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Abstract: Chain-terminating nucleoside polyphosphate compounds inhibit the ability of a viral reverse transcriptase to remove the chain terminator from the 3’ end of a blocked DNA chain. Because dinucleoside polyphosphate is produced by nucleotide-dependent DNA primer unblocking, which is catalyzed by the reverse transcriptase, it might be difficult for a virus to acquire mutations conferring resistance to this class of inhibitors without also compromising its ability to remove chain-terminating nucleoside analogues from the 3’ end of the blocked DNA chain.

Title: DINUCLEOSIDE POLYPHOSPHATE INHIBITORS OF REVERSE TRANSCRIPTASE
DINUCLEOSIDE POLYPHOSPHATE INHIBITORS OF REVERSE TRANSCRIPTASE

CROSS-REFERENCE TO RELATED APPLICATION

This application claims the benefit of provisional U.S. Application No. 60/688,332, filed June 8, 2005.

FEDERALLY-SPONSORED RESEARCH OR DEVELOPMENT

The U.S. Government has certain rights in this invention as provided for by the terms of 5-RO1-Al39973 awarded by the Department of Health and Human Services.

BACKGROUND OF THE INVENTION

This invention relates to use of chain-terminating dinucleoside polyphosphates as inhibitors of a viral reverse transcriptase (RT). Dinucleoside polyphosphates made by an enzymatic process are also provided.

One of the greatest challenges to successful long-term treatment of HIV-1 infection is the error prone nature of HIV-1 replication, resulting in development of drug resistance mutations. It is estimated that approximately 75% to 80% of failures during Highly Active Anti-Retroviral Treatment (HAART) are caused by the virus acquiring drug resistance mutations. Drug resistance is even a problem in treatment-naïve individuals, with about 25% harboring virus resistant to at least one of the three major classes of anti HIV-1 compounds: nucleoside RT inhibitors, non-nucleoside RT inhibitors (NRTIs), and protease inhibitors.

A major class of NRTI-resistance mutations (designated thymidine analogue mutations or TAMs, which includes M41L, D67N, T69S-XX, K70R, L210W, T215F or T215Y, and K219Q or K219E) confers increased removal of chain-terminating nucleotides, primarily of thymidine analogues, but also, to a lesser extent, of several other NRTIs from blocked DNA chains. The unblocking reaction occurs through transfer of the chain-terminating nucleotide to a nucleoside triphosphate acceptor in a reaction that is related to pyrophosphorolysis.
The products of the transfer reaction are a dinucleoside tetraphosphate of the form $\text{N}_{5}\text{dN}$ and an extendable primer terminus. In vitro, any NTP, dNTP, or ddNTP can serve as acceptor; whereas, in vivo, the acceptor is most likely ATP resulting in the synthesis of $\text{Ap}_{4}\text{dN}$. 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 portion of an intermediate formed during the excision reaction.

Multiple different nucleoside RT inhibitors are used in the large majority of HAART regimens. Resistance to this class of inhibitors can occur through one of two mechanisms, increased ability to discriminate against the nucleoside analogue during DNA synthesis or an increased ability to remove it from the viral DNA after it has been incorporated. The ability of HIV-1 RT to remove chain-terminating nucleoside analogues from blocked DNA chains through transfer to a nucleotide acceptor was discovered by us (Proc Natl Acad Sci USA 95:13471-13476, 1998). We found that an increase in nucleotide-dependent primer unblocking was conferred by AZT resistance mutations (Mol Cell 4:35-43, 1999), and is important for resistance to many of the nucleoside analogue inhibitors currently in use against HIV-1 infection.

Nucleotide-dependent removal of a chain terminator from a blocked DNA end by HIV-1 RT produces an unblocked, extendable DNA end and also a dinucleoside polyphosphate containing the removed nucleotide analogue linked to the nucleotide acceptor substrate through a polyphosphate chain. For example, ATP dependent removal of AZT monophosphate would yield $\text{Ap}_{4}\text{AZT}$.

While studying different aspects of the removal reaction, we discovered that it was reversible. Dinucleoside polyphosphates containing at least one 2'-deoxyribonucleoside moiety could serve as a dNTP analogue during DNA polymerization by reverse transcriptase. For example, $\text{Ap}_{4}\text{AZT}$ could serve as a dNTP analogue for incorporation of the AZT monophosphate moiety opposite a template dT position producing an AZT monophosphate-terminated DNA chain and an ATP. Interestingly, DNA polymerization by HIV-1 RTs harboring AZT resistance mutations is at least 10-fold more sensitive to inhibition by dinucleo-
side polyphosphates (containing one or two chain-terminating nucleoside analogues) than is polymerization by WT RT.

Thus, dinucleoside polyphosphates containing one or two chain-terminating nucleoside analogues represent a novel class of inhibitors with increased potency against mutant reverse transcriptases resistant to previously known inhibitors. U.S. Patents 5,049,550 and 5,681,823 describe the use of diadenosine tetraphosphates as antithrombotic agents. U.S. Patent 5,635,160 describes the use of dinucleoside polyphosphates as agonists of P2Y receptors. WO 01/12644 describes dinucleoside tetraphosphates as inhibitors of viral reverse transcriptase and viruses.

The unusual structure of dinucleoside polyphosphates makes it likely that host DNA polymerases would not recognize them as dNTP analogues or recognize them at reduced efficiency. Therefore the toxicity due to their incorporation into cellular and mitochondrial DNA might be lower than with the conventional nucleoside analogues which have been previously used as reverse transcriptase inhibitors. Furthermore, because the dinucleoside polyphosphates are produced by nucleotide-dependent unblocking of DNA primer catalyzed by a viral reverse transcriptase, it might be difficult for a virus to acquire mutations conferring resistance to this class of inhibitors without also compromising the virus’ ability to remove chain-terminating nucleoside analogues from the 3’-ends of blocked DNA primers (i.e., terminated). A long-felt need for treatment of a viral infection with lowered toxicity and/or decreased incidence of resistance as compared to conventional treatments (e.g., HAART) is addressed thereby.

The present invention is directed to an improved enzyme inhibitor of reverse transcriptase that addresses the aforementioned problems. Other advantages and improvements are described below or would be apparent from the disclosure herein.
SUMMARY OF THE INVENTION

A composition of a single or multiple different chain-terminating dinucleoside polyphosphates, as well as processes for using and making them are provided. The invention is used to inhibit viral reverse transcriptase.

In one embodiment of the invention, reverse transcriptase (RT) is inhibited in an individual infected by a virus or at risk for such infection by administering one or more dinucleoside polyphosphates to the individual in an amount sufficient to at least partially inhibit an activity of the reverse transcriptase, wherein a dinucleoside polyphosphate contains one or two chain-terminating nucleosides.

In another embodiment of the invention, an individual infected by a virus is treated by incorporating a dinucleoside polyphosphate into a DNA primer with reverse transcriptase of the virus, wherein a dinucleoside polyphosphate contains one or two chain-terminating nucleosides.

In yet another embodiment of the invention, the concentration of one or more nucleoside polyphosphates is increased to an amount which at least partially inhibits an activity of reverse transcriptase, wherein a dinucleoside polyphosphate contains one or two chain-terminating nucleosides.

The reverse transcriptase may be an enzyme derived from avian myeloblastosis virus (AMV), hepatitis B virus (HBV), human T-lymphotropic virus (HTLV), Moloney murine leukemia virus (M-MuLV), Rous sarcoma virus (RSV), or a lentivirus (e.g., bovine, feline, human, or simian immunodeficiency virus) especially HIV-1 or HIV-2. It may be wild-type (WT) or mutant enzyme, but mutants which are resistant to inhibitors of reverse transcriptase (e.g., AZT) are inhibited better than wild type.

Nucleosides combine (i) a purine base (e.g., adenine, guanine, hypoxanthine, or a modification thereof) or a pyrimidine base (cytosine, thymine, uracil, or a modification thereof) linked through an N-glycosidic bond to (ii) a 5-carbon cyclic sugar (e.g., 2',3'-dideoxyribosyl, modified 2'-deoxyribosyl missing its 3'-hydroxyl group) or an acyclic version thereof to form nucleosides such as adenosine, guanosine, inosine, cytidine, thymidine, uridine, or derivatives thereof. Homodimeric or heterodimeric dinucleosides may be linked by three to six
phosphates through phosphodiester bonds with at least the most proximal phosphodiester bond (i.e., both or only one of the bonds made between the two nucleosides and the polyphosphate linkage) being cleavable by reverse transcriptase.

At least one of the two nucleosides must act as a chain terminator which does not leave a 3'-hydroxyl end after a nascent DNA chain has been extended by reverse transcriptase. For example, the 3'-hydroxyl of the sugar may be substituted with a hydrogen (-H), azido (-N₃), amino (-NH₂), etc. at the 3' position or replaced with 2',3'-didehydro-2',3'-dideoxyribosyl. An acyclic version of ribosyl may be used as the sugar, but not substitution of arabinosyl for ribosyl. Alternatively, any chain terminator may be used as a nucleoside analogue in synthesizing dinucleoside polyphosphates. But it is preferred that both nucleosides contain the same or different chain terminator, and they be linked by four phosphates.

It is preferred that the concentration of each chain-terminating dinucleoside polyphosphate used in this invention be comparable to that of natural 2'-deoxyribonucleotide triphosphates (about 5 µM). Thus, compounds may be added or increased from 1 nM to 20 µM such as at least about 1 nM, at least about 10 nM, at least about 100 nM, at least about 500 nM, at least about 1 µM, or at least about 10 µM.

The invention also provides processes for synthesizing different dinucleoside polyphosphate compounds or derivatives thereof, as well as products made thereby. These products may then be further processing. It should be noted, however, that a claim directed to the product is not necessarily limited to these processes unless the particular steps of the process are recited in the product claim.

Another embodiment of the invention is a process for making a dinucleoside polyphosphate by incubating an unblocked DNA primer, template, and 2',3'-dideoxyribonucleoside triphosphate (ddNTP) complementary to the first single-stranded position on the template; incorporating 2',3'-dideoxyribonucleoside monophosphate (ddNMP) at the 3'-end of the DNA primer with reverse transcriptase (RT) to generate pyrophosphate (PPI) and a blocked DNA primer;
cleaving PPI with pyrophosphatase (PPase); and removing a chain-terminating nucleoside analogue from the blocked DNA primer with RT to form the dinucleoside polynucleotide and the unblocked DNA primer.

Yet another embodiment of the invention is a composition comprising at least one dinucleoside polynucleotide which is incorporated in a nascent DNA chain by reverse transcriptase and terminates further extension of the nascent DNA chain. Incorporation of a chain terminator by reverse transcriptase shifts the 3'-end of a DNA primer from an unblocked (i.e., extendable) to a blocked (i.e., terminated) state; the number of blocked DNA chains and their rate of production are determined by the parameters of the reverse transcriptase reactions (e.g., concentrations of the nucleotide, chain terminator, and enzyme components).

Further aspects of the invention will be apparent to a person skilled in the art from the following description of specific embodiments and generalizations thereto.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates a schematic for synthesis of a dinucleoside polynucleoside inhibitory compound (ddNp₄ddN). A DNA primer/template (P/T) is incubated with HIV-1 reverse transcriptase (RT) and the ddNTP complementary to the next position on the template and inorganic pyrophosphatase (PPase). After incorporation of the ddNMP the pyrophosphate (PPI) is cleaved to free phosphate by PPase, so the only available acceptor substrate for removal is the ddNTP which results in production in ddNp₄ddN and an extendable P/T, which can be chain terminated again to repeat the cycling reaction. The net effect is conversion of ddNTP starting material to ddNp₄ddN product.

Figure 2 shows the synthesis of four different dinucleoside polynucleoside inhibitory compounds. Micromolar concentrations of one of four different DNA primer/templates (see Examples for sequence) were incubated with HIV-1 RT<sup>MDR</sup>, inorganic pyrophosphatase, and mM concentrations of ddATP (lanes 1 and 5), ddCTP (lanes 2 and 6), ddGTP (lanes 3 and 7), or ddTTP (lanes 4 and 8) for 4 days at 37°C. Following heat inactivation of the reverse transcriptase,
the reaction mixture was incubated with calf intestinal alkaline phosphatase (lanes 5-8).

Figure 3 shows inhibition of DNA-dependent DNA synthesis by either ddGTP or ddGp4ddG. M13 single-stranded DNA annealed to a complementary DNA primer (2.5 nM) was incubated with HIV-1 RT<sup>AZT</sup> (100 nM), 2.5 μM of each dNTP, 50 μCi [α-<sup>32</sup>P]dGTP, and the indicated concentration of ddGTP or ddGp<sub>4</sub>ddG which was either untreated (- CIP) or pretreated with calf intestinal alkaline phosphatase (+ CIP) for 30 min at 37°C. The samples were then separated by electrophoresis through a 20% denaturing polyacrylamide gel.

Figure 4 shows the ability of HIV-1 RT<sup>MDR</sup> to use doubly-labeled Ap<sub>4</sub>ddG (A-P-P<sup>-32</sup>P-P<sup>-33</sup>P-ddG) as a dGTP analogue during DNA-dependent DNA polymerization. Ten μM WL50-33C/L32 primer/template was incubated with 2 μM HIV-1 RT<sup>MDR</sup> and 200 μM Ap<sub>4</sub>ddG (A-P-P<sup>-32</sup>P-P<sup>-33</sup>P-ddG) for the indicated time at 37°C followed by heat inactivation of the reverse transcriptase (5 min at 95°C). Some samples were then treated with 4U of calf alkaline phosphatase (CIP) (lanes marked + CIP) for 30 min followed by heat inactivation (5 min at 95°C) of the CIP. The reaction products were then separated by electrophoresis through a 20% denaturing polyacrylamide gel and the radioactivity was then visualized by phosphorimaging. Standards for free phosphate (P) and pyrophosphate (PPI) were generated by incubating [γ<sup>-32</sup>P]ATP with CIP or snake venom phosphodiesterase (SVPD), respectively.

Figure 5 shows inhibition by either ddTTP or ddT<sub>p</sub>4ddT of DNA-dependent DNA synthesis using RT<sup>WT</sup> or RT<sup>AZT</sup>. M13 single-stranded DNA annealed to a complementary DNA primer (2.5 nM) was incubated with either HIV-1 RT<sup>WT</sup> or HIV-1 RT<sup>AZT</sup> (100 nM), 2.5 μM of each dNTP, 50 μCi [α-<sup>32</sup>P]dTTP, and the indicated concentration of ddTTP or ddT<sub>p</sub>4ddT for 30 min at 37°C. The samples were then separated by electrophoresis through a 20% denaturing polyacrylamide gel.

Figure 6 shows inhibition of DNA-dependent DNA synthesis through incorporation of dDGMP from Ap<sub>4</sub>ddG by RT<sup>WT</sup>, RT<sup>AZT</sup>, or RT<sup>MDR</sup>. (A) M13 single-stranded DNA annealed to a complementary DNA primer (2.5 nM) was incubated with HIV-1 RT<sup>WT</sup> or HIV-1 RT<sup>AZT</sup> (100 nM), 2.5 μM of each dNTP, 50
μCi [α-32P]dGTP and the indicated concentration of Ap4ddG for 30 min at 37°C. The samples were separated by electrophoresis through a 20% denaturing polyacrylamide gel. (B) Percent inhibition of [32P]dGMP incorporation by Ap4ddG was determined as described in Methods. A summary of IC50s derived from several experiments is given in Table 2.

DESCRIPTION OF SPECIFIC EMBODIMENTS OF THE INVENTION

HIV-1 reverse transcriptase (RT) is one of the main targets for antiviral therapy against HIV infection. Although many effective RT inhibitors are available in clinical use, over time, resistance mutations appear in many treated individuals that limit the effectiveness of these drugs. One class of mutations (M41L, D67N, K70R, L210W, T215Y/F, and K219Q) was initially identified as conferring resistance to 3'-azido-2',3'-dideoxythymidine (AZT) (1-3). In recent years, however, it has been shown that these mutations confer resistance to 2',3'-dideoxycytidine (d4T) (4-8), as well as non-thymidine analogues, such as PMPA (9). In combination with a so-called finger insertion mutation, 69S-XX, the AZT resistance mutations confer resistance to most types of nucleoside analogues (10-12).

The molecular mechanism of resistance to AZT is due to an increased ability of the mutant enzyme to remove AZT-monophosphate (AZTMP) from blocked DNA termini (13-16). This unblocking reaction occurs through transfer of the AZTMP moiety from the primer terminus to an acceptor substrate, such as ATP, in a pyrophosphorolysis-like reaction. While removal of other chain terminators is also increased by AZT resistance mutations, the presence of physiological concentrations of dNTPs greatly inhibit removal of these nucleotide analogues (14, 17) by trapping the chain-terminated DNA chain and RT in a so-called dead-end complex (18). But the main effect of the 69S-XX finger insertion mutation seems to be a decreased sensitivity of the removal reaction to inhibition by dNTPs (9, 19, 20). The combination of both AZT resistance mutations and finger insertion mutations would therefore confer increased removal of most nucleotide analogues under physiological conditions, which would explain the resistance profile observed in phenotypic assays.
Two products are formed after nucleotide-dependent removal by reverse transcriptase of the chain terminator from a blocked DNA chain. One is the unblocked DNA chain, shortened by one base, the other is a dinucleoside polyphosphate containing the removed nucleotide analogue linked through its phosphate group to the distal phosphate of the nucleotide acceptor substrate (21). For example, ATP-dependent removal of ddAMP produces the dinucleoside polyphosphate Ap₄ddA. Like any chemical reaction, dinucleoside polyphosphate synthesis by HIV-1 RT should be a reversible reaction. In other words, HIV-1 RT should be able to use dinucleoside polyphosphates containing at least one 2'-deoxy-, or 2',3'-dideoxy nucleoside moiety as a dNTP analogue for DNA synthesis. Using Ap₄ddA as an example, HIV-1 RT could incorporate the ddAMP moiety opposite a template dTMP releasing ATP. The ability of HIV-1 RT to use certain dinucleoside polyphosphates as dNTP analogues for DNA synthesis has been reported (22).

Since HIV-1 RT containing AZT resistance mutations has an increased ability to remove chain terminators from blocked DNA ends through dinucleoside polyphosphate synthesis, it is possible that these enzymes might also use dinucleoside polyphosphates more efficiently than wild-type (WT) RT as dNTP analogues during DNA synthesis. Dinucleoside polyphosphates containing chain terminators might thus inhibit mutant RT more than WT RT.

To test this hypothesis, we synthesized and purified micromolar amounts of the homodimeric dinucleoside polyphosphates ddAp₄ddA, ddCp₄ddC, ddGp₄ddG and ddT₄ddT and tested their ability to inhibit DNA synthesis by WT and several mutants of HIV-1 RT. Compared to inhibition by ddNTPs, WT RT was fairly insensitive to inhibition by ddNp₄ddNs (15- to 74-fold higher IC₅₀ for ddNp₄ddN than for the corresponding ddNTP). In contrast, HIV-1 RT containing the 41L/215Y mutations (RT₄¹/²¹⁵) was 5- to 25-fold less sensitive to inhibition by the various ddNp₄ddNs than to inhibition by the corresponding ddNTPs, HIV-1 RT containing the 67N/70R/215Y/219Q mutations (RT₄¹²¹⁵₄) only 2- to 5-fold less sensitive to inhibition by the various ddNp₄ddNs than to inhibition by the corresponding ddNTPs, and HIV-1 RT containing the 41L/69S-AG/210W/211K/214F/215Y mutations (RT₄¹²¹⁵₄) 1- to 5-fold less sensitive to
inhibition by the various ddNp₄ddNs than to inhibition by the corresponding ddNTPs.

In contrast, mutations K65R and W88G which confer resistance to 2',3'-dideoxyinosine (dDI) (23), 2',3'-dideoxycytidine (ddC) (24), PMPA (25), abacavir (26, 27), 2',3'-dideoxy-3'-thiacytidine (3TC) (28), 2',3'-didehydro-3'-deoxythymidine (d4T) (29) and foscarnet (24, 30) or high-level resistance to foscarnet (30-32), respectively, and also confer a decrease in PPI-and nucleotide-dependent removal of chain terminators from blocked DNA ends (33, 34) conferred a decreased sensitivity to inhibition by either ddNTPs or ddNp₄ddNs, compared to WT enzyme.

It is encouraging for development of novel HIV-1 inhibitors based on these compounds that extension of a nascent DNA chain by HIV-1 RT containing an AZT-resistance mutation(s) is more sensitive to inhibition by dinucleoside polyphosphates than WT RT. These novel drugs would have the potential to at least delay or even prevent the appearance of AZT-resistance mutations or to suppress their phenotype if already present in HIV-infected individuals. Thus, combination therapy of dinucleoside polyphosphate and AZT may be practiced in situations where AZT could not be used.

A screening method may comprise administering a candidate compound to an individual, introducing a candidate compound into a cell, or incubation of a candidate compound in a cell-free DNA primer/template assay and then determining whether or not an activity of reverse transcriptase is inhibited, a decrease in unblocked chain-terminated nascent DNA chains, an increase in blocked chain-terminated nascent DNA chains, or any combination thereof. A candidate compound which is selected by such a method can potentially be used in the invention to treat an individual, inhibit reverse transcriptase-mediated DNA synthesis, prevent or at least slow virus growth, inhibit or at least reduce acquisition by the virus of resistance mutations, or any combination thereof.

Dinucleoside polyphosphate compounds may be used as a medicament or used to formulate a pharmaceutical composition with one or more of the utilities disclosed herein. For example, the pharmaceutical composition may be
administered to an individual as a formulation which is adapted for passage through the gut or blood with components known to enhance uptake by cells or tissue. Alternatively, the pharmaceutical composition may be added to culture medium. It may be administered in vitro to cells in culture, in vivo to cells in the body, or ex vivo to cells outside of an individual which may then be returned to the body of the same individual or another. The composition may be administered in a single dose or in multiple doses which are administered at different times.

Dinucleoside polyphosphate compounds may be used in the treatment of an individual in need thereof: e.g., therapy of existing viral disease in infected individuals or prophylaxis in individuals at risk thereof. The amount of the dinucleoside polyphosphate compound(s) that is administered to a subject in need of treatment, its formulation, and the timing and route of delivery is effective to at least reduce viral infection; surrogate markers thereof (e.g., immunocompetence as assayed by the number of CD4+ T cells or viral load as assayed by the number of viral RNAs); or symptoms of viral infection (e.g., lymphadenopathy, malignancies, opportunistic infections). Determination of such amounts, formulations, and timing and route of delivery is within the skill in the art.

Suitable choices in amounts and timing of doses, formulation, and routes of administration can be made with the goals of achieving a favorable response in the subject (i.e., efficacy), and avoiding undue toxicity or other harm thereto (i.e., safety). Therefore, “effective” refers to such choices that involve routine manipulation of conditions to achieve a desired effect: e.g., decrease in unblocked chain-terminated nascent DNA chains, increase in blocked chain-terminated nascent DNA chains, reduction in viral load, improvement in immunocompetence, reduction in symptoms of viral disease, or any combination thereof. An effective amount or concentration is achieved when there is at least some inhibition of reverse transcriptase or the aforementioned desired effects in an individual.

The amount of dinucleoside polyphosphate compound administered to an individual may be dependent upon factors such as bioactivity and bioavailability of the compound (e.g., half-life in the body, stability, and metabolism);
chemical properties of the compound (e.g., molecular weight, hydrophobicity, and solubility); route and scheduling of administration; and the like. It will also be understood that the specific dose level to be achieved for any particular subject may depend on a variety of factors, including age, gender, health, medical history, weight, combination therapy with one or more other antiviral agents, and severity of disease.

The composition may be formulated as a liquid for injection; tablet, capsule, or syrup for ingestion; or a micronized powder for inhalation. It may be enterally (e.g., oral) or parenterally (e.g., intravenous, intradermal, subcutaneous, or transmucosal) administered to the individual (e.g., a human patient or an animal model of disease such as an SIV-infected primate). In particular, a composition may include a single or multiple different dinucleoside polyphosphates (e.g., analogues of the four natural dinucleoside polyphosphates) in a suitable carrier or vehicle. It may be adapted for pharmaceutical use by including inactive components (e.g., USP sterile water or water for injection, cyclodextrin or other polymer, liposomes, physiological buffer and salts, excipients, preservative, stabilizers), excluding infective agents by sterilization and depyrogenation (e.g., endotoxin-free), manufacturing in a GMP facility, aseptic packaging/storing the final product, and transporting under stable conditions.

Neutral and charged lipids, sterols, and other phospholipids are known to make lipid carriers that may facilitate a cell's uptake of dinucleoside polyphosphate compounds. For example, neutral lipids are dioleoyl phosphatidylcholine (DOPC) and dioleoyl phosphatidyl ethanolamine (DOPE); an anionic lipid is dioleoyl phosphatidyl serine (DOPS); cationic lipids are dioleoyl trimethyl ammonium propane (DOTAP), dioctadecyldiamidoglycyl spermine (DOGS), dioleoyl trimethyl ammonium (DOTMA), and 1,3-dioleoyloxy-2-(6-carboxyspermyl)-propylamide tetraacetate (DOSPER). Polymers such as cationic dendrimers, polyamides, polyamidoamines, polyethylene, polypropylene glycols (PEG), polyethylenimines (PEI), or any combination thereof may be used to facilitate a cell's uptake of a dinucleoside polyphosphate compound.
Optionally, the composition may include one or more other antiviral agents: e.g., nucleoside reverse transcriptase inhibitors (NRTI like abacavir, didanosine, emtricitabine, lamivudine, stavudine, tenofovir, zalcitabine, and zidovudine); non-nucleoside reverse transcriptase inhibitors (NNRTI like delavirdine, efavirenz, and nevirapine), protease inhibitors (PI like amprenavir, atazanavir, fosamprenavir, indinavir, lopinavir, nelfinavir, ritonavir, and saquinavir), fusion inhibitors (FI such as enfuvirtide or T20 peptide), and any combination thereof. Presently recommended combination treatments are (1) efavirenz, emtricitabine or lamivudine, and tenofovir or zidovudine; (2) lopinavir, ritonavir, emtricitabine or lamivudine, and zidovudine; and (3) abacavir, lamivudine, and zidovudine. Alternatively, dinucleoside polyphosphate compounds may be separately administered before or after HAART (e.g., separated by one week to one month).

Treatment-naïve infected individuals would benefit from the antiviral effects of dinucleoside polyphosphate compounds as well as the protection they would provide against development of resistance mutations to conventional nucleoside RT inhibitors used in their treatment regimen. The dinucleoside polyphosphate compounds might be even more appealing for use in treatment-experienced or failing virally-infected individuals by possibly suppressing the resistant phenotype and thereby enhancing the antiviral effect of nucleoside analogues in these individuals who might have run out of treatment options.

EXAMPLES

Expression and Purification of HIV-1 RT. His-tagged HIV-1 RT of the wild-type (RT\textsuperscript{WT}), K65R mutant (RT\textsuperscript{K65}), W88G mutant (RT\textsuperscript{W88}), M41L/T215Y mutant (RT\textsuperscript{M41/T215}), D67N/K70R/T215Y/K219Q mutant (RT\textsuperscript{A2T}), and M41L/T69S-insertionAG/L210W/R211K/L214F,T215Y/K219Q mutant (RT\textsuperscript{MDR}) sequences were expressed and purified as previously described (35). The specific RNA-dependent DNA polymerase activities of HIV-1 RT\textsuperscript{WT}, RT\textsuperscript{K65}, RT\textsuperscript{W88}, HIV-1 RT\textsuperscript{M41/T215}, HIV-1 RT\textsuperscript{A2T}, and HIV-1 RT\textsuperscript{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]dTMP in 10 min when using poly(rA)/oligo(dT) (Amersham Biosciences) as the substrate (36). These specific activities are consistent with values reported elsewhere in the literature (36-45) and correspond to turnover numbers of 9 to 16 s⁻¹ for RTWT, 4 to 7 for RT^{MDR}, 5 to 9 for RT^{88}, 7 to 12 s⁻¹ for RT^{41/218}, 2 to 3 s⁻¹ for RT^{AZT} and 7 to 12 s⁻¹ for RT^{MDR}, and assuming that 50-90% of the enzyme molecules are active for each preparation. Oligonucleotides were obtained from Sigma Genosys; M13 DNA from New England Biolabs. [α-32P]dNTPs and [γ-32P]ATP were obtained from PerkinElmer; [α-33P]ddNTPs from Amersham Biosciences.

Synthesis and purification of homodimeric dinucleoside polyphosphates. Ten μM of L32 primer (5'-CTACTAGTTTATCCTCCATCTAGACGATACAGA-3'; SEQ ID NO:1) annealed to WL50 template (5'-GAGTGCTGAGGTCTTCTGTTATCGTCTAGATGGAGA AAAACTAGTAG-3'; SEQ ID NO:2), WL50-33G template (5'-GAGTGCTGAGGTCTTCTGTTATCGTCTAGATGGAGAA AACTAGTAG-3'; SEQ ID NO:3), WL50-33C template (5'-GAGTGCTGAGGTCTCCTAGATGGAGA AACTAGTAG-3'; SEQ ID NO:4), or WL50-33A-44T template (5'-GAGTGCTGAGGTCTTCTAATCTGGTGATCGTCTA GATGGAGAAACTAGTAG-3'; SEQ ID NO:5) was incubated with 1 μM HIV-1 RT^{MDR} and 3.2 mM ddATP and 10 μCi [α-32P]ddATP, 3.2 mM ddCTP and 10 μCi [α-33P]ddCTP, 3.2 mM ddGTP and 10 μCi [α-33P]ddGTP or 3.2 mM ddTTP and 10 μCi [α-33P]ddTTP, respectively (pretreated with thermostable pyrophosphatase as previously described (35)) in 1000 μl reaction buffer (40 mM NaHEPES, pH 7.5, 20 mM MgCl₂, 60 mM KCl, 1 mM dithiothreitol, 2.5% glycerol) containing 160 μg/ml of bovine serum albumin and 0.0005 U/μl of inorganic pyrophosphatase (Roche). The mixture was overlaid with 200 μl 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 hr, addition of 100 U CIP and incubation at 37°C for an additional 2 hr at 37°C followed by heat inactivation of the CIP 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
(TEAB) pH 8.5 (Sigma) and eluted with a gradient of TEAB 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 first incubating the dinucleoside polyphosphate with snake venom phosphodiesterase to cleave it into free nucleotides followed by spectrophotometric determination of the concentration of nucleotide. Once the concentration of the nucleotide was determined, the wavelength of maximal absorption ($\lambda_{max}$) at pH 7.0 and molar absorbency ($A_m$) could be determined for each dinucleoside polyphosphate. ddAp$_4$ddA had a $\lambda_{max}$ (pH 7.0) of 259 nm and an molar absorbency ($A_m$) of $27.9 \times 10^3$, ddCp$_4$ddC had a $\lambda_{max}$ (pH 7.0) of 270 nm and an molar absorbency ($A_m$) of $25.9 \times 10^3$, ddGp$_4$ddG had a $\lambda_{max}$ (pH 7.0) of 252 nm and an molar absorbency ($A_m$) of $21.0 \times 10^3$ and ddTp$_4$ddT had a $\lambda_{max}$ (pH 7.0) of 267 nm and an molar absorbency ($A_m$) of $18.2 \times 10^3$. The synthesis and purification of the heterodimeric dinucleoside polyphosphate Ap$_4$ddG, doubly-labeled with $^{32}$P and $^{33}$P (A-P-P-$^{32}$P-$^{33}$P-ddG) was identical to the synthesis of ddGp$_4$ddG except for the following modifications; 10 μM L32 primer annealed to WL50-33C template was incubated with 1 μM HIV-1 RT$^{MDR}$ with 10 mM ATP, 20 μCi [γ-$^{32}$P]ATP, 1 mM ddGTP and 10 μCi [α-$^{33}$P]ddGTP. Following anion exchange chromatography, Ap$_4$ddG was separated from ddGp$_4$ddG by reverse phase HPLC using a C18 column (Amersham Biosciences). The syntheses of $2'(3')$-O-(N-methylanthraniloyl)-(MANT)-Ap$_4$ddG and AppNHppddG were the same as the synthesis of Ap$_4$ddG except that MANT-ATP (Jena Bioscience) or AppNHp (Jena Bioscience), respectively, were included in the reaction instead of ATP.

Inhibition of DNA polymerization by ddNTPs or ddNp$_4$ddNs. M13 single-stranded DNA (New England Biolabs) annealed to a complementary DNA primer (5'-AAGTTGGTAACGCCAGGGTTTCCCATCAGAC-3'; SEQ ID NO:6) (2.5 nM) was incubated with excess HIV-1 RT (100 nM) in 20 μl reaction buffer containing 80 μg/ml of bovine serum albumin, 2.5 μM of each dNTP and 20 μCi of [α-$^{32}$P]dATP, [α-$^{32}$P]dCTP, [α-$^{32}$P]dGTP, or [α-$^{32}$P]dTTP in the absence or presence of varying concentrations of ddATP or ddAp$_4$ddA; ddCTP or ddCp$_4$ddC; ddGTP or ddGp$_4$ddG; or ddTTP or ddTp$_4$ddT, respectively, at
37°C for 30 min. The reaction was terminated by heating at 90°C for 5 min. Five μl of reaction mixture was mixed with 5 μl gel loading dye and then 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 quantitation of incorporated radioactive nucleotide, the remaining 15 μl 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% TCA containing 20 mM sodium pyrophosphate followed by two 5 min washes in ethanol at 4°C. The precipitable radioactivity in each sample was quantitated by scintillation counting. The amount of inhibition of DNA synthesis was plotted versus inhibitor concentration using Sigmaplot 8.0 to obtain the apparent IC_{50} (IC_{50APP}). The IC_{50APP} is a relative number that depends on many factors in addition to the ability of the enzyme to discriminate between incorporation of a chain terminator versus incorporation of natural nucleotides, such as the concentration of dNTPs used (the higher the dNTP levels, the higher the IC_{50APP} due to competition between incorporation of the nucleotide analogue and the dNTP) as well as the average number of nucleotides incorporated in the absence of inhibitor, i.e., the activity of the enzyme (the higher the average number of incorporated nucleotides in the absence of inhibitor the lower the IC_{50APP} since each chain-termination event will result in a loss of a higher number of potential incorporation events (46)). In order to compare IC_{50S} of different enzymes we therefore normalized the IC_{50APPs} to the relative activity of each enzyme compared to the activity of HIV-1 RT^{WT} (IC_{50} = IC_{50APP} x Activity of HIV-1 RT/ Activity of HIV-1 RT^{WT}).

Synthesis and Purification of Homodimeric Dinucleoside Polyphosphates Containing 2',3'-Dideoxyribonucleosides.

A scheme for synthesis of dinucleoside polyphosphates is illustrated in Fig. 1. Micromolar concentrations of a DNA primer/template (P/T) are incubated with mM concentrations of the 2',3'-dideoxyribonucleoside triphosphate (ddNTP), a small amount of the [α-32P]ddNTP complementary to the first single-stranded position on the template, micromolar concentrations of HIV-1 RT^{MDR},
and inorganic pyrophosphatase (PPase). HIV-1 RT<sup>MDR</sup> contains M41L/T69S-insertionAG/L210W/R211K/L214F/T215Y/K219Q mutations and has a high level of ATP-dependent removal activity (20). Incorporation of the 2',3'-dideoxyribonucleoside monophosphate into the primer terminus leads to chain termination. Since the pyrophosphate (PPI) generated by the incorporation is cleaved by pyrophosphatase, the only substrate for removal of the chain terminator from the blocked DNA end is the ddNTP, leading to formation of the dinucleoside polyphosphate ddNp₄ddN and an unblocked P/T. Over time, this reaction converts some of the ddNTP into ddNp₄ddN, as seen in Fig. 2. Since there are no exposed phosphate groups on dinucleoside polyphosphates, the compounds are resistant to treatment with calf intestinal alkaline phosphatase (CIP) while the remaining ddNTP is sensitive to CIP treatment (Fig. 2). After CIP treatment, the ddNp₄ddN was separated from free phosphate and 2',3'- dideoxyribonucleoside by anion-exchange chromatography. They may be produced in nanomole quantities. Alternatively, heterodimers can be synthesized by including a different 2',3'- dideoxyribonucleoside triphosphate to the incubation and then separating homodimers from heterodimers.

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 measured the ability of these compounds, compared to ddNTPs, to inhibit DNA-dependent DNA synthesis using M13 P/T as 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 portion 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 to allow quantitation of incorporated radioactivity. Incorporation of ddNMP from either ddNTP or ddNp₄ddN would result in chain termination which would lead to a decrease in the total amount of incorporated radioactive nucleotide and the appearance of chain-terminated
products. Fig. 3 shows the ability of either ddGTP or ddGp$_4$ddG to inhibit DNA polymerization by HIV-1 RT$^{AZT}$. In the absence of inhibitor (lane 1), the large majority of M13 primers are 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 2-5), there is a concomitant decrease in the amount of total incorporated radioactive label and a decrease in the average length of the extended products which are now chain terminated at G-incorporation sites. A similar pattern is observed at increasing concentrations of ddGp$_4$ddG (lanes 6-9), although the enzyme seems to be slightly less sensitive to inhibition by ddGp$_4$ddG than to inhibition by ddGTP. Since it was possible that the inhibition observed by ddGp$_4$ddG was due to contaminating ddGTP, we also tested the ability of ddGTP and ddGp$_4$ddG that had been pretreated with CIP to inhibit DNA polymerization. As can be seen in Fig. 3, pretreatment of ddGTP with CIP abolished its inhibitory activity (lanes 10-13), while pretreated ddGp$_4$ddG still retained its ability to inhibit DNA synthesis by HIV-1 RT$^{AZT}$ (lanes 14-17). Therefore, HIV-1 RT can use dinucleoside polyphosphates as dNTP analogues during DNA synthesis, in agreement with previously reported results (22).

There are two possible reaction mechanisms, a direct and an indirect one, for the ability of HIV-1 RT to use dinucleoside polyphosphates as dNTP analogues. In the direct mechanism, the dinucleoside tetraphosphate itself serves as the dNTP analogue. After incorporation of a nucleotide monophosphate moiety from the dinucleoside tetraphosphate into the primer terminus this pathway would yield one nucleotide triphosphate. In the indirect pathway the RT first cleaves the dinucleoside tetraphosphate into a nucleoside monophosphate and a nucleoside triphosphate, and subsequently uses the nucleoside triphosphate as the dNTP analogue. This pathway would yield one nucleotide monophosphate and one pyrophosphate (PPi) for each incorporation event. To distinguish between these two possibilities, the ability of HIV-1 RT$^{AZT}$ to use doubly labeled Ap$_4$ddG (A-P-P$^{32P}$.P$^{33P}$-ddG) as a dGTP analogue was measured. As can be seen in Fig. 4, increased incubation times lead to an increased amount of the labeled product L32-ddGM$^{33P}$, indicating that Ap$_4$ddG
can serve as a dGTP analogue. Concomitant with the increase in L32-ddGM\(^{32}\)P was an increase in \([\gamma^{32}\text{P}]\text{ATP} \text{ and, importantly, no increase in}\ PPI. The absence of PPI clearly indicates that ddGMP is incorporated into the primer terminus by HIV-1 RT directly from Ap\(_4\)ddG. In addition, a similar incorporation efficiency of ddGMP from Ap\(_4\)ddG was observed when the reaction was performed in the presence of CIP indicating that no CIP-sensitive intermediate was formed during the reaction further supporting the direct incorporation mechanism.

Next, we compared the sensitivity of DNA polymerization by WT and mutant RTs to inhibition by ddNTPs or ddNp\(_4\)ddNs. As can be seen in Fig. 5, increasing concentrations of ddTTP led to a concomitant decrease in the amount of incorporated \([^{32}\text{P}]\text{dTMP}\). HIV-1 RT\(^{WT}\) and RT\(^{AZT}\) appear to be approximately equally sensitive to inhibition by ddTTP. DNA polymerization by HIV-1 RT\(^{AZT}\), however, seems more sensitive to inhibition by ddTp\(_4\)ddT than is DNA polymerization by HIV-1 RT\(^{WT}\). Experiments similar to that shown in Fig. 5 were conducted and the degree of inhibition of incorporated, labeled nucleotide by ddNTPs or ddNp\(_4\)ddNs was quantitated in order to obtain the sensitivity (IC\(_{50}\)) of DNA polymerization by either WT or mutant RTs to ddATP or ddAp\(_4\)ddA (Table 1, panel A); ddCTP or ddCP\(_4\)ddC (Table 1, panel B); ddGTP or ddGp\(_4\)ddG (Table 1, panel C); or ddTTP or ddTp\(_4\)ddT (Table 1, panel D)).

HIV-1 RT\(^{WT}\) was 15- to 74-fold less sensitive to inhibition by ddNp\(_4\)ddNs than to inhibition by the corresponding ddNTP. Of the dinucleoside polyphosphates tested, the enzyme was the most sensitive to inhibition by ddCP\(_4\)ddC and ddGp\(_4\)ddG (IC\(_{50}\)s \text{~} 2 \mu M) and the least sensitive to inhibition by ddTp\(_4\)ddT (IC\(_{50}\) = 12 \mu M).

HIV-1 RT\(^{35}\) was also much more (19- to 64-fold) sensitive to inhibition by ddNTPs than inhibition by ddNp\(_4\)ddNs and more sensitive to inhibition by ddCP\(_4\)ddC (IC\(_{50}\) = 11 \mu M) and ddGp\(_4\)ddG (9.5 \mu M) than to inhibition by ddTp\(_4\)ddT (IC\(_{50}\) = 42 \mu M) or ddAp\(_4\)ddA (IC\(_{50}\) > 36 \mu M). Compared to RT\(^{WT}\), DNA polymerization by RT\(^{35}\) was 4- to 9-fold less sensitive to inhibition by ddNTPs and 5- to 7-fold less sensitive to inhibition by ddNp\(_4\)ddNs.
DNA polymerization by HIV-1 RT\textsuperscript{88} was 25- to 88-fold less sensitive to inhibition by ddNp\textsubscript{4}ddN than to inhibition by the corresponding ddNTP. Of the dinucleoside polyphosphates tested, it was more sensitive to inhibition by ddCp\textsubscript{4}ddC (IC\textsubscript{50} = 9.2 µM) and ddGp\textsubscript{4}ddG (IC\textsubscript{50} = 6.0 µM) than to inhibition by ddTp\textsubscript{4}ddT (IC\textsubscript{50} = 34 µM) or ddAp\textsubscript{4}ddA (IC\textsubscript{50} > 15 µM). HIV-1 RT\textsuperscript{88} was 1.8- to 4.8-fold less sensitive to inhibition by ddNTPs, and 3.5- to 5.1-fold less sensitive to inhibition by ddNp\textsubscript{4}ddNs than was RT\textsuperscript{WT}.

Compared to RT\textsuperscript{WT}, RT\textsuperscript{85} and RT\textsuperscript{88}, the enzymes containing AZT resistance mutations, RT\textsuperscript{41/215}, RT\textsuperscript{AZT} and RT\textsuperscript{MDR}, were relatively more sensitive to inhibition by ddNp\textsubscript{4}ddNs. HIV-1 RT\textsuperscript{41/215} was only 12- to 15-fold less sensitive to inhibition by ddNp\textsubscript{4}ddN than by the corresponding ddNTP. As had been observed with the other enzymes, HIV-1 RT\textsuperscript{41/215} was also more sensitive to inhibition by ddCp\textsubscript{4}ddC (IC\textsubscript{50} = 1.1 µM) and ddGp\textsubscript{4}ddG (0.39 µM) than to inhibition by ddTp\textsubscript{4}ddT (IC\textsubscript{50} = 3.3 µM) or ddAp\textsubscript{4}ddA (IC\textsubscript{50} = 2.9 µM). Compared to RT\textsuperscript{WT}, DNA polymerization by RT\textsuperscript{41/215} was about equally (0.74- to 1.9-fold) sensitive to inhibition by ddNTPs but 1.9- to 4.5-fold more sensitive to inhibition by ddNp\textsubscript{4}ddN.

DNA polymerization by HIV-1 RT\textsuperscript{AZT} was only 1.8- to 4.7-fold less sensitive to inhibition by ddNp\textsubscript{4}ddNs than by the corresponding ddNTP. Of the dinucleoside polyphosphates tested, the enzyme was most sensitive to inhibition by ddCp\textsubscript{4}ddC (IC\textsubscript{50} = 0.18 µM) or ddGp\textsubscript{4}ddG (IC\textsubscript{50} = 0.20 µM) and least sensitive to inhibition by ddTp\textsubscript{4}ddT (IC\textsubscript{50} = 0.92 µM). Compared to RT\textsuperscript{WT}, RT\textsuperscript{AZT} was about equally (0.82- to 1.7-fold) sensitive to inhibition by ddNTPs but 9- to 12-fold more sensitive to inhibition by ddNp\textsubscript{4}ddNs.

DNA polymerization by HIV-1 RT\textsuperscript{MDR} was about as sensitive, or slightly more sensitive (0.95- to 4.8-fold) to inhibition by ddNTPs than to inhibition by ddNp\textsubscript{4}ddNs. Of the dinucleoside polyphosphates tested, it was most sensitive to inhibition by ddGp\textsubscript{4}ddG (IC\textsubscript{50} = 0.20 µM) and least sensitive to inhibition by ddAp\textsubscript{4}ddA (IC\textsubscript{50} = 1.2 µM). Compared to RT\textsuperscript{WT}, RT\textsuperscript{MDR} was 1.5- to 3.2-fold less sensitive to inhibition by ddNTPs, but 7.5- to 35-fold more sensitive to inhibition by ddNp\textsubscript{4}ddNs.
Therefore, HIV-1 RT\textsuperscript{WT}, RT\textsuperscript{85}, and RT\textsuperscript{88} were much more sensitive (15-83 fold) to inhibition by ddNTPs than by ddNp\textsubscript{4}ddNs. RT\textsuperscript{85} and RT\textsuperscript{88} were less sensitive to inhibition by either ddNTPs or ddNp\textsubscript{4}ddNs than RT\textsuperscript{WT}. TAM-containing reverse transcriptases (e.g., RT\textsuperscript{41/215}, RT\textsuperscript{AZT}, and RT\textsuperscript{MDR}) had increased sensitivity to ddNp\textsubscript{4}ddNs compared with RT\textsuperscript{WT} but there was little difference in sensitivity to ddNTPs. The ratio for inhibition of DNA synthesis [IC\textsubscript{50} for ddNp\textsubscript{4}ddN]/[IC\textsubscript{50} for ddNTP] was 15-72 for RT\textsuperscript{WT}, 4.8-15 for RT\textsuperscript{41/215}, 1.8-4.7 for RT\textsuperscript{AZT}, and 0.95-4.8 for RT\textsuperscript{MDR}. Of the dinucleoside tetraphosphates tested, all six enzymes were more sensitive to inhibition by ddCp\textsubscript{4}ddC and ddGp\textsubscript{4}ddG. The IC\textsubscript{50}s for ddTp\textsubscript{4}ddT and ddAp\textsubscript{4}ddA were 1.2- to 8.5-fold higher than for ddCp\textsubscript{4}ddC and ddGp\textsubscript{4}ddG.

We characterized the ability of Ap\textsubscript{4}ddG or Ap\textsubscript{4}ddG-derived compounds containing modifications of the sugar or the phosphate chain to inhibit DNA polymerization (Fig. 6 and Table 2). RT\textsuperscript{MDR} used Ap\textsubscript{4}ddG as a dGTP analogue even more efficiently than ddGp\textsubscript{4}ddG, while RT\textsuperscript{WT} was about 4-fold less sensitive to Ap\textsubscript{4}ddG than to ddGp\textsubscript{4}ddG resulting in a 50-fold higher sensitivity of DNA polymerization by RT\textsuperscript{MDR} than polymerization by RT\textsuperscript{WT} to inhibition by Ap\textsubscript{4}ddG (Fig. 6, Tables 1 and 2). With MANT-Ap\textsubscript{4}ddG as inhibitor, the difference in sensitivity between these enzymes was over 100-fold. DNA polymerization by RT\textsuperscript{MDR} was also sensitive to inhibition by AppNHppddG (IC\textsubscript{50} = 1.7 µM), a compound to which all other enzymes tested were highly resistant (IC\textsubscript{50}s ≥ 28 µM).

In summary, TAMs and the T69S-XX insertion mutation, which confer an increase in ATP-dependent removal of nucleotide analogues from blocked DNA ends, conferred a greater ability of the mutant RTs, than of WT RT, to use dinucleoside polyphosphates as dNTP analogues during DNA synthesis which resulted in greater sensitivity of HIV-1 RT containing these mutations than of WT enzyme to inhibition by ddNp\textsubscript{4}ddNs. In contrast, mutations K65R and W88G which are associated with decreased PPI-and nucleotide-dependent removal of chain terminators from blocked DNA ends (33, 34) conferred a decreased sensitivity to inhibition by either ddNTPs or ddNp\textsubscript{4}ddNs, compared to WT enzyme.
Here, we have shown that HIV-1 RT can use dinucleoside tetraphosphates containing 2',3'-dideoxyribonucleoside analogues as substrates for DNA synthesis, resulting in chain termination, and that DNA polymerization by enzymes containing AZT resistance mutations is up to 30-fold more sensitive to inhibition by ddNp₄ddNs than is DNA polymerization by WT enzyme.

These results are consistent with our knowledge of nucleotide-dependent removal by reverse transcriptase of chain terminators from blocked ends. Since HIV-1 RT catalyzes nucleotide-dependent removal of a chain terminator resulting in the production of an unblocked primer/template and a dinucleoside polyphosphate (21), the reverse reaction, that is, incorporation of a dideoxyribo-nucleoside monophosphate from a dinucleoside polyphosphate resulting in chain termination should also be catalyzed by HIV-1 RT. In addition, since the efficiency ($k_{cat}/K_m$) of ATP-dependent removal of ddAMP from blocked DNA ends by HIV-1 RTₐZT and HIV-1 RTₐMDR are increased by 16-fold and 32-fold, respectively, compared to removal by HIV-1 RTₐWT (20), it is perhaps not unexpected that these mutant enzymes can use ddNp₄ddNs more effectively than RTₐWT as dNTP analogues during DNA polymerization, and that therefore DNA polymerization by HIV-1 RTₐZT and HIV-1 RTₐMDR are up to 12-fold and 33-fold, respectively, more sensitive to inhibition by ddNp₄ddNs than is DNA polymerization by RTₐWT.

The idea of using dinucleoside polyphosphates as anti-HIV-1 agents has been proposed by several groups (47, 48). We propose improvements herein to overcome an obstacle presented by charges on dinucleoside polyphosphate compounds, which greatly limits their cellular uptake. Liposomes or other carriers could be formulated with chain-terminating dinucleoside polyphosphates to facilitate their entry into cells and tissues. Secondly, dinucleoside polyphosphates could be modified to decrease their charge and to increase their hydrophobicity. A similar approach has been used to increase the bioavailability of the pyrophosphate analogue phosphonoformic acid (foscarinet) by attaching lipophilic hydrocarbon chains to it (49-51). Similarly, lipophilic hydrocarbon (e.g., acyl) chains could be substituted for one or more hydroxyls
of the polyphosphate linkage between dinucleosides. Such prodrugs would enter the cell and be activated by cellular esterases to be used as chain terminators by reverse transcriptase.

It has also been proposed that modified, non-cleavable, dinucleoside polyphosphates have the potential to act as HIV-1 RT inhibitors (48). The advantage of this type of compound would be that by modifying the polyphosphate chain the cellular uptake could be greatly improved. The drawback would be that the compounds could no longer cause chain termination, but would instead act as reversible RT inhibitors. It is possible that dinucleoside polyphosphate compounds could be made that have high binding affinity for RT, but it would seem that the optimal inhibitor of RT, based on our study of dinucleoside polyphosphates, should have high affinity binding, be incorporated efficiently leading to chain termination, and contain a chain terminator that is removed inefficiently from the blocked DNA chain.

A major advantage of inhibitors based on dinucleoside polyphosphates is their potential to be more potent against HIV-1 harboring AZT resistance mutations than against WT. Our results clearly indicate that DNA polymerization by HIV-1 RT containing AZT resistance mutations, either in the absence or presence of the 69S-XX finger insertion mutations, is more sensitive to inhibition by ddNp4ddNs than is polymerization by WT RT. It is important, however, to realize that under physiological conditions (i.e. mM concentrations of dNTPs), the AZT-resistant RT mutants would also remove the incorporated chain terminator much more efficiently than WT RT. The overall effect should therefore be an increased number of incorporated chain terminators, from ddNp4ddNs, and increased number of removed chain terminators from blocked DNA ends by the mutant enzymes compared to WT RT. The net effect should be that the mutants would be slightly more sensitive to inhibition by ddNp4ddNs than WT RT, since each incorporation/removal event would interrupt DNA polymerization. Of the dinucleoside polyphosphates tested in our studies, ddGp4ddG and ddCp4ddC were more efficient than ddAp4ddA and ddTp4ddT against all enzymes. This suggests that the ability to form a G-C base pair greatly improves the ability of RT to use dinucleoside polyphosphates as dNTP
analogues, suggesting that dinucleoside polyphosphates containing cytosine or guanosine analogues might be more efficient against virus than compounds containing adenosine or thymidine analogues. As mentioned earlier, the efficacy of dinucleoside polyphosphates as inhibitors against both WT and mutant RTs would also be increased if they contained nucleoside analogues that would be inefficiently removed, either directly or through the ability to easily form a dead-end complex with the next complementary dNTP.

Incorporation of ddGMP or ddCMP using ddGp₄ddG or ddCp₄ddC as 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 RT⁰⁻¹ and RT⁰⁺ was greatest when MANT-Ap₄ddG was used as substrate suggesting that RT⁰⁻¹ is better able to accommodate a bulky substituent on the ribose than is RT⁰⁺. AppNHppddG was a poor substrate for chain termination with all of the enzymes tested suggesting that the precise structure of the phosphate chain is important.

It may be possible to take advantage of these results 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 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 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. Either or both of these disadvantages may be addressed by adding modifying groups to the phosphate residues (52-54). On the other hand, compounds may be identified with favorable uptake and stability characteristics that have high affinity for the Np₄ddN-binding surface but that are not
substrates for chain termination. Such compounds might, nonetheless, be effective inhibitors of the mutant RT.

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 provides 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.

In conclusion, dinucleoside polyphosphates containing nucleoside analogues have great promise as novel viral inhibitors. If the bioavailability of the compounds could be improved, nucleoside analogues could be administered as dinucleoside polyphosphates, instead of nucleosides. This would have several advantages; it would bypass the need for phosphorylation, which could improve the efficacy of poorly phosphorylated compounds and could improve their efficacy against AZT resistant mutants, as previously mentioned. AZT resistance mutations confer resistance to many of the nucleoside analogues currently in clinical use. By using compounds that are at least as efficient against mutant virus as against WT virus, it might be possible to delay, or perhaps even prevent, the appearance of AZT resistance mutations in treatment of naïve HIV-1 infected individuals and suppress the AZT-resistant phenotype in individuals harboring AZT-resistant virus. By including RT inhibitors derived from dinucleoside polyphosphates in the treatment regimen against virus, it might therefore be feasible to enhance both the efficacy and durability of chain-terminating nucleoside analogues.

The patents, patent applications, books, and other publications cited herein (including the following) are incorporated by reference in their entirety.

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An "isolated" chemical compound (e.g., a dinucleoside polyphosphate or derivative thereof) is at least partially purified from a cell or tissue (e.g., sub-cellular fraction) or a chemical or enzymatic synthesis (e.g., in vitro reactions). For example, the isolated chemical compound may be at least about 90%, at least about 95%, or at least about 99% of the total weight of nucleosides and/or at least partially purified from other chemically-similar solutes (e.g., other types of nucleoside or non-nucleoside analogues). Purification may be achieved by biochemical techniques such as chromatography or crystallization; in vitro synthesis may require only a minimum of purification if the yield of the reactions is great. As a general matter, solvent (e.g., water) and functionally inert chemicals (e.g., salts and buffers) are disregarded when determining purity. A pharmaceutical composition may include inactive components such as carriers
(e.g., polymers, liposomes), excipients, preservatives, stabilizers, and vehicles such as those which are known in the art. It will preferably be isolated from any contaminants (e.g., endotoxin, infectious agents) at a level approved by a government regulatory authority and appropriate for human or animal administration.

An “analogue” has a related but different chemical structure as compared to the four natural 2'-deoxyribonucleosides (i.e., adenosine, cytidine, guanosine, and thymidine), and substantially equivalent function (e.g., incorporation and termination) as utilized by reverse transcriptase. It may have a different binding affinity for reverse transcriptase. Analogues of the invention will be incorporated into nascent DNA chains and will prevent further extension by blocking the 3'-end of the nascent DNA chain with a chain terminator.

The term “treatment” refers to, inter alia, reducing or alleviating one or more symptoms of viral disease in an infected individual or reducing incidence of infection in an individual at risk thereof (e.g., known exposure to an infectious agent). For a given individual, improvement in a symptom, its worsening, regression, or progression may be determined by an objective or subjective measure. Surrogate markers such as immunocompetence (e.g., counting the number of CD4+ T cells) or viral load (e.g., counting the number of viral RNAs) may be assessed for their improvement or lack of disease progression. Treatment may also involve combination with other existing modes of treatment and antiviral agents. Thus, combination treatment may be practiced.

In stating a numerical range, it should be understood that all values within the range are also described (e.g., between two and seven also includes every integer value from three to six as well as all intermediate ranges such as four to five). The term “about” may refer to the statistical uncertainty associated with a measurement or the variability in a numerical quantity which a person skilled in the art would understand does not affect operation of the invention or its patentability.

All modifications and substitutions that come within the meaning of the claims and the range of their legal equivalents are to be embraced within their scope. A claim using the transition “comprising” allows the inclusion of other
elements to be within the scope of the claim; the invention is also described by such claims using the transitional phrase “consisting essentially of” (i.e., allowing the inclusion of other elements to be within the scope of the claim if they do not materially affect operation of the invention) and the transition “consisting” (i.e., allowing only the elements listed in the claim other than impurities or inconsequential activities which are ordinarily associated with the invention) instead of the “comprising” term. Any of these three transitions can be used to claim the invention.

It should be understood that an element described in this specification should not be construed as a limitation of the claimed invention unless it is explicitly recited in the claims. Thus, the granted claims are the basis for determining the scope of legal protection instead of a limitation from the specification which is read into the claims. In contradistinction, the prior art is explicitly excluded from the invention to the extent of specific embodiments that would anticipate the claimed invention or destroy novelty.

Moreover, no particular relationship between or among limitations of a claim is intended unless such relationship is explicitly recited in the claim (e.g., the arrangement of components in a product claim or order of steps in a method claim is not a limitation of the claim unless explicitly stated to be so). All possible combinations and permutations of individual elements disclosed herein are considered to be aspects of the invention. Similarly, generalizations of the invention’s description are considered to be part of the invention.

From the foregoing, it would be apparent to a person of skill in this art that the invention can be embodied in other specific forms without departing from its spirit or essential characteristics. The described embodiments should be considered only as illustrative, not restrictive, because the scope of the legal protection provided for the invention will be indicated by the appended claims rather than by this specification.
Table 1. Inhibition of HIV-1 RT-mediated DNA-dependent DNA synthesis by (A) ddATP or ddAp₄ddA, (B) ddCTP or ddCp₄ddC, (C) ddGTP or ddGp₄ddG, and (D) ddTTP or ddTp₄ddT.

### A

<table>
<thead>
<tr>
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<th>IC₅₀ ddATP (µM)</th>
<th>IC₅₀ ddAp₄ddA (µM)</th>
<th>Relative IC₅₀ ddAp₄ddA/ddATP</th>
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<tbody>
<tr>
<td>RT&lt;sup&gt;WT&lt;/sup&gt;</td>
<td>0.11 ± 0.008</td>
<td>6.5 ± 0.38</td>
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<td>RT&lt;sup&gt;65&lt;/sup&gt;</td>
<td>0.59 ± 0.094</td>
<td>&gt; 36</td>
<td>&gt; 61</td>
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<tr>
<td>RT&lt;sup&gt;88&lt;/sup&gt;</td>
<td>0.31 ± 0.13</td>
<td>&gt; 15</td>
<td>&gt; 48</td>
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<tr>
<td>RT&lt;sup&gt;41/215&lt;/sup&gt;</td>
<td>0.22 ± 0.034</td>
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<td>13</td>
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<tr>
<td>RT&lt;sup&gt;AZT&lt;/sup&gt;</td>
<td>0.16 ± 0.014</td>
<td>0.65 ± 0.12</td>
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<tr>
<td>RT&lt;sup&gt;MDR&lt;/sup&gt;</td>
<td>0.25 ± 0.025</td>
<td>1.2 ± 0.18</td>
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### B

<table>
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<tr>
<th></th>
<th>IC₅₀ ddCTP (µM)</th>
<th>IC₅₀ ddCp₄ddC (µM)</th>
<th>Relative IC₅₀ ddCp₄ddC/ddCTP</th>
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<td>RT&lt;sup&gt;WT&lt;/sup&gt;</td>
<td>0.079 ± 0.0092</td>
<td>2.1 ± 0.35</td>
<td>27</td>
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<tr>
<td>RT&lt;sup&gt;65&lt;/sup&gt;</td>
<td>0.44 ± 0.045</td>
<td>11 ± 1.6</td>
<td>25</td>
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<tr>
<td>RT&lt;sup&gt;88&lt;/sup&gt;</td>
<td>0.28 ± 0.056</td>
<td>9.2 ± 1.2</td>
<td>33</td>
</tr>
<tr>
<td>RT&lt;sup&gt;41/215&lt;/sup&gt;</td>
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<td>1.1 ± 0.086</td>
<td>12</td>
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<tr>
<td>RT&lt;sup&gt;AZT&lt;/sup&gt;</td>
<td>0.10 ± 0.0082</td>
<td>0.18 ± 0.022</td>
<td>1.8</td>
</tr>
<tr>
<td>RT&lt;sup&gt;MDR&lt;/sup&gt;</td>
<td>0.12 ± 0.010</td>
<td>0.28 ± 0.028</td>
<td>2.3</td>
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### C

<table>
<thead>
<tr>
<th></th>
<th>IC$_{50}$ ddGTP (µM)</th>
<th>IC$_{50}$ ddGp$_4$ddCG (µM)</th>
<th>Relative IC$_{50}$ ddGp$_4$ddG/ddGTP</th>
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<tr>
<td>RT$^{WT}$</td>
<td>0.12 ± 0.0055</td>
<td>1.8 ± 0.20</td>
<td>15</td>
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<td>RT$^{65}$</td>
<td>0.49 ± 0.1</td>
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<td>RT$^{88}$</td>
<td>0.24 ± 0.036</td>
<td>6.0 ± 0.92</td>
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<td>RT$^{41/215}$</td>
<td>0.082 ± 0.010</td>
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<td>4.8</td>
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<tr>
<td>RT$^{AZT}$</td>
<td>0.092 ± 0.017</td>
<td>0.20 ± 0.026</td>
<td>2.2</td>
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<tr>
<td>RT$^{MDR}$</td>
<td>0.21 ± 0.050</td>
<td>0.20 ± 0.017</td>
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### D

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<th>IC$_{50}$ ddTTP (µM)</th>
<th>IC$_{50}$ ddTp$_4$ddT (µM)</th>
<th>Relative IC$_{50}$ ddTp$_4$ddT/ddTTP</th>
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<tr>
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<td>0.12 ± 0.018</td>
<td>8.6 ± 2.0</td>
<td>72</td>
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<td>RT$^{65}$</td>
<td>0.66 ± 0.12</td>
<td>42 ± 14</td>
<td>64</td>
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<tr>
<td>RT$^{88}$</td>
<td>0.41 ± 0.046</td>
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Inhibition of DNA polymerization on M13 P/T was measured as described in the legends to Figs. 3 and 5, and then plotted versus the inhibitor concentration.
using Sigmaplot 8.0 to obtain the apparent IC₅₀s. They were normalized to the relative specific activity for each enzyme.

Table 2. Inhibition of HIV-1 RT-mediated DNA-dependent DNA synthesis by Ap₄ddG, MANT-Ap₄ddG, or AppNHppddG.

<table>
<thead>
<tr>
<th></th>
<th>IC₅₀ Ap₄ddG (µM)</th>
<th>IC₅₀ MANT-Ap₄ddG (µM)</th>
<th>IC₅₀ AppNHppddG (µM)</th>
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</thead>
<tbody>
<tr>
<td>RT&lt;sup&gt;WT&lt;/sup&gt;</td>
<td>6.8 ± 0.70</td>
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<td>RT&lt;sup&gt;67/70&lt;/sup&gt;</td>
<td>1.4 ± 0.10</td>
<td>2.5 ± 0.37</td>
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<td>RT&lt;sup&gt;41/215&lt;/sup&gt;</td>
<td>1.95 ± 0.31</td>
<td>3.8 ± 0.26</td>
<td>&gt; 37</td>
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<tr>
<td>RT&lt;sup&gt;AZT&lt;/sup&gt;</td>
<td>0.74 ± 0.049</td>
<td>0.83 ± 0.091</td>
<td>28 ± 8.5</td>
</tr>
<tr>
<td>RT&lt;sup&gt;MDR&lt;/sup&gt;</td>
<td>0.13 ± 0.027</td>
<td>0.13 ± 0.13</td>
<td>1.7 ± 0.58</td>
</tr>
</tbody>
</table>

IC₅₀s were obtained as described in Table 1.
WHAT IS CLAIMED IS:

1. A method of inhibiting reverse transcriptase (RT) in an individual infected by a virus or at risk for such infection, said method comprising:
administering one or more dinucleoside polyphosphates to said individual in an amount sufficient to at least partially inhibit an activity of said reverse transcriptase, wherein a dinucleoside polyphosphate contains one or two chain-terminating nucleosides.

2. A method of treating an individual infected by a virus, said method comprising:
incorporating a dinucleoside polyphosphate into a DNA chain with reverse transcriptase of said virus, wherein a dinucleoside polyphosphate contains one or two chain-terminating nucleosides.

3. A method of inhibiting a reverse transcriptase (RT), said method comprising:
increasing the concentration of one or more nucleoside polyphosphates to an amount which at least partially inhibits an activity of a reverse transcriptase, wherein a dinucleoside polyphosphate contains one or two chain-terminating nucleosides.

4. The method of Claim 3, wherein said concentration is from 1 nM to 20 μM.

5. The method of any one of Claims 1-4, wherein said reverse transcriptase is an HIV-1 reverse transcriptase.

6. The method of Claim 5, wherein said HIV-1 reverse transcriptase contains one or more AZT resistance mutations.
7. The method of any one of Claims 1-6, wherein at least one of the dinucleosides contains 2',3'-dideoxyribosyl.

8. The method of Claim 7, wherein said 2',3'-dideoxyribosyl is linked to a purine or a pyrimidine base.

9. The method of any one of Claims 1-8, wherein the dinucleosides are linked by four phosphates.

10. The method of Claim 9, wherein the polyphosphates are linked through cleavable phosphodiester bonds.

11. The method of any one of Claim 1-10, wherein the dinucleosides are identical.

12. The method of any one of Claim 1-10, wherein the dinucleosides are different.

13. The method of any one of Claims 1-12 further comprising administering one or more anti-viral agents.

14. The method of Claim 13, wherein at least one, two, three or four anti-viral agents are administered and said anti-viral agents are selected from the group consisting of nucleoside RT, non-nucleoside RT, protease, and fusion inhibitors.

15. Use of at least one dinucleoside polyphosphate containing one or two chain-terminating nucleosides in preparation of a medicament for administration to an individual infected by a virus or at risk for such infection.

16. A process of making a dinucleoside polyphosphate, said process comprising:
(a) incubating an unblocked DNA primer, template, and 2',3'-dideoxynucleoside triphosphate (ddNTP) complementary to a first single-stranded position on the template;
(b) incorporating 2',3'-dideoxynucleoside monophosphate (ddNMP) at the 3'-end of the DNA primer with reverse transcriptase (RT) to generate pyrophosphate (PPI) and a blocked DNA primer;
(c) cleaving PPI with pyrophosphatase (PPase); and
(d) removing a chain-terminating nucleoside analogue from the blocked DNA primer with RT to form the dinucleoside polyphosphate and the unblocked DNA primer.

17. A composition comprising at least one dinucleoside polyphosphates which is incorporated in a nascent DNA chain by reverse transcriptase and terminates further extension of the nascent DNA chain.
FIG. 4
<table>
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<110>  Peter R. MEYER
        Walter A. SCOTT

<120>  Dinucleoside Polyphosphate Inhibitors of Reverse Transcriptase

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<150>  US 60/688,332
<151>  2005-06-08

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Artificial Sequence

WL50-33A-44T template

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Artificial Sequence

DNA primer

aagttgggta acgcacagggt tttcccagtc acgac 35