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Toxicity of Nucleoside Analogues Used to Treat AIDS and the Selectivity of the Mitochondrial DNA Polymerase†

Harold Lee,‡ Jeremiah Hanes,‡ and Kenneth A. Johnson*

Institute for Cell and Molecular Biology, Department of Chemistry and Biochemistry, University of Texas at Austin, 2500 Speedway #A4800, Austin, Texas 78712

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ABSTRACT: Incorporation of nucleoside analogues by the mitochondrial DNA polymerase has been implicated as the primary cause underlying many of the toxic side effects of these drugs in HIV therapy. Recent success in reconstituting recombinant human enzyme has afforded a detailed mechanistic analysis of the reactions governing nucleotide selectivity of the polymerase and the proofreading exonuclease. The toxic side effects of nucleoside analogues are correlated with the kinetics of incorporation by the mitochondrial DNA polymerase, varying over 6 orders of magnitude in the sequence zalcitabine (ddC) > didanosine (ddI metabolized to ddA) > stavudine (d4T) ≫ lamivudine (3TC) > tenofovir (PMPA) > zidovudine (AZT) > abacavir (metabolized to carbovir, CBV). In this review, we summarize our current efforts to examine the mechanistic basis for nucleotide selectivity by the mitochondrial DNA polymerase and its role in mitochondrial toxicity of nucleoside analogues used to treat AIDS and other viral infections. We will also discuss the promise and underlying challenges for the development of new analogues with lower toxicity.

In 1992, the Food and Drug Administration (FDA)[1] approved zalcitabine (dideoxycytosine, ddC) for the treatment of AIDS, the first drug to receive approval under the accelerated review process. However, subsequent clinical use revealed severe toxic side effects that limit the widespread use of ddC (*1*, *2*). In a 1993 hepatitis B clinical trial, five patients died from toxic side effects of another analogue, fialuridine (FIAU). Studies following the FIAU clinical trial pointed to mitochondrial poisoning as the basis for the severe toxicity (*3*, *4*). We have now shown that the toxicities of nucleoside analogues are correlated with their kinetics of incorporation by the human mitochondrial DNA (mtDNA) polymerase, and have established an in vitro enzyme assay that can successfully identify those compounds with the highest potential for mitochondrial toxicity on a quantitative scale (*5*). New insights gained by analysis of the structure and function of the mitochondrial DNA polymerase offer

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* To whom correspondence should be addressed. Phone: 512−471−0434. Fax: 512−471−0435. E-mail: kajohnson@mail.utexas.edu.

‡ Contributed equally to the manuscript.

[1] Abbreviations: HIV RT, human immunodeficiency virus-1 reverse transcriptase; Pol γ, DNA polymerase γ; dNTP, deoxynucleoside triphosphate; dNTP; ddNTP, dideoxynucleoside triphosphate; AnaTP, nucleoside analogue triphosphate; E, enzyme; FDA, Food and Drug Administration; d4T, 2′,3′-didehydro-2′,3′-dideoxythymidine; ddA, 2′,3′-dideoxyadenosine; ddI, 2′,3′-dideoxyinosine; AZT, 3′-azido-2′,3′-dideoxythymidine; FIAU, 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil; CBV, (−)-*cis*-2-amino-1,9-dihydro-9-(4-hydroxymethyl)-2-cyclopenten-1-yl)-6H-purine-6-one; PMPA, (R)-9-(2-phosphonylmethoxypropyl)adenine. The suffixes -MP or -TP are added to the drug abbreviations to indicate their monophosphate or triphosphate forms, respectively.

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the promise for more effective and less toxic drugs needed for the long-term maintenance of HIV infections.

Tremendous success has been achieved in AIDS treatment based upon a therapy including nucleoside and nonnucleoside inhibitors of reverse transcriptase in combination with inhibitors of HIV protease. Nucleoside analogue reverse transcriptase inhibitors (NRTIs) continue to be essential components of this highly active antiretroviral therapy (HAART) combination regimen (*6*). This aggressive therapy effectively slows the rate of viral replication sufficiently to reduce the viral load and thereby also slow the rate of evolution of new, resistant forms of the virus. However, since there is no cure and resistant variants of the virus still emerge, the treatment of HIV infections continues to be one of long-term maintenance compounded by the evolution of resistance by HIV on one hand and limited by toxic side effects of the drugs on the other. Nucleoside analogues target reverse transcriptase (RT) and are incorporated into the viral genome during replication, but are not rapidly removed because RT lacks a proofreading exonuclease, although they may be slowly removed by pyrophosphorolysis (*7*). The clinically observed toxicity of NRTIs varies widely across this series of related compounds and is manifested at various sites physiologically, but appears to result from inhibition of mtDNA replication (*8*).

The nuclearly encoded DNA polymerase γ (Pol γ) is responsible for the replication and repair of the mitochondrial genome, and consists of two polypeptides: a catalytic subunit of 140 kDa containing both the polymerase and exonuclease sites, and a noncatalytic accessory protein of 54 kDa which improves processivity by increasing the rate of polymerization (*9*). In recent work, we have succeeded in reconstituting human Pol γ from the two individually expressed subunits and have fully characterized the fidelity of the polymerase, including the contribution of the 3′-5′ proofreading exonuclease (*9*−*11*). Moreover, we have characterized the kinetics of incorporation and removal of nucleoside analogues used to treat AIDS and have shown that the specificity constant for incorporation is correlated with the toxicity of the analogues (*5*). Much to our surprise, not all NRTIs were efficiently removed by the proofreading exonuclease, contributing to the more extreme toxicity of some analogues.

Currently, there are seven NRTIs approved by the United States FDA for use in combating HIV infection. In Figure 1, we show the structures of the seven FDA-approved nucleoside analogues in addition to FIAU and two other related analogues, ddA which is the active metabolic product of ddI (*12*), and the (+) isomer of 3TC which is not approved for use. Formally, PMPA is a nucleotide analogue, not a nucleoside; but to simplify the nomenclature in this review, we will refer to the class of drugs as nucleoside analogues or NRTIs. The analogues are ranked, as shown by the bar graph, by their order of *potential* toxicity based upon kinetic measurements of the rates of incorporation by the human mtDNA polymerase, versus their rates of removal by its proofreading exonuclease. As we will explain in more detail below, the calculation of the toxicity index is based upon the premise that the primary cause of the toxicity results from inhibition of mtDNA replication, and is computed to represent the relative increase in time required to replicate the mitochondrial genome based upon the probability of incorporation of each drug and the time it takes for the proofreading exonuclease to remove it after incorporation. The toxicity index varies widely for the nine analogues

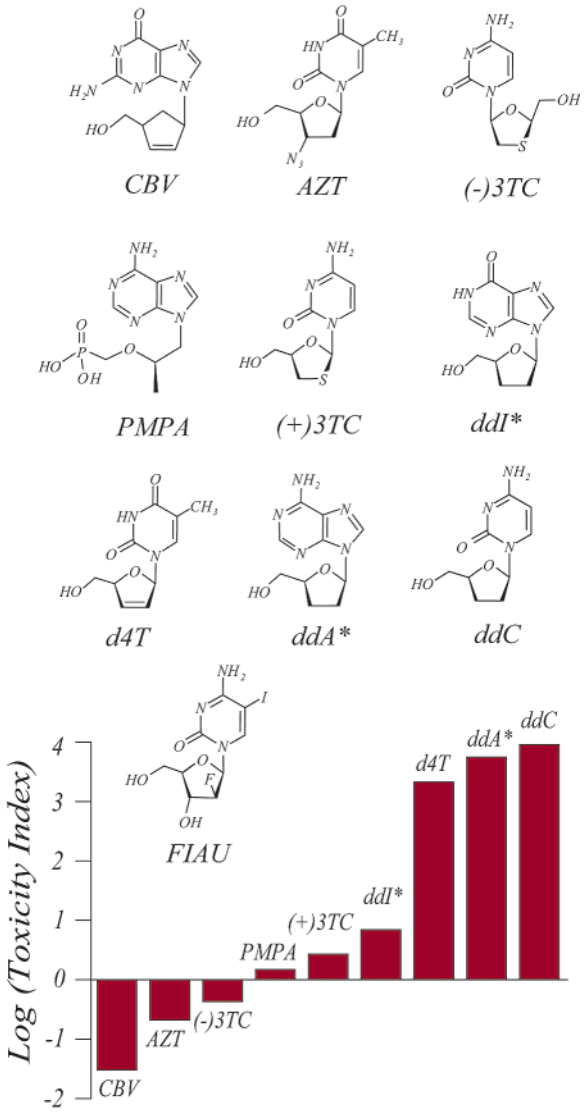

FIGURE 1: Toxicity and structures of nucleoside analogues. The structures of various nucleoside analogues are shown over a bar graph showing mitochondrial toxicity on a log scale spanning 6 orders of magnitude. There are currently seven FDA-approved nucleoside/nucleotide analogues, zalcitabine (ddC), lamivudine ((−)3TC), didanosine (ddI which is metabolized to ddA), zidovudine (AZT), stavudine (D4T), abacavir (prodrug form of CBV), and tenofovir (PMPA). We also show (+) 3TC (β-D-(+)-2′,3′-dideoxy-3′-thiacytadine), ddA (2′,3′-dideoxyadenosine), and 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodoracil (FIAU) (see inset), which led to the deaths of five patients in a hepatitis B clinical trial; unlike other analogues, FIAU is not a chain terminator, and it is extended rather than removed 60% of the time, leading to severe toxicity probably due to mutagenesis of the mitochondrial genome (*5*). The calculation of the toxicity index is described in the text. Note that the definition given here differs slightly from that used previously (*5*) in that we have removed the value of 1 so that now the *index* refers to a fractional increase in time rather than the fold increase in time required to replicate the mitochondrial genome. For example, the 1.2-fold increase for (−)3TC is now reported as 0.2-fold (20%) increase in time. We also have introduced the results of a more accurate measurement of the rate of exonuclease removal of ddC (0.0004 s^{-1}, J. Hanes and K. A. Johnson, unpublished results), and corrected minor math errors in the previous report of the toxicity index (*5*). The values for the toxicity index in order from CBV to ddC are 0.03, 0.20, 0.40, 1.5, 2.6, 6.8, 2000, 5600, and 9000.

shown, and it is presented on a log scale spanning 6 orders of magnitude in Figure 1.

Several features of the analogues are immediately apparent. The drugs that show the highest clinical toxicity, most notably ddC, d4T, and ddA (metabolic product of ddI) clearly stand out in our assays. In contrast, perhaps with the exception of AZT, the drugs with the lowest toxicity index (CBV, (−)3TC, and PMPA) have produced the lowest clinical toxicity (*13*). Our in vitro assay clearly identifies those analogues with the highest and lowest toxicity, and therefore further studies are likely to prove useful in the screening and in the design of new drugs based upon a greater understanding of the mechanistic and structural determinants governing nucleotide selectivity by Pol γ.

In this review, we will first summarize the evidence pointing to mitochondria as the basis for toxicity, and then examine the variations in the kinetics of incorporation of each nucleoside analogue leading to different levels of toxicity. We also will examine the structural features of the analogues that correlate with toxicity, and some unusual aspects of the kinetics of incorporation and exonuclease removal that underlie our calculation of the toxicity index.

Mitochondrial Toxicity in Vivo. The mitochondrial genome is comprised of 16 569 base pairs encoding genes for 13 proteins involved in oxidative phosphorylation, 22 tRNAs, and 2 rRNAs. Administration of nucleoside analogues used as anti-HIV agents causes various levels of duration-dependent mitochondrial dysfunction, which may be a result of three interrelated mechanisms: mtDNA depletion, oxidative stress, and mtDNA mutations (*8*). It has been proposed that these mechanisms work in a sequential manner beginning with energy decline resulting from mtDNA depletion, leading to related events of oxidative stress, which can cause damage to mtDNA, ultimately leading to mutations in the mitochondrial genome.

Effectiveness and toxicity of any given nucleoside analogue may be the results of many factors including uptake, transport, metabolic activation, incorporation, and degradation, all of which serve to establish the concentration of the active form of the drugs at the sites of action. As a result of these many factors that influence bioavailability and energy demand, toxicity is cell-, tissue-, and organ-specific, even though the primary cause may be a constant (*8*). Side effects resulting from the toxicity of NRTIs are reminiscent of heritable mitochondrial diseases. Patients treated with one or more nucleoside analogues may experience a variety of side effects including peripheral neuropathy, cardiac and skeletal muscle myopathy, pancreatitis, and bone marrow suppression (*14*).

Each of the nucleoside analogues are administered orally in an inactive form and require subsequent phosphorylation by host cell kinases to the triphosphate form, which can then act as substrates for RT and possibly other DNA polymerases. Some variability is seen in the intracellular levels of phosphorylated nucleotide analogues in different individuals and as a function of dosage, but generally reach concentrations in the 1−20 nM range (*15*).

Dose-dependent peripheral neuropathy is the major treatment-limiting adverse effect of nucleoside analogues resulting in pain and numbness primarily in the distal lower extremities (reviewed in ref *16*). Neuropathy is commonly seen following treatment with ddC, d4T and ddI, but has not consistently been attributed to AZT, 3TC, or CBV (given as the prodrug abacavir). Analysis of biopsies from patients experiencing peripheral neuropathy induced by ddC has revealed reductions of up to 80% in mtDNA content and a high frequency of mitochondria with abnormal ultrastructure (*2*). Peripheral neuropathy occurs in 34% of patients receiving ddC and is manifested approximately 6−8 weeks after starting therapy, often as the first indicator of mitochondrial toxicity, but it is usually reversed upon withdrawal of the offending NRTI.

Lactic acidosis is an often fatal outcome of extreme mitochondrial toxicity and can be prevented by withdrawing NRTIs at the first signs of mitochondrial toxicity. Lactic acidosis has been seen most commonly in patients receiving stavudine (d4T) and/or didanosine (ddI), and in one study all patients developing hyperlactatemia were receiving d4T (reviewed in ref *16*). Although these studies are biased by the percentages of drugs used most commonly among the patients in the study, they clearly point to d4T and ddI as causing severe mitochondrial toxicity.

Extended treatment often leads to what has been termed a "fat redistribution syndrome" that is characterized by several clinical and metabolic phenomena that include abnormalities in lipid handling, hyperlipidemia, and altered body fat distribution, namely, loss of subcutaneous fat in the face and periphery, combined with localized intra-abdominal fat gain and breast enlargement. Although some theories have suggested that protease inhibitors cause the fat redistribution syndrome, theories positing a role for NRTIs focus on the differential impact of mitochondrial toxicity on peripheral white fat cells which have a low mitochondrial reserve (reviewed by ref *16*). Switching from d4T to AZT or CBV produces benefits in normalizing lipid metabolism. Combined with our mitochondrial toxicity index, these observations support a role for mtDNA polymerase inhibition as the primary site of action leading to the fat redistribution syndrome.

Myopathy was the first clinical toxicity associated with NRTI therapy, but it is now observed less frequently due to reduced doses of NRTIs. Skeletal and cardiac myopathy was reported predominantly following AZT treatment, but it is clearly dose-dependent, and lower doses of AZT have markedly reduced the occurrence of myopathy (reviewed in refs *8* and *16*)). This may be consistent with the observations that AZT inhibits cell growth more potently than it induces mitochondrial toxicity in cell culture. The selective impact of AZT on muscles cells has been suggested to involve inhibition of succinate transport (*17*), but other evidence supports DNA replication as the primary site of action (*8*). Contrasting with other nucleoside analogues, AZT causes bone marrow toxicity which is thought to be due to inhibition of one or more of the chromosomal DNA polymerases (*18*), but in vivo studies have revealed ultrastructural changes in mitochondria caused by AZT (*19*, *20*). These data taken together may explain some of the confusion regarding the toxicity of AZT. Our toxicity index suggests that AZT is one of the least toxic NRTIs, and much of the reported toxicity may stem in part from the higher doses used in early trials, and in part from inhibition at sites other than the mtDNA polymerase. In addition, AZT toxicity is cumulative over time and this may reflect an additive effect on mtDNA replication. Alternatively, it has recently been shown that

significant levels of d4T-TP can be measured in peripheral blood mononuclear cells of patients receiving AZT therapy (*21*). Thus, it is possible that the toxic effects observed after AZT treatment may be due to its conversion to d4T.

In summary, although there may be additional sites of action leading to toxic side effects of NRTIs, a majority of the toxic side effects result from a primary site of action on the mtDNA polymerase. Certainly, the ranking of the potential for mitochondrial toxicity seen by our in vitro enzyme assays (Figure 1) recapitulates the ranking of the toxicities of drugs seen clinically (*13*). Moreover, given this information, we can now examine more closely the features of the polymerase structure and dynamics that govern the nucleotide selectivity underlying the widely varying discrimination against the NRTIs.

Mitochondrial DNA Polymerase Structure and Fidelity: Quantifying Selectivity. Polymerase γ is a DNA-dependent DNA polymerase possessing a 5′- 3′ polymerase activity and a 3′-5′ exonuclease activity, both dependent upon divalent metal ions (*22*). The polymerase is a member of the family A DNA polymerases, along with T7 DNA polymerase and *Escherichia coli* DNA polymerase I, and although there is, currently, no crystal structure of Pol γ, it is presumed to have the right-hand structure, characteristic of related DNA polymerases (*23*).

Reconstituted human Pol γ is able to copy DNA at an average rate of 37 bases per second (*10*), with an average processivity of ~1800 bases copied per binding event (*9*), and an overall average error frequency of one in 60 million. The net fidelity is the product of a two-step selectivity mechanism during polymerization comparable to that seen by other polymerases (*24*) plus the contribution of the proofreading exonuclease. Ground-state nucleotide binding, utilizing base-pair free energy, contributes an average selectivity of 200 to the fidelity by distinguishing correct versus mismatched base pairs. The rate-limiting incorporation following nucleotide binding contributes an additional average selectivity factor of 1500. These two steps combine to achieve an overall average selectivity against mismatches of 300 000 during polymerization. The proofreading 3′-5′ exonuclease contributes an additional factor of approximately 200 by selectively removing mismatches leading to an overall fidelity of 1 error per 60 million bases copied (*11*). This fidelity is sufficient to afford propagation of the mitochondrial genome, but it is at least 100−1000-fold lower than the fidelity of replication of the nuclear genome (*25*).

Evaluation of the overall fidelity of replication of the mitochondrial genome is complicated by the approximately 1000−5000 copies per cell. Most individuals with defects in mtDNA have a mixed population of wild-type and mutated mitochondria, a phenomenon known as intracellular heteroplasmy (*26*). For a number of reasons, it is reasonable to suspect that the Pol γ need not have evolved a high fidelity to fulfill its biological mission and therefore, it may be most susceptible to poisoning by nucleoside analogues among the polymerases responsible for replication. Lower fidelity enzymes are known to participate in repair processes (*27*), but they may be less likely to cause significant NRTI toxicity because they are responsible for such a small fraction of the DNA replication.

For polymerases that have been studied in the most detail, the evidence supports a rate-limiting conformational change defining k_2, which is then followed by fast incorporation at a rate k_3 according to the scheme below:

$$\mathrm{E{\cdot}DNA}_n + \mathrm{dNTP} \underset{\text{ground-state binding}}{\overset{K_d}{\rightleftharpoons}} E_{\text{open}}{\cdot}\mathrm{DNA}_n{\cdot}\mathrm{dNTP} \underset{\text{conformational change}}{\overset{k_2}{\rightleftharpoons}} E_{\text{closed}}{\cdot}\mathrm{DNA}_n{\cdot}\mathrm{dNTP} \underset{\text{chemistry}}{\overset{k_3}{\rightleftharpoons}} \mathrm{E{\cdot}DNA}_{n+1}{\cdot}\mathrm{PP}_i$$

Correct nucleotide binding proceeds via a two-step mechanism, whereby fast nucleotide binding is followed by a rate-limiting conformational change preceding a fast incorporation reaction (*10*, *24*). According to this scheme, measurement of the time- and concentration-dependence of a single incorporation reaction by rapid kinetic methods allows quantification of the ground-state substrate dissociation constant, K_d, and the maximum rate of polymerization, k_{pol}. The rate of incorporation may be limited by k_2, but in general is a function of both k_2 and k_3. Because the ground-state binding is in a rapid equilibrium and there are no rate-limiting steps after incorporation (at least during the fast kinetics reaction phase), the measured ratio of k_{pol}/K_d is equal to k_{cat}/K_m, the *specificity constant* for incorporation.

The steady-state parameter, k_{cat}/K_m, is defined as the *specificity constant* because it quantifies the relative rates of reaction of competing substrates. However, accurate measurements of k_{cat}/K_m in the steady state are nearly impossible to make due to the processive nature of DNA polymerases. Attempts to measure k_{cat} and K_m are dominated by the slow release of DNA from the polymerase such that each number is altered by a factor approaching the value of the processivity, which for Pol γ is 1800 (*9*). Although in theory, the systematic errors in the individual parameters k_{cat} and K_m cancel in calculating the ratio, the attempts to measure k_{cat}/K_m in the steady state are usually in error by a factor of 100 or more (for example, compare ref *28* with ref *5*). Given the importance of reliable estimates of selectivity of nucleoside analogues, we will not discuss further measurements made by steady-state methods because they have been shown to produce artifactual results (*5*, *10*) and a complete understanding of the kinetics of the reaction provides a sound rationale for dismissing the steady-state measurements.

Kinetics of Incorporation and Removal by Pol γ and the Toxicity of NRTIs. We have inspected the in vitro incorporation and excision of nucleoside analogues using recombinant exonuclease deficient (E200A) and wild-type Pol γ to quantify the potential mitochondrial toxicities of each analogue. For each nucleotide, we measured the *specificity constant* $= k_{pol}/K_d$ and then calculated the *discrimination* as the ratio of the specificity constant for the correct dNTP relative to that for the analogue triphosphate (AnaTP):

$$D = \text{discrimination} = (k_{pol}/K_d)_{\text{dNTP}}/(k_{pol}/K_d)_{\text{AnaTP}}$$

The discrimination times the ratio of concentrations of dNTP relative to the analogue triphosphate defines the relative rates of incorporation of the correct dNTP relative to the analogue triphosphate (*5*) as competing substrates: dNTP rate/AnaTP rate $= D$[dNTP]/[AnaTP].

The specificity constants of the nine nucleoside analogues inspected for Pol γ varied more than 500 000-fold for the series ddC > ddA (ddI) > FIAU > d4T ≫ (+)3TC ≫ (−)-

3TC > PMPA > AZT > CBV (*5*). Note that FIAU is not a chain terminator, and its incorporation can result in internalization into the DNA as discussed below, possibly causing a severe form of mitochondrial toxicity due to the accumulation of mutations caused by mispairing with stably incorporated FIAU (*29*).

After the chain terminators are incorporated, they can be removed by the proofreading exonuclease. Using recombinant wild-type human Pol γ, the rate of excision of each of the analogues was measured directly. The rates of excision were in the range of 0.06−0.0004 s^{-1} for the series FIAU > (+)3TC ∼ (−)3TC > CBV > AZT > PMPA ∼ d4T > ddA (ddI) (*5*). The rate of removal of ddCMP from the primer has recently been measured more accurately than previously reported to give a value of 0.0004 s^{-1} (J. Hanes and K. A. Johnson, unpublished results).

To combine the contributions of the kinetics of incorporation and exonuclease removal to evaluate the net toxicity of each analogue, we computed the toxicity index, calculated by the equation:

$$\text{toxicity index} = (k_{cat}/k_{exo})([\text{AnaTP}]/[\text{dNTP}])/4D$$

where k_{cat} represents the maximum rate of polymerization of the natural dNTP, k_{exo} represents the rate of excision of the chain terminator by the proofreading exonuclease, D is the discrimination, [dNTP] is the natural nucleotide concentration, and [AnaTP] is the concentration of analogue triphosphate. By this definition, the toxicity index gives the relative increase in the time required to replicate the mitochondrial genome based upon the rates of incorporation and removal of the chain terminators.

For the data shown in Figure 1, the toxicity index is calculated assuming that the ratio, [AnaTP]/[dNTP] = 1. Clearly, this is not a valid assumption, but it is useful in that it puts the analogues on a single scale with the understanding that changes in the ratio of concentrations [AnaTP]/[dNTP] translate linearly to the calculated toxicity index. Simultaneous measurements of ddATP and dATP following administration of didanosine has given a ratio of ddATP/dATP = 0.04−0.08 (*30*). Similarly, following treatment with stavudine, the d4T-TP/dTTP ratio was 0.2−0.6. Taking the average values, the toxicity index can be corrected to yield values of 1100 and 940 for didanosine and stavudine, respectively. Once reliable estimates of the [AnaTP]/[dNTP] ratios are available for all analogues, the toxicity index can be corrected to account for all intracellular concentrations. The resultant toxicity of the compounds may be further reduced by lower concentrations inside the mitochondria.

The toxicity index serves to put the all of the analogues on a common scale representing the potential for toxicity once the analogues enter the mitochondria. It recapitulates the order of toxicity seen clinically (*13*) and clearly points to ddC, ddI, and d4T as the most likely to cause significant toxic side effects. Deviations in comparing the relative clinical toxicities to the in vitro toxicity index can be used to spotlight possible differences in the rates of phosphorylation and/or transport of the various analogues into the mitochondria or may point to alternative mechanisms of toxicity.

The analogue with the highest toxicity is ddC, and this toxicity arises because of two features revealed by our kinetic studies. First, it is readily incorporated, exhibiting a discrimination of only 2.9; that is, dCTP would be incorporated only 2.9-fold faster than ddCTP if they were present at the same concentration. Second, after it is incorporated, ddCMP is removed slowly from the DNA at a rate <0.0004 s^{-1} (half-life of >30 min). These two factors combine to give a toxicity index of 9000. This means that it will take 9000-fold longer to replicate the mitochondrial genome if the concentrations of ddCTP and dCTP are equal in the mitochondria. Although many factors may limit the concentration of ddCTP in the mitochondria leading to lower toxicity than suggested by this number, it clearly points to the high potential for problems due to treatment with ddC.

At the other end of the spectrum, CBVTP shows a discrimination of 902 000 and it is removed at a rate of 0.0016 s^{-1} (half-life of 7 min) after it is incorporated, giving CBV a very low toxicity index of only 0.03. This implies that there will be only a 3% increase in the time required to replicate the mitochondrial genome if the concentration of CBVTP inside the mitochondria is equal to the concentration of dGTP. This clearly points to CBV as a desirable analogue that can be readily excluded from toxic side effects due to incorporation into the mtDNA. Even if it is efficiently phosphorylated and transported into the mitochondria, it will not cause significant side effects due to inhibition of mtDNA replication.

The toxicity index is displayed on a log scale in Figure 1 to span the 6 orders of magnitude difference in potential mitochondrial toxicity seen for the different nucleoside analogues. Although this scale corresponds well with the order of toxicity seen clinically (*13*), it is difficult to compare directly because of the lack of a correspondingly quantitative basis for measuring clinical toxicity. Moreover, it is reasonable to suppose that at the extremes of our scale, other factors may come in to play that will tend to compress the scale from both ends. At the high end, the toxicity may be limited by the reactions necessary to phosphorylate the nucleosides and transport them into the mitochondria that will reduce the concentrations of the triphosphate forms within the mitochondria. At the low end, the low predicted mitochondrial toxicity may then permit dosages and time of treatment that then lead to other toxic side effects. For example, our results may suggest that the toxicity of AZT could be due to factors other than the mtDNA polymerase. Effectively, AZT may be below the threshold of mitochondrial toxicity and therefore, extended treatment with AZT leads to other toxic side effects due to interactions at other sites. Alternatively, there may be sites on the mtDNA genome that are more susceptible to the accumulation of AZT, or the formation of d4T from AZT may account for the observed toxicity of AZT (*21*). This important possibility warrants further investigation.

It must be borne in mind that measurements of polymerase kinetics in vitro provide a quantitative assessment for the *potential* for mitochondrial toxicity of the nucleoside analogues. Our assays can easily and efficiently guide the screening and search for new compounds, and can eliminate those compounds with the highest potential for toxicity early on during the drug discovery process, but they cannot substitute for extended toxicity studies in animal model systems that are typically performed later in the process.

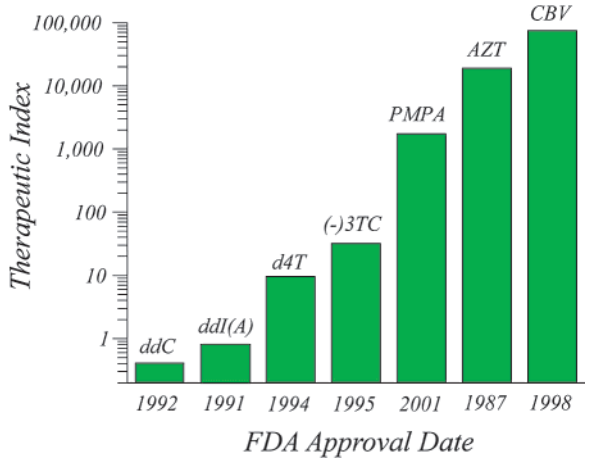

FIGURE 2: Therapeutic index of nucleoside analogues. The theoretical, enzyme-based therapeutic index is presented on a log scale for the seven nucleoside analogues approved by the FDA. The therapeutic index is calculated as the ratio of the discrimination by RT divided by the discrimination by Pol γ as described in the text and in ref *5*. The year in which each analogue was approved for AIDS treatment by the FDA is shown under each bar.

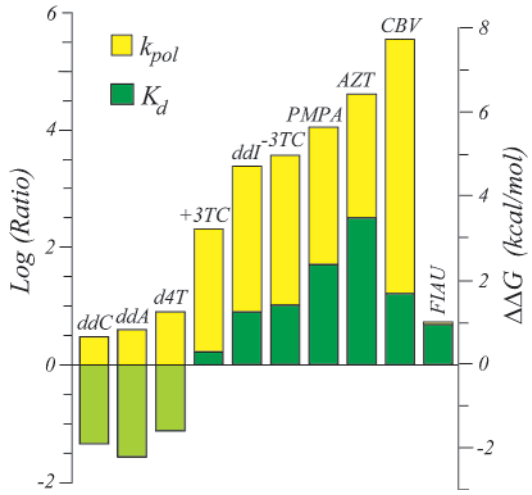

FIGURE 3: Contributions of K_d and k_{pol} to discrimination. The ratio of K_d values for each analogue triphosphate divided by the K_d for the normal dNTP K_d^{AnaTP}/K_d^{dNTP}, and the corresponding ratios of k_{pol} values $k_{pol}^{dNTP}/k_{pol}^{AnaTP}$ are shown. The values are displayed on a log scale to represent the contributions of ground-state binding (K_d, darker color) and rate of polymerization (k_{pol}, lighter color) to the net discrimination (k_{pol}/K_d), represented by the sum of the two. For those analogues that bind more tightly than the normal nucleotide, the bar for the K_d contribution is negative and therefore is superimposed on the selectivity factor resulting from k_{pol} to get the net discrimination. On the right-hand axis, the same information is displayed on a free energy scale where $\Delta\Delta G = RT \ln(R)$, where R is the ratio of K_d or k_{pol} values. Formally, the free energy scale for the ratio of rates equates to $\Delta\Delta G^{\ddagger}$, differences in the free energy of activation. Note that on this scale, a positive $\Delta\Delta G$ equals the free energy difference favoring the normal nucleotide over the analogue, whereas a negative value that the analogue is favored over the normal nucleotide. Values of K_d and k_{pol} ratios are from ref *5*.

Therapeutic Index of Nucleoside Analogues. The other most important feature in the search for new inhibitors of HIV RT is the *therapeutic index*, which is a ratio of the toxic dose at which side effects become significant relative to the effective dose required for inhibition of HIV RT. It is desirable to attempt to compute this index based upon in vitro measurements using the isolated enzymes so that this information may be obtained early during the drug discovery process. We show in Figure 2, the theoretical therapeutic index, based upon the relative discrimination by Pol γ compared to the discrimination by HIV RT against each of the nucleoside analogues. In computing the therapeutic index, we divide the enzyme discrimination parameters for each nucleotide for HIV RT by the corresponding discrimination by Pol γ. These values are based upon the average discrimination observed with DNA and RNA templates with HIV RT since the selectivity by HIV RT is slightly different when copying an RNA template leading to a slightly different therapeutic index, and we have averaged the values to create Figure 2. We have also shown in Figure 2 the year during which each NRTI received FDA approval for treatment of AIDS. It is encouraging to note the general upward trend with time.

The calculated therapeutic index for ddC is less than unity because ddC is a better substrate for Pol γ than HIV RT, and this is clearly a problem for its therapeutic usefulness. The fact that ddC was seen to be effective enough to receive FDA approval may be taken as evidence that the concentrations of ddCTP in the cytoplasm may reach levels higher than in the mitochondria to achieve a differential inhibition of HIV RT over Pol γ. However, the theoretical therapeutic index clearly points to difficulties that are expected to arise during treatment with ddC. At the other end of the spectrum, our calculated therapeutic index clearly identifies the most effective inhibitors ((−)3TC, PMPA, AZT, and CBV) as defined by a preference for incorporation by HIV RT over Pol γ ranging from 32-fold for (−)3TC to 74 000-for CBV. The enzymologic therapeutic index shows the reverse order of toxicity seen in the calculated toxicity index, but the scale spans only 5 orders of magnitude, due to variable rates of incorporation by HIV RT for different analogues.

As a ratio of ratios, the therapeutic index is no longer susceptible to uncertainties in the concentrations of the analogue triphosphates relative to the normal dNTPs, except as they may pertain to different concentrations in different cellular compartments. Since HIV replicates in the cytoplasm where nucleoside analogues are phosphorylated, and it is likely that transport into the mitochondrial will limit rather than increase the concentration of the analogues in the mitochondria, it is expected that our numbers will only underestimate the biological therapeutic index. Moreover, because our calculated therapeutic index does not take into account the rates of proofreading excision after incorporation, it may overestimate the toxicity of those analogues that are excised very rapidly. Each of these factors will tend to underestimate the therapeutic index.

Kinetic Parameters Governing Nucleotide Selectivity. Selectivity is a function of the two-step process whereby rapid equilibrium binding of the nucleoside triphosphate is followed by rate-limiting reactions leading to incorporation such that nucleotide discrimination is the product of the selectivity in each step. Analysis of the two contributions to the discrimination against each NRTI is shown in Figure 3, where we illustrate the ratios of the K_d and k_{pol} values for each NRTI relative to the corresponding normal nucleotide. Figure 3 is informative because it illustrates the individual contributions of the ground state binding and the maximum

rates of polymerization to the net selectivity. It immediately reveals that the three inhibitors with the highest mitochondrial toxicity (ddC, ddA, and d4T) all bind more tightly than the corresponding normal dNTP, and this tighter binding is largely responsible for their greater toxicity. In contrast, the four best NRTI's (3TC, PMPA, AZT, and CBV) all bind more weakly than the corresponding normal dNTP and show an additional, additive discrimination during incorporation. Although ddI appears to be a less toxic alternative, it is metabolized to ddA, which is the active form of the drug (*29*). There is little discrimination against FIAU, but its toxicity is even greater than would be indicated on this scale because it is not a chain terminator. As will be discussed separately below, FIAU is readily extended to become internalized during polymerization.

Inspection of the structures of the nucleoside analogues is also revealing. Although several NRTIs are given as prodrugs, the final active metabolic product in each case is a normal base connected to a modified ribose ring, perhaps better described as a ribose mimic. The least toxic compounds are those with the largest changes to the structure of the ribose mimic. CBV contains a double bond and lacks an oxygen in the ring; AZT has an azido group replacing the 3′OH; (−)3TC contains a sulfur substitution and has the opposite stereochemistry; and PMPA has no ring at all, substituting an ether linkage that is one atom shorter from the nitrogen of the base to the phosphorus.

In contrast, the simple dideoxy analogues, ddC and ddA, and the sole addition of a double bond to the ring in d4T, apparently do not provide sufficiently large differences in structure to be excluded from the dNTP binding pocket at the active site of Pol γ. Moreover, they bind more tightly than the normal dNTP increasing their toxicity. These data suggest that there are residues at the active site of Pol γ that allow a tighter interaction with dideoxynucleotides. The structural basis for the tighter binding of ddNTP to Pol γ remains to be determined. However, it is notable that a single tyrosine/phenyalanine variation in related polymerases is responsible for a large variation in the discrimination against dideoxynucleotides in that mutation of Y526F in T7 DNA polymerase increases discrimination against ddNTPs by 2000-fold (*31*). Interestingly, Pol γ has a tyrosine at position 951 corresponding in sequence alignment with Y526 in T7 DNA polymerase. Thus, it is predictable, but not yet established that a similar mutation in Pol γ may produce a similar change to improve selectivity against ddNTPs.

One caveat needs to be added to our ranking of AZT. The kinetics of incorporation of AZTTP were unusual in that the amplitude of the pre-steady-state burst of the reaction increased with increasing nucleotide concentration, while the rate of incorporation was constant (*5*). This suggests that the chemistry of incorporation is reversibly linked to nucleotide binding, and this is unlike the kinetics seen with other nucleotides. The unusual kinetics of incorporation for AZTTP raises questions regarding the apparent specificity constant and toxicity index reported here. Although the numbers reported here represent our best estimate of the selectivity by Pol γ against AZTTP, our observations point to the fact that AZT is different from the other nucleoside analogues. In addition, the toxicity of AZT may be attributable to its intracellular conversion to d4T (*21*), an observation consistent with the cross-resistance seen in d4T- and AZT-treated patients (*32*), but needing further investigation to substantiate.

The initial clinical trial with 1-(2′-deoxy-2′-fluoro-beta-D-arabinofuranosyl)-5-iodoridine (FIAU) on chronic HBV infected patients resulted in the death of five patients (*3*). The toxicity that arose from FIAU was apparent clinically by myopathy, lactic acidosis, pancreatitis, and hepatic steatosis. On the basis of the evident mitochondrial toxicity of FIAU, a comparative in vitro study using mammalian DNA polymerases α, β, γ, δ, and ϵ purified from various tissues revealed that while FIAUMP was incorporated into the nascent DNA chain by all the polymerases, Pol γ was the most efficient (*33*). One significant difference between FIAU and other nucleoside analogues is the presence of a 3′-OH creating an opportunity for FIAUMP to be stably incorporated into the mitochondrial genome in vivo. In our studies, FIAUTP was incorporated at a rate of 24 s^{-1}, which was identical to dTTP (*10*) and bound only 5-fold more weakly, defining the net discrimination of only 5. Since FIAU has a 3′-OH, we examined the capability of Pol γ to continue elongation on top of FIAUMP relative to the rate of its removal by the proofreading exonuclease. Using a primer 3′-terminated with FIAUMP, the next correct nucleotide, dCTP, was incorporated at a rate of 0.13 s^{-1}, while the proofreading excision rate was 0.06 s^{-1}. These data indicate that approximately 68% of the time FIAUMP was internalized by continued extension rather than removed. Once internalized into the mtDNA, FIAUMP may likely serve as a potent mutagen.

Lamivudine ((−)3TC) is the only analogue used clinically with an unnatural L (−) configuration, which has been shown to be more effective and less toxic than the D (+) isomer (*34*). Our experiments with (+)3TC-TP and (−)3TC-TP help explain this trend. Pol γ is highly discriminating on the basis of stereochemistry with a 16-fold higher discrimination against (−)3TC-TC compared to (+)3TC-TP, due largely to the 6−8-fold difference in K_d. The rates of excision were 2-fold slower than removal of a correctly base paired primer 3′-terminated with dCMP. Although (−)3TC may slow the rate of mitochondrial replication, the toxic effects are likely minimized due to the low rate of incorporation by Pol γ. Clinically, (−)3TC appears to produce fewer side effects than the other nucleoside analogues and has shown little evidence of mitochondrial toxicity (*35*). Indeed, there has recently been an emergence of even more potent L-configuration nucleoside analogues in light of the toxicity profile of (−)3TC, which are promising antiviral agents (*36*).

The most recently approved analogue, PMPA, has been reported to have low potential of producing mitochondrial dysfunction (*37*). The structure of PMPA is quite different from the other nucleoside analogues in that there is no ribose ring, which, like the ribonucleoside analogue acyclovir, makes the observed incorporation of PMPApp (PMPA diphosphate) by any polymerase a surprising discovery. The maximum rate of polymerization was similar to that of d4TTP at 0.21 s^{-1}, but the ground-state binding was 50-fold weaker than the natural nucleotide (dATP), which means that Pol γ discriminates strongly against PMPA compared to dATP (11 400).

Data obtained for CBVTP, the active form of the drug abacavir, showed that its incorporation was overall the most strongly discriminated against by Pol γ, $k_{pol}/K_d = 0.00014$

μM^{-1} s^{-1} and $D = 902\ 777$, respectively. Excision CBVMP occurred at a rate only slightly slower than normal nucleotides, further contributing to the low predicted toxicity which is consistent with a growing body of clinical data.

CONCLUSION

The data summarized in this review suggest that the key to discovering new NRTI's will rest with the ability to synthesize novel compounds that are accepted readily as substrates by HIV RT but not Pol γ, and that the most important features are the modifications to the ribose ring mimic with a preference for more severe alterations in structure. With the exception of PMPA, the least toxic analogues are those that are more bulky, which is perhaps reminiscent of a recurrent theme in enzymology whereby greater selectivity is afforded by steric repulsion of a larger substrate than by loss of free energy of interaction from a smaller substrate. The most important limitation is the likelihood that HIV RT can evolve the ability to discriminate against any new inhibitors. In an abstract sense, one could argue that if Pol γ has a structure that can discriminate against a given inhibitor while retaining efficient incorporation of the normal nucleotide, it should be possible for HIV RT to evolve toward a similar structural solution to the problem of discrimination. The only caveat to this logic is that HIV RT must be able to copy both RNA and DNA templates and therefore may have a more open or flexible active site to accommodate both templates. However, recent results have shown that Pol γ is able to efficiently copy RNA (*38*) and that the mitochondrial genome appears to contain a substantial portion of RNA (*39*). Thus, selective advantage of Pol γ may ultimately depend on its proofreading activity.

Initially theories regarding the potential for chain terminators to selectively inhibit HIV RT were based upon the postulate that most eukaryotic polymerases contain efficient proofreading exonuclease activities. The selectivity of the proofreading exonuclease toward removal of mismatches is based upon the slowing of the rates of extension on top of a mismatch, which then leads to a higher kinetic probability of excision versus extension. Therefore, it was reasonable to expect that chain terminators would be efficiently removed. This proved not to be the case, and we were surprised at the slow rate at which most analogues were removed after their incorporation into a growing DNA. Since there is a significant variability in the rates of exonuclease excision of some NRTIs, it is possible that future designs could incorporate features that might enhance the rates of excision. Further studies will be needed to define the parameters governing the rate of the exonuclease for different analogues.

Despite the successes of the current combination therapy, there remains a continuing need to invent new, less toxic drugs. Much of the severe toxicity of nucleoside analogues appears to be attributable to incorporation into mtDNA by Pol γ. Moreover, we can now identify those compounds with the highest potential for mitochondrial toxicity before they go into the clinic, thereby avoiding the pain and suffering caused by the most severe side effects. We are now working to define the parameters that govern mitochondrial toxicity, and this new knowledge can then guide the search for newer, less toxic drugs to be used to combat HIV and other viral infections. Future drug design efforts should be facilitated by a detailed understanding of the function of the human mitochondrial polymerase, as it pertains to the incorporation of nucleotide analogues into the mitochondrial genome.

NOTE ADDED IN PROOF:

An eighth NRTI was recently approved by the FDA, emtricitabine (FTC), which is a fluorine-substituted analogue of 3TC. We will examine its toxicity index as soon as the manufacturer provides us with a sample.

REFERENCES

1. Lewis, W., and Dalakas, M. C. (1995) *Nat. Med. 1*, 417−422.
2. Dalakas, M. C., Semino-Mora, C., and Leon-Monzon, M. (2001) *Lab. Invest. 81*, 1537−1544.
3. McKenzie, R., Fried, M. W., Sallie, R., Conjeevaram, H., Di Bisceglie, A. M., Park, Y., Savarese, B., Kleiner, D., Tsokos, M., Luciano, C., et al. (1995) *N. Engl. J. Med. 333*, 1099−1105.
4. Colacino, J. M. (1996) *Antiviral Res. 29*, 125−139.
5. Johnson, A. A., Ray, A. S., Hanes, J., Suo, Z., Colacino, J. M., Anderson, K. S., and Johnson, K. A. (2001) *J. Biol. Chem. 276*, 40847−40857.
6. Squires, K. E. (2001) *Antivir. Ther. 6 Suppl 3*, 1−14.
7. Ray, A. S., Murakami, E., Basavapathruni, A., Vaccaro, J. A., Ulrich, D., Chu, C. K., Schinazi, R. F., and Anderson, K. S. (2003) *Biochemistry 42*, 8831−8841.
8. Lewis, W. (2003) *Antiviral Res. 58*, 189−197.
9. Johnson, A. A., Tsai, Y., Graves, S. W., and Johnson, K. A. (2000) *Biochemistry 39*, 1702−1708.
10. Johnson, A. A., and Johnson, K. A. (2001) *J. Biol. Chem. 276*, 38090−38096.
11. Johnson, A. A., and Johnson, K. A. (2001) *J. Biol. Chem. 276*, 38097−38107.
12. Kewn, S., Hoggard, P. G., Henry-Mowatt, J. S., Veal, G. J., Sales, S. D., Barry, M. G., and Back, D. J. (1999) *AIDS Res. Hum. Retroviruses 15*, 793−802.
13. Kakuda, T. N. (2000) *Clin. Ther. 22*, 685−708.
14. Lewis, W., Haase, C. P., Raidel, S. M., Russ, R. B., Sutliff, R. L., Hoit, B. D., and Samarel, A. M. (2001) *Lab. Invest. 81*, 1527−1536.
15. Becher, F., Pruvost, A., Gale, J., Couerbe, P., Goujard, C., Boutet, V., Ezan, E., Grassi, J., and Benech, H. (2003) *J. Mass Spectrom. 38*, 879−890.
16. Moyle, G. (2000) *Clin. Ther. 22*, 911−936.
17. Pereira, L. F., Oliveira, M. B., and Carnieri, E. G. (1998) *Cell Biochem. Funct. 16*, 173−181.
18. W. B. Parker. Cheng, Y. C. J. (1994) *NIH Res. 6*, 57−61.
19. Lewis, W., Papoian, T., Gonzalez, B., Louie, H., Kelly, D. P., Payne, R. M., and Grody, W. W. (1991) *Lab. Invest. 65*, 228−236.
20. Lewis, W., Gonzalez, B., Chomyn, A., and Papoian, T. (1992) *J. Clin. Invest. 89*, 1354−1360.
21. Becher, F., Pruvost, A. G., Schlemmer, D. D., Creminon, C. A., Goujard, C. M., Delfraissy, J. F., Benech, H. C., and Grassi, J. J. (2003) *AIDS 17*, 555−561.
22. Graves, S. W., Johnson, A. A., and Johnson, K. A. (1998) *Biochemistry 37*, 6050−6058.
23. Steitz, T. A. (1999) *J. Biol. Chem. 274*, 17395−17398.
24. Johnson, K. A. (1993) *Annu. Rev. Biochem. 62*, 685−713.
25. Khaidakov, M., Heflich, R. H., Manjanatha, M. G., Myers, M. B., and Aidoo, A. (2003) *Mutat. Res. 526*, 1−7.
26. Van Camp, G., and Smith, R. J. (2000) *Clin. Genet. 57*, 409−414.
27. Goodman, M. F. (2002) *Annu. Rev. Biochem. 71*, 17−50.
28. Lim, S. E., and Copeland, W. C. (2001) *J. Biol. Chem. 276*, 23616−23623.
29. Periclou, A. P., Nandy, P., and Avramis, V. I. (2000) *In Vivo 14*, 377−388.
30. Becher, F., Pruvost, A., Goujard, C., Guerreiro, C., Delfraissy, J. F., Grassi, J., and Benech, H. (2002) *Rapid Commun. Mass Spectrom. 16*, 555−565.
31. Tabor, S., and Richardson, C. C. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 6339−6343.
32. Lafeuillade, A., and Tardy, J. C. (2003) *AIDS Rev. 5*, 80−86.
33. Lewis, W., Meyer, R. R., Simpson, J. F., Colacino, J. M., and Perrino, F. W. (1994) *Biochemistry 33*, 14620−14624.

34. Chang, C. N., Doong, S. L., Zhou, J. H., Beach, J. W., Jeong, L. S., Chu, C. K., Tsai, C. H., Cheng, Y. C., Liotta, D., and Schinazi, R. (1992) *J. Biol. Chem. 267*, 13938−13942.
35. Perry, C. M., and Faulds, D. (1997) *Drugs 53*, 657−680.
36. Chen, S. H. (2002) *Curr. Med. Chem. 9*, 899−912.
37. Birkus, G., Hitchcock, M. J., and Cihlar, T. (2002) *Antimicrob. Agents Chemother. 46*, 716−723.
38. Murakami, E., Feng, J. Y., Lee, H., Hanes, J., Johnson, K. A., and Anderson, K. S. (2003) *J. Biol. Chem. 278*, 36403−36409.
39. Yang, M. Y., Bowmaker, M., Reyes, A., Vergani, L., Angeli, P., Gringeri, E., Jacobs, H. T., and Holt, I. J. (2002) *Cell 111*, 495−505.

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