased excision determines the resistance profile of HIV-1 K65R RT. AIDS 19, 971–976.

Yap, S.H., Sheen, C.W., Fahey, J., Zanin, M., Tyssen, D., Lima, V.D., Wynhoven, B., Kuiper, M., Sluis-Cremer, N., Harrigan, P.R., Tachedjian, G., 2007. N348I in the connection domain of HIV-1 reverse transcriptase confers zidovudine and nevirapine resistance. PLoS Med. 4, e335.