Chain-Terminating Dinucleoside Tetraphosphates Are Substrates for DNA Polymerization by Human Immunodeficiency Virus Type 1 Reverse Transcriptase with Increased Activity against Thymidine Analogue-Resistant Mutants[▽]

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Nucleoside reverse transcriptase inhibitors are an important class of drugs for treatment of human immunodeficiency virus type 1 (HIV-1) infection. Resistance to these drugs is often the result of mutations that increase the transfer of chain-terminating nucleotides from blocked DNA termini to a nucleoside triphosphate acceptor, resulting in the generation of an unblocked DNA chain and synthesis of a dinucleoside polyphosphate containing the chain-terminating deoxynucleoside triphosphate analogue. We have synthesized and purified several dinucleoside tetraphosphates (ddAp₄ddA, ddCp₄ddC, ddGp₄ddG, ddTp₄ddT, Ap₄ddG, 2'-(3')-O-(N-methylanthraniloyl)-Ap₄ddG, and AppNHppddG) and show that these compounds can serve as substrates for DNA chain elongation and termination resulting in inhibition of DNA synthesis. Thymidine analogue-resistant mutants of reverse transcriptase are up to 120-fold more sensitive to inhibition by these compounds than is wild-type enzyme. Drugs based on the dinucleoside tetraphosphate structure could delay or prevent the emergence of mutants with enhanced primer unblocking activity. In addition, such drugs could suppress the resistance phenotype of mutant HIV-1 that is present in individuals infected with resistant virus.

Chronic treatment of human immunodeficiency virus type 1 (HIV-1)-infected individuals with nucleoside reverse transcriptase inhibitors (NRTIs) such as 3'-azido-2',3'-dideoxythymidine (AZT, or zidovudine) usually leads to selection of drug resistance mutations in the viral reverse transcriptase (RT) that may reduce or abolish the effectiveness of NRTI therapy (9, 11, 13, 14, 34). A major class of NRTI resistance mutations (designated thymidine analogue mutations, or TAMs, which include M41L, D67N, T69S-XX, K70R, L210W, T215F or Y, and K219Q or E) confers increased removal of chain-terminating nucleotides, primarily thymidine analogues and also, to a lesser extent, several other NRTIs, from blocked DNA chains (1, 3, 15, 16, 18, 21–23, 27). This unblocking reaction occurs through transfer of the chain-terminating nucleotide to a nucleoside triphosphate (NTP) acceptor in a reaction that is related to pyrophosphorolysis (23). The products of the transfer reaction are a dinucleoside tetraphosphate of the form Np₄ddN and an extendable primer terminus. In vitro, any NTP, deoxynucleoside triphosphate (dNTP), or dideoxyribonucleoside triphosphate (ddNTP) can serve as acceptor, whereas in vivo the acceptor is most likely ATP, resulting in the synthesis of Ap₄ddN (31). The mechanism by which TAMs give rise to primer unblocking has not been determined, but it has been proposed on the basis of crystal structure and substrate specificity data that the residues affected by TAMs play a role in interaction with the ATP substrate or with the adenosine por-

tion of an intermediate formed during the excision reaction (3, 5, 22, 24).

The widespread use of AZT and other NRTIs for antiretroviral therapy has resulted in a high prevalence of mutant HIV-1 containing TAMs, which were present in more than 25% of patients failing therapy in a recent survey (6). Therapies that are effective against these mutant viruses will continue to be needed. The increased ability of RT containing TAMs to utilize NTPs as substrates for primer unblocking suggests that it may be possible to target inhibitors to the mutant enzymes using the Np₄ddN interaction surface.

We have used an enzymatic strategy to synthesize several different dinucleoside tetraphosphates containing ddNTP components, and we show that HIV-1 RT can use these compounds as substrates for DNA elongation resulting in chain termination. Mutant RTs containing TAMs are as much as 120-fold more sensitive than wild-type (WT) RT to chain termination by these compounds. Dinucleoside tetraphosphates provide novel tools for further studies to explore the effects of TAMs on HIV-1 RT, and they may serve as a foundation for the development of new drugs that target DNA synthesis and nucleotide excision by this enzyme.

MATERIALS AND METHODS

Expression and purification of HIV-1 RT. His-tagged HIV-1 RT containing wild-type (RT^{WT}), K65R (RT^{K65R}), W88G (RT^{W88G}), M41L/T215Y (RT^{M41L/T215Y}), D67N/K70R/T215Y/K219Q (RT^{AZT}) or M41L/T69S-insertionAG/L210W/R211K/L214F/T215Y/K219Q mutations (RT^{MDR}) were expressed and purified as previously described (26). The specific RNA-dependent DNA polymerase activities of HIV-1 RT^{WT}, RT^{K65R}, RT^{W88G}, RT^{M41L/T215Y}, RT^{AZT}, and RT^{MDR} were 35,000, 16,000, 19,000, 27,000, 7,600, and 27,000 U/mg, respectively, where 1 U is equal to the amount of enzyme required for incorporation of 1.0 nmol of [³H]dTTP in 10 min when using poly(rA)/oligo(dT)

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(Amersham Biosciences) as the substrate (32). These specific activities are consistent with values reported elsewhere in the literature (4, 7, 12, 17, 32). Oligonucleotides were obtained from Sigma Genosys; M13 DNA was from New England Biolabs. [α - 32 P]dNTPs and [γ - 32 P]ATP were obtained from PerkinElmer, and [α - 33 P]ddNTPs and [α - 32 P]ddATP were from Amersham Biosciences (GE Healthcare).

Synthesis and purification of dinucleoside tetraphosphates. A 10 μ M concentration of L32 primer (5'-CTACTAGTTTCTCCATCTAGACGATACCAG A-3') was annealed to an equal amount of one of the templates listed below and incubated with 1 μ M HIV-1 RT^{MDR} and the ddNTP corresponding to the first unpaired base on the primer/template (P/T). Each reaction was carried out in 1,000 μ l of reaction buffer (40 mM Na-HEPES, pH 7.5, 20 mM MgCl₂, 60 mM KCl, 1 mM dithiothreitol, 2.5% glycerol) containing 160 μ g of bovine serum albumin (BSA) and 0.5 U of inorganic pyrophosphatase (Roche Applied Science). Templates and ddNTPs used were as follows: WL50 template (5'-GAG TGCTGAGGTCTTCATTCTGGTATCGTCTAGATGGAGAAAAGTAGTA G-3') with 3.2 mM ddATP and 10 μ Ci of [α - 33 P]ddATP; WL50-33G template (5'-GAGTGCTGAGGTCTTCAGTCTGGTATCGTCTAGATGGAGAAAAGTAGTAG-3') with 3.2 mM ddCTP and 10 μ Ci of [α - 33 P]ddCTP; WL50-33C template (5'-GAGTGCTGAGGTCTTCAGTCTGGTATCGTCTAGATGGAGAAAAGTAGTAG-3') with 3.2 mM ddGTP and 10 μ Ci of [α - 33 P]ddGTP; and WL50-33A-34T template (5'-GAGTGCTGAGGTCTTCATCTGGTATCGTCTAGATGGAGAAAAGTAGTAG-3') with 3.2 mM ddTTP and 10 μ Ci of [α - 33 P]ddTTP. All nucleotides were pretreated with thermostable pyrophosphatase as previously described (26). The mixture was overlaid with 200 μ l of mineral oil and incubated at 37°C for 7 days. After heat inactivation of the RT at 90°C for 5 min, 100 U of calf intestinal alkaline phosphatase (CIP) (New England Biolabs) was added, followed by incubation at 37°C for 2 h. One hundred units of CIP was added a second time, and incubation continued at 37°C for an additional 2 h, followed by heat inactivation at 90°C for 10 min. The CIP-treated products were loaded onto a 5-ml anion exchange column, HiTrap Q HP (Amersham Biosciences) pre-equilibrated with 10 mM triethylammonium bicarbonate buffer pH 8.5 (Sigma), and eluted with a gradient of triethylammonium bicarbonate buffer up to 1 M. Fractions containing 33 P-labeled dinucleoside polyphosphates were vacuum dried and resuspended in reaction buffer. The concentration of each compound was determined by incubation first with snake venom phosphodiesterase to cleave dinucleoside polyphosphates to free nucleotides, followed by spectrophotometric determination of the total concentration of nucleotide. The synthesis and purification of the dinucleoside tetraphosphate Ap₄ddG, doubly labeled with 32 P and 33 P (A-P-P- 32 P- 33 P-ddG), were identical to the synthesis and purification of ddGp₄ddG except for the following modifications: 10 μ M L32 primer annealed to WL50-33C template was incubated with 1 μ M HIV-1 RT^{MDR} with 10 mM ATP, 20 μ Ci of [γ - 32 P]ATP, 1 mM ddGTP, and 10 μ Ci of [α - 33 P]ddGTP. Following anion exchange chromatography, Ap₄ddG was separated from ddGp₄ddG by reverse-phase high-pressure liquid chromatography using a C₁₈ column (Amersham). The synthesis of 2'-(3'-O-(N-methylanthraniloyl)-(MANT)-Ap₄ddG and AppNHppddG was the same as the synthesis of Ap₄ddG except that MANT-ATP (Jena Bioscience) or AppNHp (Jena Bioscience), respectively, was included in the reaction instead of ATP.

Inhibition of DNA polymerization by ddNTPs or ddNp₄ddNs. M13 single-stranded DNA (New England Biolabs) annealed to a DNA primer (5'-AAGTT GGGTAACGCCAGGGTTTCCAGTCACGAC-3') (2.5 nM) was incubated with excess HIV-1 RT (100 nM) in 20 μ l of reaction buffer containing 80 μ g/ml of BSA; a 2.5 μ M concentration of each dNTP; and 20 μ Ci of [α - 32 P]dATP, [α - 32 P]dCTP, [α - 32 P]dGTP, or [α - 32 P]dTTP in the absence or presence of various concentrations of ddATP or ddAp₄ddA, ddCTP or ddCp₄ddC, ddGTP or ddGp₄ddG, or ddTTP or ddTp₄ddT, respectively, at 37°C for 30 min. The reaction was terminated by heating at 90°C for 5 min. Five microliters of reaction mixture was mixed with 5 μ l of gel loading dye and separated by electrophoresis through a 10% denaturing polyacrylamide gel. The radioactivity of the products in the dried gel was visualized by exposure on KODAK BioMax MR Film. For quantification of incorporated radioactive nucleotide, the remaining 15 μ l of the reaction mixture was spotted on 3MM Whatman filter circles and soaked three times (45 min, 30 min, and 30 min) at 4°C in large excess of 5% trichloroacetic acid containing 20 mM sodium pyrophosphate, followed by two 5-min washes in ethanol at 4°C. The precipitable radioactivity in each sample was quantified by scintillation counting. The amount of inhibition of DNA synthesis was plotted versus inhibitor concentration using SigmaPlot 8.0 to obtain the 50% inhibitory concentration (IC₅₀). IC₅₀s for mutant enzymes were adjusted for differences in activity between mutant and WT RT to correct for effects due to the formation of products of different average lengths (28) using the following formula: IC₅₀ = (uncorrected IC₅₀ $\times$ mutant RT activity)/WT RT activity.

FIG. 1. Schematics for synthesis of ddNp₄ddNs. A 10 μ M concentration of DNA P/T was incubated with a 3.2 mM concentration of the ddNTP (a small portion of which was α - 33 P labeled) that is complementary to the first single-stranded position on the template, 1 μ M multinucleoside-resistant HIV-1 RT (HIV-1 RT^{MDR}), and 0.5 units of inorganic pyrophosphatase. Incorporation of the 2',3'-dideoxynucleoside monophosphate into the primer terminus leads to chain termination. The PP_i generated by the incorporation was cleaved by pyrophosphatase, and the chain terminator was transferred from the blocked DNA to ddNTP, leading to formation of the dinucleoside polyphosphate ddNp₄ddN and unblocked P/T available for another round of synthesis.

Electrophoretic mobility shift assay. L31 primer (5'-CTACTAGTTTCTCC ATCTAGACGATACCAG-3') annealed to a fourfold excess of WL50-33C DNA template was terminated with [32 P]ddAMP as previously described (23). A 5 nM concentration of L31-[32 P]ddAMP/WL50-33C was incubated with 100 nM HIV-1 RT and various concentrations of either ddGTP or ddGp₄ddG, as indicated in the legend of Fig. 4, in 10 μ l of reaction buffer containing 80 μ g/ml BSA for 15 min at 37°C. Samples were incubated at 4°C for 5 min, followed by the addition of 3 μ l of heparin and loading dye (0.03 U heparin/ μ l, 30% sucrose, 0.25% bromophenol blue, and 0.25% xylene cyanol) in reaction buffer to dissociate binary complexes. The samples were separated by electrophoresis through an 8% native polyacrylamide gel at 200 V for 1 h at 4°C using Tris-taurine buffer (90 mM Tris–30 mM taurine). The radioactivity was quantitated by phosphorimaging. The percentage of DNA in complex, after deduction of complex formed in the absence of dNTP, was plotted against the ligand concentration. The apparent dissociation constant [$K_{D(\text{app})}$] for dead-end complex was obtained by fitting the data to a one-ligand binding curve using SigmaPlot 8.0.

RESULTS

Synthesis and purification of dinucleoside tetraphosphates containing identical 2',3'-dideoxynucleoside moieties (ddNp₄ddN). A recycling reaction using multinucleoside-resistant HIV-1 RT (HIV-1 RT^{MDR}) containing M41L/T69S-insertionAG/L210W/R211K/L214F/T215Y/K219Q mutations (which has a high level of ATP-dependent removal activity [16, 21]) was used for synthesis of dinucleoside tetraphosphates as outlined in Fig. 1 and in the Materials and Methods section. Over time, this reaction converted a portion of the ddNTP into ddNp₄ddN (Fig. 2A). The reaction mixture was treated with CIP to degrade the remaining ddNTP. The ddNp₄ddN, which is resistant to CIP cleavage, was separated from free phosphate and 2',3'-dideoxynucleosides by

FIG. 2. Synthesis and purification of ddNp₄ddNs. (A) A 10 μ M concentration of one of four different DNA P/Ts (see Materials and Methods for sequences) was incubated with HIV-1 RT^{MDR}, inorganic pyrophosphatase, and 3.2 mM ddATP (lanes 1 and 5), ddCTP (lanes 2 and 6), ddGTP (lanes 3 and 7), or ddTTP (lanes 4 and 8) for 7 days at 37°C. Following heat inactivation of the RT, the reaction mixture was incubated with CIP (lanes 5 to 8). (B) The CIP-treated reaction mixture shown in lane 5 in panel A was loaded onto an anion exchange column and eluted with a 10 to 1,000 mM gradient of triethylammonium bicarbonate (TEAB) buffer, pH 8.5. Aliquots of reaction mixtures or selected column fractions were separated by electrophoresis through 20% urea-polyacrylamide gel electrophoresis.

anion exchange chromatography (Fig. 2B). The structures of four dinucleoside polyphosphates are shown in Fig. 3.

Inhibition of HIV-1 RT-mediated DNA synthesis by ddNTPs and ddNp₄ddNs. In order to test the ability of WT and mutant HIV-1 RT to use ddNp₄ddNs as dNTP analogues, we determined the inhibition of DNA-dependent DNA synthesis by these compounds using M13 P/T as a substrate. DNA synthesis was measured by incorporation of [³²P]dNMP from [α -³²P]dNTP. Incorporation of ddNMP from either ddNTP or ddNp₄ddN resulted in chain termination, leading to a decrease in the total amount of incorporated radioactive nucleotide and the appearance of shorter, chain-terminated products. A por-

FIG. 3. Structures of four of the dinucleoside tetraphosphates that were synthesized.

tion of the reaction mixture was separated by denaturing gel electrophoresis to visualize the extended products, while the majority was spotted onto filter circles and precipitated with trichloroacetic acid to allow quantification of incorporated radioactivity. Figure 4A shows the ability of either ddGTP or ddGp₄ddG to inhibit DNA polymerization by HIV-1 RT containing D67N/K70R/T215Y/K219Q mutations (HIV-1 RT^{AZT}). In the absence of inhibitor (Fig. 4, lane 1), a large majority of M13 primers were extended to a similar position, observed as a strong radioactive band at the top of the gel. In the presence of increasing concentrations of ddGTP (lanes 2 to 5), there was a concomitant decrease in the amount of total incorporated radioactive label and a decrease in the average lengths of the extended products that were chain terminated at G-incorporation sites. A similar pattern was observed at increasing concentrations of ddGp₄ddG (lanes 6 to 9), although the enzyme was slightly less sensitive to inhibition by ddGp₄ddG than to inhibition by ddGTP. Pretreatment of ddGTP with CIP abolished its inhibitory activity (lanes 10 to 13), while similar treatment of ddGp₄ddG had minimal affect on its ability to inhibit DNA synthesis (lanes 14 to 17). Therefore, ddGp₄ddG was an efficient substrate for DNA synthesis by HIV-1 RT^{AZT}. Figure 4B shows that heparin-resistant complex formed with ddAMP-terminated primer/template by RT^{AZT} and RT^{WT} had similar stability with ddGTP, but the complexes formed with ddGp₄ddG were about threefold more stable with RT^{AZT} than with RT^{WT} (K_D of 0.26 μ M versus 0.80 μ M). While the thymidine analogue mutations selectively enhanced interaction with the tetraphosphate substrate, the affinity with the mutant enzyme was still about fourfold less than for ddGTP, in agree-

FIG. 4. Inhibition of DNA synthesis and formation of stable complexes with RT^{WT} and RT^{AZT} by ddGTP or ddGp₄ddG. (A) M13 single-stranded DNA annealed to a cDNA primer (2.5 nM) was incubated with HIV-1 RT^{AZT} (100 nM), a 2.5 μM concentration of each dNTP, 50 μCi of [α -³²P]dGTP, and the indicated concentration of ddGTP or ddGp₄ddG that was either untreated (–) or had been pretreated with CIP (+) for 30 min at 37°C. The samples were separated by 20% urea-polyacrylamide gel electrophoresis. (B) Electrophoretic mobility shift assay for the ability of RT^{WT} or RT^{AZT} to form a stable dead-end complex (DEC) with ddAMP-terminated primer/template. Enzyme was incubated with WL50-33C/L31-[³²P]ddAMP template/primer in the presence of the indicated concentration of either ddGTP or ddGp₄ddG, and stable complexes were separated from free DNA by nondenaturing electrophoresis as described in Materials and Methods. K_D values were determined from plots of the percentage of total radioactivity in complex versus substrate concentration. DNA P/T sequence near the primer terminus is given at the bottom of the panel.

ment with the sensitivity of this enzyme to inhibition by ddGTP (Fig. 4A).

Mechanism for transfer of ddNMP from a dinucleoside tetraphosphate onto the primer terminus by HIV-1 RT. There are two possible reaction mechanisms, a direct and an indirect one, for the ability of HIV-1 RT to use dinucleoside tetraphosphates as substrates for ddNMP incorporation. In the direct mechanism, the dinucleoside tetraphosphate itself serves as the dNTP analogue. After incorporation of a ddNMP moiety from Np₄ddN into the primer terminus, this pathway would yield one molecule of NTP. In the indirect pathway, the RT first cleaves the Np₄ddN to yield a molecule of NMP and a molecule of ddNTP, which would subsequently be incorporated at the primer terminus. This pathway would yield one molecule of NMP and one molecule of PP_i for each incorporation event. To distinguish between these two possibilities, the ability of HIV-1 RT^{AZT} to use doubly labeled Ap₄ddG (A-P-P-³²P-³³P-ddG) as a dGTP analogue was measured. As can be seen in Fig. 5, increased incubation times led to an increased amount of the labeled product L32-ddGM-³³P, indicating that Ap₄ddG can serve as a dGTP analogue. Concomitant with the

increase in L32-ddGM-³³P was an increase in [γ -³²P]ATP and, importantly, no increase in PP_i, indicating that ddGMP was incorporated into the primer terminus by HIV-1 RT directly from Ap₄ddG. In addition, a similar incorporation efficiency of ddGMP from Ap₄ddG was observed when the reaction was performed in the continuous presence of CIP (data not shown), indicating that no CIP-sensitive intermediate was formed during the reaction, which further supports the direct incorporation mechanism.

Sensitivity of DNA polymerization by WT and mutant HIV-1 RTs to inhibition by ddNTPs, ddNp₄ddNs, or Np₄ddNs. The IC₅₀s were determined for DNA polymerization by WT and mutant RTs for ddATP, ddAp₄ddA, ddCTP, ddCp₄ddC, ddGTP, ddGp₄ddG, ddTTP, and ddTp₄ddT (Table 1). HIV-1 RT^{WT}, RT^{K65R}, and RT^{W88G} were much more sensitive (15- to 83-fold) to inhibition by ddNTPs than by ddNp₄ddNs. RT^{K65R} and RT^{W88G} were less sensitive to inhibition by either ddNTPs or ddNp₄ddNs than RT^{WT}. RTs containing TAMs (RT^{M41L/T215Y}, RT^{AZT}, and RT^{MDR}) had increased sensitivity to ddNp₄ddNs compared with RT^{WT}, but there was little difference in sensitivity to ddNTPs. The ratio

FIG. 5. Products formed during incorporation of ddGMP from doubly labeled Ap₄ddG. A 10 μ M concentration of unlabeled WL50-33C/L32 template/primer was incubated with 2 μ M HIV-1 RT^{MDR} and 200 μ M Ap₄ddG (A-P-P-³²P-³³P-ddG) for the indicated times at 37°C, followed by heat inactivation of the RT (5 min at 95°C). Some samples were then treated with 4 U of CIP (+CIP) for 30 min, followed by heat inactivation (5 min at 95°C) of the CIP. The reaction products were separated by 20% urea-polyacrylamide gel electrophoresis, and the radioactivity was visualized by phosphorimaging. Standards for free phosphate and PP_i were generated by incubating [γ -³²P]ATP with CIP or snake venom phosphodiesterase (SVPD), respectively.

for inhibition of DNA synthesis (IC_{50} for ddNp₄ddN/ IC_{50} for ddNTP) was 15- to 72-fold for RT^{WT}, 4.8- to 25-fold for RT^{M41L/T215Y}, 1.8- to 4.7-fold for RT^{AZT}, and 0.95- to 4.8-fold for RT^{MDR}. Of the dinucleoside tetraphosphates tested, all six enzymes were more sensitive to inhibition by ddCp₄ddC and ddGp₄ddG. The IC_{50} s for ddTp₄ddT and ddAp₄ddA were 1.2- to 8.5-fold higher than for ddCp₄ddC and ddGp₄ddG.

Next, we characterized the ability of Ap₄ddG or Ap₄ddG-derived compounds containing modifications of the sugar or phosphate chains to inhibit DNA polymerization (Fig. 6 and Table 2). RT^{MDR} used Ap₄ddG as a dGTP analogue even more efficiently than ddGp₄ddG, while RT^{WT} was about four-fold less sensitive to Ap₄ddG than to ddGp₄ddG, resulting in a 50-fold higher sensitivity of DNA polymerization by RT^{MDR} than polymerization by RT^{WT} to inhibition by Ap₄ddG (Fig. 6 and Tables 1 and 2). With MANT-Ap₄ddG the difference in sensitivity between these enzymes was over 100-fold. DNA polymerization by RT^{MDR} was also sensitive to inhibition by AppNHppddG (IC_{50} of 1.7 μ M), a compound to which all other enzymes tested were highly resistant (IC_{50} s of ≥ 28 μ M).

In summary, TAMs and the T69S-XX insertion mutation, which confer an increase in ATP-dependent removal of nucleotide analogues from blocked DNA, also conferred an in-

creased ability to use dinucleoside polyphosphates as dNTP analogues during DNA synthesis. This resulted in greater sensitivity of HIV-1 RT containing these mutations to inhibition by ddNp₄ddNs. In contrast, mutations K65R and W88G, which are associated with decreased PP_i- and nucleotide-dependent removal activity (25, 30), conferred decreased sensitivity to inhibition by either ddNTPs or ddNp₄ddNs.

DISCUSSION

Our results show that AZT-resistant HIV-1 RTs that contain TAMs and have enhanced nucleotide excision activity also have increased ability to use dinucleoside tetraphosphates of the form Np₄ddN or ddNp₄ddN as substrates for DNA elongation and chain termination. By contrast, when ddNTPs were provided as substrates, the efficiency of chain termination was about the same for TAM-containing RTs and WT RT. The difference between WT and mutant RTs was greatest when MANT-Ap₄ddG was used as a substrate. Chain termination with this substrate by RT^{MDR} was 120-fold more efficient than by RT^{WT}. Chain termination with MANT-Ap₄ddG by RT^{AZT} was 19-fold more efficient than by RT^{WT}. Other dinucleoside tetraphosphate compounds showed smaller but still substantial differences between mutant and WT RT. Incorporation of ddNMP occurred without prior formation of a ddNTP intermediate, in agreement with a previous report (33). Our results

TABLE 1. Inhibition of HIV-1 RT-mediated DNA-dependent DNA synthesis by ddNTPs or ddNp₄ddNs

ddN ^a	Enzyme	IC_{50} (μ M) ^b		IC_{50} (ddNp ₄ ddN)/ IC_{50} (dNTP) [WT/mutant] ^c
		ddNTP (WT/mutant) ^c	ddNp ₄ ddN (WT/mutant) ^c	
ddA	RT ^{WT}	0.11 $\pm$ 0.01 (1.0)	6.5 $\pm$ 0.4 (1.0)	61 (1.0)
	RT ^{K65R}	0.59 $\pm$ 0.09 (0.19)	>36 (<0.18)	>61 (<1.0)
	RT ^{W88G}	0.31 $\pm$ 0.13 (0.35)	>15 (<0.43)	>48 (<1.3)
	RT ^{M41L/T215Y}	0.22 $\pm$ 0.03 (0.50)	2.9 $\pm$ 1.0 (2.2)	13 (4.7)
	RT ^{AZT}	0.16 $\pm$ 0.01 (0.69)	0.65 $\pm$ 0.12 (10)	4.2 (15)
	RT ^{MDR}	0.25 $\pm$ 0.03 (0.44)	1.2 $\pm$ 0.2 (5.4)	4.8 (13)
ddC	RT ^{WT}	0.079 $\pm$ 0.009 (1.0)	2.1 $\pm$ 0.4 (1.0)	27 (1.0)
	RT ^{K65R}	0.44 $\pm$ 0.05 (0.18)	11 $\pm$ 2 (0.19)	25 (1.1)
	RT ^{W88G}	0.28 $\pm$ 0.06 (0.28)	9.2 $\pm$ 1.2 (0.23)	33 (0.82)
	RT ^{M41L/T215Y}	0.095 $\pm$ 0.012 (0.83)	1.1 $\pm$ 0.1 (1.9)	12 (2.2)
	RT ^{AZT}	0.10 $\pm$ 0.01 (0.79)	0.18 $\pm$ 0.02 (12)	1.8 (15)
	RT ^{MDR}	0.12 $\pm$ 0.01 (0.66)	0.28 $\pm$ 0.03 (7.5)	2.3 (12)
ddG	RT ^{WT}	0.12 $\pm$ 0.01 (1.0)	1.8 $\pm$ 0.2 (1.0)	15 (1.0)
	RT ^{K65R}	0.49 $\pm$ 0.10 (0.24)	9.5 $\pm$ 1.2 (0.19)	19 (0.79)
	RT ^{W88G}	0.24 $\pm$ 0.04 (0.50)	6.0 $\pm$ 0.9 (0.30)	25 (0.60)
	RT ^{M41L/T215Y}	0.082 $\pm$ 0.010 (1.5)	0.39 $\pm$ 0.11 (4.6)	4.8 (3.1)
	RT ^{AZT}	0.092 $\pm$ 0.017 (1.3)	0.20 $\pm$ 0.03 (9.0)	2.2 (6.8)
	RT ^{MDR}	0.21 $\pm$ 0.05 (0.57)	0.20 $\pm$ 0.02 (9.0)	0.95 (16)
ddT	RT ^{WT}	0.12 $\pm$ 0.02 (1.0)	8.6 $\pm$ 2.0 (1.0)	72 (1.0)
	RT ^{K65R}	0.66 $\pm$ 0.12 (0.18)	42 $\pm$ 14 (0.20)	64 (1.1)
	RT ^{W88G}	0.41 $\pm$ 0.05 (0.29)	34 $\pm$ 12 (0.25)	83 (0.87)
	RT ^{M41L/T215Y}	0.13 $\pm$ 0.02 (0.92)	3.3 $\pm$ 0.4 (2.6)	25 (2.9)
	RT ^{AZT}	0.20 $\pm$ 0.02 (0.60)	0.92 $\pm$ 0.09 (9.3)	4.7 (15)
	RT ^{MDR}	0.34 $\pm$ 0.06 (0.35)	0.34 $\pm$ 0.02 (25)	1.0 (72)

^a ddN indicates the dideoxynucleoside component of ddNTP and both dideoxynucleoside components of ddNp₄ddN compounds tested as inhibitors of DNA synthesis.

^b IC_{50} s were determined on M13 P/T and adjusted for equivalent specific activity for each enzyme as described in Materials and Methods. Results are the means of three experiments $\pm$ standard deviations.

^c Ratio of IC_{50} for RT^{WT} to the IC_{50} for each of the enzymes.

FIG. 6. Inhibition of DNA-dependent DNA synthesis through incorporation of ddGMP from Ap_4ddG by RT^{WT} , RT^{AZT} , or RT^{MDR} . (A) M13 single-stranded DNA annealed to a DNA primer (2.5 nM) was incubated with HIV-1 RT^{WT} or HIV-1 RT^{AZT} (100 nM), a 2.5 μM concentration of each dNTP, 50 μCi of $[\alpha\text{-}^{32}\text{P}]\text{dGTP}$, and the indicated concentrations of Ap_4ddG for 30 min at 37°C . The samples were separated by 20% urea-polyacrylamide gel electrophoresis. (B) Percent inhibition of $[\text{P}^{32}]\text{dGMP}$ incorporation by Ap_4ddG was determined as described in Materials and Methods. A summary of IC_{50} s derived from several experiments is given in Table 2.

provide tools for further mechanistic studies and development of drugs that target this class of HIV-1 mutants.

Our results suggest that dinucleoside tetraphosphates have the ability to interact with two adjacent nucleoside binding surfaces on RT: one identified as the binding site for the incoming dNTP in the polymerase reaction and the second identified by the residues mutated in TAMs and lying just distal to the end of the phosphate chain of the incoming dNTP. The dNTP binding site is known from the crystal structure of $\text{RT}^{\text{WT}}\text{-P/T-dTTP}$ ternary complex (10), and the locations for the second nucleoside and additional phosphate residue have been inferred by modeling in an ATP molecule with the β and γ phosphates superimposed on the γ and β phosphates of the dTTP (3, 5). In the proposed structure, the adenine base forms $\pi\text{-}\pi$ interactions with tyrosine or phenylalanine side chains at

residue 215 in the mutant RT. Tests of excision substrate specificity also provide support for this model (24). The enhanced rate of Np_4ddN -mediated chain termination by TAM-containing RTs can be explained by more favorable binding of Np_4ddN to the mutant enzyme, as observed with ddGp_4ddG (Fig. 4). While these results support the ability of the tetraphosphates to interact with adjacent sites on RT, our data indicate that functional binding of ddGTP is favored over ddGp_4ddG for TAM-containing RTs such as RT^{AZT} . This suggests that binding of Np_4ddN compounds to the ddNTP site is severely restricted in RT^{WT} and that TAMs confer increased ability to bind these compounds, possibly by removing steric constraints or by contributing binding interactions that can compensate for limited accessibility of the tetraphosphate compounds to the binding site. The enhanced rate of nucleotide-dependent excision by TAM-containing RTs is largely due to effects on catalytic rate that occur after substrate binding since TAMs have little effect on the K_D for the nucleotide substrate (29). It has been proposed that the T215Y or F mutations increase the rate of nucleophilic attack by controlling the alignment of the pyrophosphate component of the acceptor substrate with respect to the bond to be cleaved in the nascent DNA chain (8, 19, 20). Similar considerations may explain the ability of these mutations to enhance the use of dinucleoside tetraphosphates as substrates for incorporation.

Incorporation of ddGMP or ddCMP using ddGp_4ddG or ddCp_4ddC as a substrate was more efficient than incorporation of ddAMP or ddTMP using the corresponding dinucleoside tetraphosphate substrates. This suggests that a G-C base pair was preferred during the reaction. The difference in ddGMP incorporation between RT^{MDR} and RT^{WT} was greatest when MANT- Ap_4ddG was used as a substrate, suggesting that RT^{MDR} is better able to accommodate a bulky substituent on the ribose than is RT^{WT} . AppNHppddG was a poor substrate for chain termination with all of the enzymes tested, indicating that the precise structure of the phosphate chain is important.

These results suggest that it may be possible to identify bifunctional compounds that have high affinity for RT and preferentially bind to the TAM-containing enzymes. Dinucleoside tetraphosphate derivatives that contain hydrolyzable phosphate links have the advantage that they may be able to bypass the need for intracellular phosphorylation to lead to chain

TABLE 2. Inhibition of HIV-1 RT-mediated DNA-dependent DNA synthesis by Ap_4ddG , MANT- Ap_4ddG , or AppNHppddG

Enzyme	IC_{50} (μM) ^a		
	Ap_4ddG (WT/mutant) ^b	MANT- Ap_4ddG (WT/mutant) ^b	AppNHppddG (WT/mutant) ^b
RT^{WT}	6.8 ± 0.7 (1.0) ^b	16 ± 5 (1.0)	>50 (1.0)
$\text{RT}^{\text{D67N/K70R}}$	1.4 ± 0.1 (4.9)	2.5 ± 0.4 (6.4)	>41 (NC) ^c
$\text{RT}^{\text{M41L/T215Y}}$	1.95 ± 0.31 (3.5)	3.8 ± 0.3 (4.2)	>37 (NC)
RT^{AZT}	0.74 ± 0.05 (9.2)	0.83 ± 0.09 (19)	28 ± 9 (>1.8)
RT^{MDR}	0.13 ± 0.03 (52)	0.13 ± 0.13 (120)	1.7 ± 0.6 (>29)

^a IC_{50} s were determined as in Table 1. Results are the means of three experiments $\pm$ standard deviations.

^b Ratio of IC_{50} for RT^{WT} to the IC_{50} for each of the enzymes.

^c NC, not calculated.

termination. This would expand the range of nucleoside structures that can be used for chain termination by HIV-1 since current NRTIs are limited to molecules that are phosphorylated by cellular nucleoside kinases. On the other hand, the presence of phosphate residues may limit the ability of these compounds to enter mammalian cells and increase their sensitivity to degradative enzymes such as phosphodiesterases. However, modification of the phosphate residues might improve bioavailability and stability of dinucleoside polyphosphates (2).

The ability of dinucleoside tetraphosphates to serve as substrates for DNA chain elongation and termination by HIV-1 RT and the strong preference of these compounds for use by TAM-containing mutant enzymes provide a useful tool for analysis of the effects of these mutations on RT activities. In addition, our studies provide evidence for an interaction surface on the mutant enzymes that recognizes both nucleoside components of the Np₄ddN structure and may be an attractive target for future drug development.

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