Antiretroviral nucleosides, deoxynucleotide carrier and mitochondrial DNA: evidence supporting the DNA pol gamma hypothesis

William Lewis, Emory University
James J Kohler, Emory University
Seyed H. Hosseini, Emory University
Chad P. Haase, Emory University
William C. Copeland, National Institute of Environmental Health Sciences
Rachelle J. Bienstock, National Institute of Environmental Health Sciences
Tomika Ludaway, Emory University
Jamie McNaught, Emory University
Rodney Russ, Emory University
Tami Stuart, Emory University

Only first 10 authors above; see publication for full author list.

Journal Title: AIDS
Volume: Volume 20, Number 5
Publisher: Lippincott, Williams & Wilkins | 2006-03-21, Pages 675-684
Type of Work: Article | Post-print: After Peer Review
Publisher DOI: 10.1097/01.aids.0000216367.23325.58
Permanent URL: http://pid.emory.edu/ark:/25593/fjm7c

Final published version:

Copyright information:
© 2006 Lippincott Williams & Wilkins, Inc.

Accessed March 2, 2020 8:27 PM EST
Antiretroviral nucleosides, deoxynucleotide carrier and mitochondrial DNA: evidence supporting the DNA pol γ hypothesis


From the Department of Pathology, Emory University, Atlanta, Georgia, and the National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina, USA.

Abstract

Design— Nucleoside reverse transcriptase inhibitors (NRTIs) exhibit mitochondrial toxicity. The mitochondrial deoxynucleotide carrier (DNC) transports nucleotide precursors (or phosphorylated NRTIs) into mitochondria for mitochondrial (mt)DNA replication or inhibition of mtDNA replication by NRTIs. Transgenic mice (TG) expressing human DNC targeted to murine myocardium served to define mitochondrial events from NRTIs in vivo and findings were corroborated by biochemical events in vitro.

Methods— Zidovudine (3′-azido-2′,3′-deoxythymidine; ZDV), stavudine (2′, 3′-didehydro-2′, 3′-deoxythymidine; d4T), or lamivudine ((−)-2′-deoxy-3′-thiacytidine; 3TC) were administered individually to TGs and wild-type (WT) littermates (35 days) at human doses with drug-free vehicle as control. Left ventricle (LV) mass was defined echocardiographically, mitochondrial ultrastructural defects were identified by electron microscopy, the abundance of cardiac mtDNA was quantified by real time polymerase chain reaction, and mtDNA-encoded polypeptides were quantified.

Results— Untreated TGs exhibited normal LV mass with minor mitochondrial damage. NRTI monotherapy (either d4T or ZDV) increased LV mass in TGs and caused significant mitochondrial destruction. Cardiac mtDNA was depleted in ZDV and d4T-treated TG hearts and mtDNA-encoded polypeptides decreased. Changes were absent in 3TC-treated cohorts. In supportive structural observations from molecular modeling, ZDV demonstrated close contacts with K947 and Y951 in the DNA pol γ active site that were absent in the HIV reverse transcriptase active site.

Conclusions— NRTIs deplete mtDNA and polypeptides, cause mitochondrial structural and functional defects in vivo, follow inhibition kinetics with DNA pol γ in vitro, and are corroborated by molecular models. Disrupted pools of nucleotide precursors and inhibition of DNA pol γ by specific NRTIs are mechanistically important in mitochondrial toxicity.

Keywords

mitochondria; deoxynucleotide carrier; nucleoside reverse transcriptase inhibitor; DNA polymerase; AIDS; transgenic; cardiomyopathy; heart
Introduction

The mitochondrial transporter family (SLC25) includes inner mitochondrial membrane transport proteins that move molecules into the matrix. The deoxynucleotide carrier (DNC) is a member that imports nucleotide precursors for mitochondrial (mt)DNA replication, but offers a route for mitochondrial uptake of phosphorylated nucleoside reverse transcriptase inhibitors (NRTIs), including zidovudine (3'-azido-2',3'-azido-deoxythymidine; ZDV) and stavudine (2',3'-didehydro-2', 3'-deoxythymidine; d4T). DNC is characterized [1–3] and DNC mutations cause neurological diseases [4,5]. DNC was suggested to link mitochondrial toxicity of NRTIs to their mitochondrial import [1]. Recently, human DNC was expressed transgenically in the murine heart [6]. The DNC transgenic mouse (TG) is a tool to dissect mitochondrial toxicity of specific NRTIs of combined antiretroviral regimens (HAART).

NRTI mitochondrial toxicity limits treatment of HIV infection (reviewed in [7]). NRTI-sparing regimens may obviate some mitochondrial manifestations (reviewed in [8]), but also are limited. Studies here establish that ZDV and d4T (monotherapy, human therapeutic doses) cause mitochondrial structural and functional defects in vivo. Conversely, lamivudine ((−)-2'-deoxy-3’-thiacytidine; 3TC) had no deleterious effects. These data corroborate the ‘DNA pol γ hypothesis’ [9] by establishing a mechanistic connection between NRTIs (ZDV or d4T) in vivo, mtDNA depletion and mitochondrial ultrastructural defects. DNA pol γ inhibition kinetics with NRTI triphosphates in vitro [10–13] correlate with molecular modeling studies and with in vivo data. Taken together, in vitro and in vivo findings support the DNA pol γ hypothesis, the concept frequently invoked to explain mitochondrial toxicity of NRTIs in patients [9].

Materials and methods

Generation of α-MyHC/DNC transgenic mice

Established methods were employed essentially as described previously [14] and applied to the DNC TGs as recently described [6].

Treatment protocols

Procedures complied with Emory IACUC and NIH guidelines. The drugs were from the manufacturers or from the Emory Center for AIDS Research Pharmacology Core (Raymond Schinazi, VA Medical Center, Decatur, Georgia, USA). Dosing was done by daily gavage (morning) at doses that resemble human therapy. Doses used were ZDV = 0.22 mg/day; d4T = 0.0285 mg/day; 3TC = 0.11 mg/day or vehicle controls [6]. At day 35, measurements were made, animals were terminated, and samples retrieved and stored.

Genotyping

For the TG line α-MyHC/DNC the presence of the transgene was detected in the founders and their offspring using Southern blotting and polymerase chain reaction (PCR) essentially as previously reported [14].

Echocardiography in DNC transgenic and wild-type mice

For echocardiography (ECHO) observations in the protocols with ZDV monotherapy cohort, TG + ZDV, n = 8; TG + vehicle, n = 9; wild-type (WT) + ZDV, n = 7; WT +, n = 9. For protocols containing d4T, TG + d4T, n = 6; TG + vehicle, n = 4; WT + d4T, n = 7; WT + vehicle, n = 6. For 3TC monotherapy TG + 3TC, n = 8; TG + vehicle, n = 6; WT + 3TC, n = 7; WT + vehicle, n = 4. ECHO studies were performed in age and gender-matched (littermate) WT and TGs as previously reported [15].
Fine structure pathological evaluations with transmission electron microscopy images of mitochondrial damage

Ultrastructure \((n = 12)\) was evaluated using transmission electron microscopy (EM). Sections (approximately 1 mm cubes) were rapidly fixed in diluted Karnovsky’s fixative and processed for EM as previously reported \([15]\) and examined for intramitochondrial lamellar bodies, cristae reduplication, and intramitochondrial paracrystals as reported in human ZDV-induced skeletal muscle mitochondrial myopathy \([16]\). Damaged mitochondria were defined as having loss or dissolution of \(\geq 25\%\) of cristae in addition to above features.

Mitochondrial DNA and nuclear DNA quantitation in heart tissue using real time polymerase chain reaction

Methods employed were based on modifications of those used by others \([17,18]\). Total DNA was extracted from unfixed heart tissue (10–20 mg) using a Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, Wisconsin, USA) following the manufacturer’s protocol. DNA concentration was determined by using a plate reader spectrophotometer (Molecular Devices, Carlsbad, California, USA) and DNA was diluted in ddH\(_2\)O for amplification.

DNA sequences for primers and probes were adapted from other reports \([18]\). Mouse mitochondrial DNA polymerase gamma (ASPG) and mouse mitochondrial cytochrome oxidase subunit 1 (COX) were quantified individually. The mitochondrial forward primer (5'-TCGTTG ATTATTIC TCAACCAATCA-3') and reverse primer (5'-GCTCTCC AATTATTATTGTGTTA CTATGA-3') were used to amplify the target segment of COXI gene. Hybridization probes were 3'-flouresceine (5'-AACCAGGTGCACTTTAGGAGATGACCF-3') and 5'-LC Red 640 3'-phosphate-blocked (5'-AATTATTACATGTATCGTA ACTGCCCATGCP-3') for this gene. The nuclear forward primer (5'-GGAGGAGGCGACTTTCCTACG-3') and reverse primer (5'-GAAGACCTGCTCCCTG AACAC-3') were used to amplify the ASPG gene. A 3'-flouresceine labeled oligonucleotide (5'-GGCCTTTGG ACCTTTGGGTGTAG-F3') and a 5'-LC Red 640 3'-phosphate-blocked (5'-GTTACGAAAGAACCTAGC CTCACAGTGGTCP-3') oligonucleotide were used as hybridization probe for ASPG.

Amplification was performed in a LightCycler 2.0 (Roche Applied Biosystems, Indianapolis, Indiana, USA) and consisted of a denaturation step (10.0 min), 45 cycles of amplification (95°C for 0 s, 60°C for 10 s followed by single fluorescence acquisition, and 72°C for 5 s), a melting curve (95°C for 30 s, 60°C for 30 s and 95°C for 0 s with a ramping rate of 0.1°C/s), and a cooling cycle (40°C). Temperature ramping rates were 20°C/s unless noted. Standard DNA curves for quantification of the LC products were employed. Both mitochondrial and nuclear target sequences were PCR amplified and cloned into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, California, USA) following the manufacturer’s protocol. Serial dilutions were made from products and the copy number was calculated based on the molecular weight of plasmid plus the insert and PCR reactions were performed to construct the standard curve for mitochondrial and nuclear DNA respectively. Samples were run in duplicate. PCR products of mtDNA and nuclear \((n)\) DNA were quantified by using the corresponding external standard.

Immunoblotting of mitochondrial complex I

Methods employed resembled those used recently by the authors \([6,13]\).

Molecular modeling of nucleoside reverse transcriptase inhibition of DNA pol \(\gamma\)

The previously developed catalytic active site model of DNA pol \(\gamma\) \([19,20]\) was employed to identify structural factors responsible for differential incorporation of HIV-RT NRTIs and to explain \textit{in vivo} findings. The Accelerys Insight II molecular modeling software package.
(Accelrys, San Diego, California, USA) was used for superimposition of the analogs into the active site model. The protein backbone of functionally and structurally conserved residues between the DNA pol \( \gamma \) model and an X-ray structure of HIV-RT with ZDV-MP-terminated template-primer with the 3' primer end position at the pre-translocation site, [21] PDB:1N6Q were superimposed in order to fit the phosphorylated form of ZDV into the DNA pol \( \gamma \) model. From this superimposition, the position of the ZDV-MP within the DNA pol \( \gamma \) catalytic site was determined and distances and interactions with residues involved in catalysis were determined. Based on the position of ZDV-MP within the DNA pol \( \gamma \) active site, lamivudine and stavudine were fitted within the DNA pol \( \gamma \) model active site and the interactions with surrounding residues of catalytic importance were evaluated comparatively.

### Statistical analysis

For mtDNA quantitation, data were expressed as the ratio of mean value of the mtDNA measurement to the mean value of nDNA divided by 1000 and the resultant values are expressed as mean \( \pm \) standard error. A value of \( P < 0.05 \) was considered statistically significant. Echo-cardiographic determinations from all groups were compared by analysis of variance (ANOVA) [15].

### Results

#### Echocardiographic data from transgenic mice and nucleoside reverse transcriptase inhibitor monotherapy

The DNC TG exhibited no ECHO phenotype in absence of NRTI treatment. In ZDV protocols, the LV mass (normalized; mg/g body weight) of TG + ZDV mice was 1.34 \( \pm \) 0.08 in comparison with 0.97 \( \pm \) 0.04 in TG + vehicle, a 38% increase (Fig. 1a; \( P < 0.05 \)). The LV mass of WT + ZDV was 1.10 \( \pm \) 0.06 in comparison with 0.97 \( \pm \) 0.04 in WT + vehicle (\( P = \text{NS} \)).

Analogous findings were obtained with d4T. The LV mass of TG + d4T was 1.32 \( \pm \) 0.09 in comparison with 1.12 \( \pm \) 0.08 in TG + vehicle, an 18% increase above untreated TG (\( P < 0.001 \) ANOVA). For WT + d4T, the LV mass was 0.84 \( \pm \) 0.03 in comparison with 0.97 \( \pm \) 0.05 in WT + vehicle (\( P = \text{NS} \); ANOVA; Fig. 1b). In contrast to findings from either treatment protocol above, TGs and WT treated with 3TC showed no change in LV mass (data not shown).

#### Ultrastructural features of mitochondria in TG hearts

An EM feature of cardiac-targeted expression of human DNC in TG cardiac mitochondria was reduplicated mitochondrial cristae (Fig. 2). Treatment of TGs with either ZDV or d4T increased the ultrastructural damage (described below) and correlated with increased LV mass. In contrast, 3TC treatment resulted in no ultrastructural changes above controls.

NRTI treatment of TGs amplified the deleterious changes in cardiac mitochondria. Mitochondrial cristae dissolution (Fig. 2a, b, bottom right panels), accumulation of radiodense, intramitochondrial amorphous material (Fig. 2a, b, bottom right panel, heavy arrow), and conspicuous defects in matrix density occurred in hearts of TGs that were treated with either ZDV or d4T. Mitochondrial ultra-structural defects (found with ZDV or d4T), and their absence with another NRTI (3TC; data not shown), together suggested that the damage resulted directly from administration of ZDV and d4T individually.

#### mtDNA/nDNA relative abundance

mtDNA depletion has been considered a hallmark of NRTI toxicity [7]. Using real-time PCR, the abundance of mtDNA (relative to nDNA) was determined in cardiac samples from the treatment protocols (\( n \geq 7 \) per cohort). The results indicated that decreased mtDNA/nDNA ratio was found in TG hearts in comparison with WT littermates. Transgenic expression of

---

*AIDS*. Author manuscript; available in PMC 2007 March 21.
human DNC yielded a net average decrease from 5.6 (WT) to 4.8 (TG) relative units (−14.2%; Fig. 3; \(P < 0.05\)). With ZDV or d4T monotherapy, each TG cohort (i.e., ± NRTI) exhibited a significant decrease in mtDNA/nDNA ratio in comparison with untreated WTs resulting in a loss of as much as 2.3 relative units (−42.9% Fig. 3a, b; \(P < 0.001\)). In contrast, 3TC caused no change in mtDNA/nDNA ratio (Fig. 3c; \(P = \text{NS}\)).

**Mitochondrial complex I immunoblot quantitation**

Mitochondrial fractions from hearts of vehicle-treated TGs demonstrated increased abundance of Complex I (data not shown). With ZDV treatment of the TGs, a dramatic decrease in the abundance of Complex I occurred in cardiac mitochondria (\(P < 0.01\)). This ZDV-related change was absent in corresponding WT cohorts (\(P = \text{NS}\)).

**Comparison of inhibition kinetic data obtained with DNA pol \(\gamma\) and phosphorylated zidovudine, stavudine and lamivudine**

Data from our previous studies and from those of others [11–13,22] were reviewed to determine the biological relationship between enzyme inhibition kinetics of DNA pol \(\gamma\) *in vitro*, and the observed in-vivo changes of mitochondrial destruction and mtDNA depletion *in vivo*. When compared with findings *in vivo*, kinetic data reflecting efficacy of a given NRTI triphosphate as an inhibitor of DNA pol \(\gamma\) *in vitro* (i.e., lower \(K_i\)) related directly to its efficacy *in vivo* as a mitochondrial toxin (e.g., mtDNA/nDNA ratio, ECHO, or EM; Table 1). Lamivudine’s modest incorporation into nascent chain mtDNA, coupled with its exonucleolytic cleavage, helped explain the absence of in-vivo mitochondrial toxicity of 3TC.

**Molecular modeling of the interaction between DNA pol \(\gamma\) and nucleoside reverse transcriptase inhibitors at the active site of the enzyme**

The molecular basis for the difference between DNA pol \(\gamma\) inhibition and incorporation into DNA is partially provided by molecular modeling of these analogs into the active site of DNA \(\gamma\) (Fig. 4a). Using this model [19,20], the phosphorylated form of ZDV was fitted into the active site. This DNA pol \(\gamma\) model was compared by superimposition with the structure of the pre-and post-insertion complexes of HIV-RT with ZDV-MP primer-terminated DNA solved by Sarafianos and colleagues [21]. The pre-translocation site is the site of the incoming dNTP (in processive polymerization the elongation DNA primer is translocated from the pre- to post-translocation site). The superimposition of the DNA pol \(\gamma\) structural model and HIV-RT crystal structure was performed by superimposition of residues structurally and catalytically conserved between the respective DNA pol \(\gamma\) and HIV-RT enzymes. These included polymerase Motif A residues (DNA pol \(\gamma\) 882-900 with RT 102–120): polymerase Motif C residues (DNA pol \(\gamma\) 1128-1141 with RT 178-191); DNA pol \(\gamma\) R943 with RT K65, DNA pol \(\gamma\) K947 with RT K72 and DNA pol \(\gamma\) Y951 with RT Q151. In the backbone position of the residues involved in binding dNTP and metals (Mg+2) (Residues D890, D891 to E895 in DNA pol \(\gamma\) that corresponds to D110, V111 to Y115 in HIV RT in Motif A and D185 (HIV-RT)/D1135 (DNA pol \(\gamma\)), the D in the YMDD/HDVE Motif C superimpose and are structurally conserved with respect to their relative positions within the respective polymerase active sites. This finding provided a structural explanation for incorporation of these NRTIs into mtDNA by DNA pol \(\gamma\).

In the DNA pol \(\gamma\) model, the azido group of ZDV causes steric hindrance with residues Tyr951 and Lys947. In contrast, the structurally equivalent residues in HIV-RT (Gln151 and Arg72) provide adequate space for this azido group. This observed difference is partly accounted for by the smaller size of Gln compared to Tyr and by the fact that the R72 side chain in HIV-RT is on the other side of the helix whereas Lys947 in DNA pol \(\gamma\) faces into the active site. This analysis provides a structural explanation for the apparent paradox seen with Y951A DNA pol

*AIDS*. Author manuscript; available in PMC 2007 March 21.
γ mutant (but not the wild-type or Y951F mutant of DNA pol γ) incorporation of ZDV with improved efficiency [23].

Fig. 4b illustrates the differences in binding by d4T, 3TC, and ZDV and explains differential incorporation by DNA pol γ on a structural basis. For emphasis, d4T-TP is a relatively good DNA pol γ inhibitor in vitro (whereas ZDV-TP and 3TC-TP are only moderate inhibitors). A comparison between d4T and ZDV in the binding pocket illustrates the steric hindrance caused by the ZDV azido group. In contrast, d4T tightly fits into the DNA pol γ active site. DNA pol γ readily incorporates d4T and other dideoxynucleotides into mtDNA with efficiency of native nucleotides. These facts correlate with the Ki (Table 1). In the case of 3TC, the large sulfur is positioned in ways similar to that of ZDV’s azido group. This accounts for the similarity in moderate inhibition of DNA pol γ by these latter NRTIs (in comparison to that of d4T) and is due to steric conflicts with K947 and Y951.

Discussion

Targeted cardiac transgenic mice are useful to define features of cardiac dysfunction [24], features of AIDS heart disease [25], and the toxicity of NRTIs in AIDS [7,26,27]. In the present study, mitochondrial structural damage worsened and accompanied LV enlargement, mtDNA depletion, and decreased expression of Complex I gene products in DNC TGs treated with ZDV or d4T monotherapy. However, these changes were absent with TGs treated with either 3TC monotherapy or with vehicle.

In recent studies, DNC TGs treated with HAART combination therapy (that included ZDV or d4T) revealed cardiac dysfunction whereas treatment with a NRTI-sparing HAART regimen caused no such cardiac changes [6]. Taken together, those data pointed to a pathogenetic role for ZDV or d4T in the observed effects in vivo. Experiments here with NRTI monotherapy causally linked them to mitochondrial toxicity in vivo and related injury to mtDNA depletion and ultrastructural defects. The extreme toxicity of fialuridine (FIAU) in vitro and in vivo [10,28,29] precluded its inclusion in the present studies. In the TG model here, DNC appears to affect homeostasis of native nucleotides whose stoichiometry is further disrupted by the NRTIs. This model may have significant utility in evaluating mechanisms of mitochondrial toxicity of NRTIs which may be pathogenetically implicated in lipodystrophy [30–33].

A pathogenetic nexus of events occurs with the relative abundance of native nucleotides and NRTIs in mitochondria. These moieties serve as mtDNA precursors (i.e. native nucleotides) or inhibitors of DNA pol γ (NRTIs disrupt mtDNA replication, and lead to mtDNA depletion). Genetic ‘mtDNA depletion syndromes’ [34,35] cause mitochondrial and cytoplasmic nucleotide pools to be disturbed and may be used here to support this reasoning. It follows that ‘acquired mtDNA depletion’ is pharmacologically based [7,9]. The shared phenotype between both acquired and genetic mtDNA depletion rests in mitochondrial dysfunction, ultrastructural abnormalities, and organ dysfunction.

A principal focus of many groups [11,12,22,23,28,36–39] has been the exploration of mitochondrial toxicity of NRTIs in vivo and in vitro with an important focus to treat side effects of NRTI therapy such as lipodystrophy [33,40]. NRTIs become toxins at a ‘threshold concentration’ in mitochondria where they may compete with native moieties. As such, they may inhibit intramitochondrial phosphorylation of native nucleotides (by competing as substrates for kinases) and ultimately inhibit DNA pol γ. Both NRTI mono and triphosphates may serve as inhibitors of DNA pol γ (Table 1; [10–13,41]).

Attention in early experiments focused on inhibition of DNA pol γ (reviewed in [42]) because of clinical [16], enzyme kinetic (reviewed in [22,43]), and biological data [44,45] that supported the role of enzyme inhibition. NRTIs were linked to altered mtDNA replication.
The ‘DNA pol γ hypothesis’ [9] was strengthened to add oxidative stress and mtDNA mutations to a ‘mitochondrial dysfunction hypothesis’ [7,27], but the importance of intramitochondrial availability of NRTIs (and their phosphates), and homeostasis of mitochondrial pools of native nucleotides requires emphasis. Intra-mitochondrial abundance of native nucleotides and NRTIs affect mtDNA replication (and inhibition of the mtDNA replicase) at the level of the nucleotide substrate for DNA pol γ and some kinases. Pools of native nucleotides in mitochondria could be altered by the presence of NRTIs and their phosphorylated products on the basis of mass action [48].

Enzyme kinetics previously performed indicated that d4T-TP is an inhibitor of DNA pol γ [13], is utilized by DNA pol γ in vitro as efficiently as natural deoxynucleotides, whereas ZDV-TP and 3TC-TP are only moderate inhibitors of DNA chain elongation ([11,12,22]; Table 1). The inefficient excision of d4T and ZDV from DNA predicts persistence in vivo following successful incorporation [12]. Removal of 3′-terminal 3TC residues is 50% as efficient as natural 3′-termini. The DNA pol γ exonuclease activity is inhibited by ZDV-MP concentrations occurring in cells [41]. Although inhibitory effects of NRTIs relate to their incorporation and DNA chain termination, the persistence of these analogs in DNA and inhibition of exonucleolytic proofreading may contribute to mitochondrial toxicity. 3TC-TP is among those least likely to be incorporated yet among those most efficiently removed. These points help to explain aspects of the observation of lower mitochondrial toxicity of 3TC in vivo. Conversely, ZDV-TP is least likely to be incorporated by DNA pol γ. However, once ZDV-MP is incorporated, it is inefficiently removed by the DNA pol γ exonuclease. Inefficiency of removal of ZDV-MP may explain mtDNA depletion in that case. One subcellular defense against NRTI toxicity is DNA pol γ’s 3′→5′ exonuclease. Exonucleolytic function [49,50] is inhibited by nucleoside 5′-monophosphates [51] and by NRTI monophosphates [41].

3TC-TP is among those least likely to be incorporated yet among those most efficiently removed. These points help to explain aspects of the observation of lower mitochondrial toxicity of 3TC in vivo. Conversely, ZDV-TP is least likely to be incorporated by DNA pol γ. However, once ZDV-MP is incorporated, it is inefficiently removed by the DNA pol γ exonuclease. Inefficiency of removal of ZDV-MP may explain mtDNA depletion in that case. One subcellular defense against NRTI toxicity is DNA pol γ’s 3′→5′ exonuclease. Exonucleolytic function [49,50] is inhibited by nucleoside 5′-monophosphates [51] and by NRTI monophosphates [41].

ZDV may have other cellular targets. ZDV has been shown to inhibit thymidine kinase 2 (TK2) thus preventing the complete conversion of thymidine to dTTP [52]. Our recent finding in rat tissues suggest that dTTP concentrations are critical and rate limiting to mitochondrial DNA replication [48], to development of DNA precursor asymmetries in mammalian tissue mitochondria, and to mutagenesis through reduced replication fidelity [48]. Further reduction in dTTP pools by inhibition of TK2 may help explain the reduced mtDNA content in ZDV-treated cells and tissues.

In summary, TGs that expressed human DNC in the murine heart helped define in vivo mitochondrial toxicity from ZDV, d4T, and 3TC. The LV mass increased in TGs treated with either d4Tor ZDV, but not with 3TC. This was associated with decreased abundance of mtDNA and of polypeptides encoded by mtDNA, and with mitochondrial destruction. Our previous studies demonstrated the effects of transgenically expressed HIV [53] on the murine heart, along with effects of cardiac-targeted, specific HIV gene products [14,54]. Here, experimental results demonstrate that NRTIs cause mitochondrial side effects in vivo in doses used in humans. These results follow predicted interactions of the respective NRTIs at the DNA pol γ active site and the kinetic data in vitro. Last, these studies suggest that nucleotide and NRTI intramitochondrial homeostasis, as influenced by DNC, impacts toxicity of NRTIs.

Acknowledgements
Sponsorship: This work was supported by DHHS, NIH NHLBI R01 HL072707, R01 HL059798, and R01 HL063666 to W.L. and intramural NIH funds to W.C.C and R.J.B. Brian Hoit is thanked for his insightful comments on this manuscript.
References


38. Feng JY, Murakami E, Zorca SM, Johnson AA, Johnson KA, Schinazi RF, et al. Relationship between antiviral activity and host toxicity: comparison of the incorporation efficiencies of 2',3'-dideoxy-5-


Fig. 1. Quantitative analysis of echocardiographic images: left ventricular (LV) mass was calculated in a blinded fashion, code was broken, and data tabulated from the (a) zidovudine (ZDV), and (b) stavudine (d4T) monotherapy groups.

Data were normalized to body weight (mg/g) and plotted as mean ± SEM. In either transgenic (TG) protocol with nucleoside reverse transcriptase inhibitors (a, b), the TG cohort treated revealed increased LV mass after 35 days ($P < 0.05$; ANOVA). In the cohort treated with lamivudine (3TC) monotherapy, no significant difference in LV mass was found (not shown). BW, body weight; CMC, vehicle control; DNC, deoxynucleotide carrier; WT, wild type.
Fig. 2. Electron microscope views of mitochondria from transgenic (TG) and wild-type (WT) hearts: deoxynucleotide carrier (DNC) overexpression in the heart caused mitochondrial cristae reduplication (TG; a, b).

Addition of nucleoside reverse transcriptase inhibitors [either zidovudine (ZDV) or stavudine (d4T); 35 days] resulted in mitochondrial destruction, loss of cristae, and amorphous deposits (arrow in a, b). In contrast, no change in cardiac mitochondria was seen between vehicle and lamivudine treatment in the DNC TG or WT (not shown). Original magnification on electron microscope: 26 000 (marker indicates 1 μm).
Fig. 3. Real time polymerase chain reaction (PCR) mitochondrial (mt)DNA/nuclear (n)DNA ratios with nucleoside reverse transcriptase inhibitor treatment: transgenic (TG) and wild type (WT) cohorts were treated with (a) zidovudine (ZDV), (b) stavudine (d4T) or (c) lamivudine (3TC) monotherapy or with vehicle control for 35 days. Tissue samples were analyzed using real-time PCR. A decrease in mtDNA/nDNA ratio was found with both ZDV and d4T monotherapy (a, b) but not with 3TC monotherapy (c). TG exhibited a decreased mtDNA/nDNA ratio in comparison with WT. *P < 0.05; **P < 0.01; ***P < 0.001; NS, not significant.
The active site in DNA pol γ is shown in purple inserting ZDV-MP. This is superimposed over the equivalent location in HIV-RT (green). The azido group on ZDV has close contacts with K947 and Y951. These contacts do not occur in HIV-RT because the equivalent residues in HIV-RT, R72 is in the other direction off the helix, and Q151 is a smaller residue than Y951 and is not positioned as close to the ZDV azido group. (b) Superimposition of ZDV-MP (green), D4T-MP (red), and 3TC-MP (blue) in the active site model of DNA pol γ. D4T-MP fits into the active site similar to ddTMP or dTMP.
Table 1
Inhibition of DNA pol γ by phosphorylated nucleoside reverse transcriptase inhibitors (NRTIs).

<table>
<thead>
<tr>
<th>NRTI triphosphate</th>
<th>$K_i$ (reported; m mol/l)</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zidovudine</td>
<td>$K_i = 1.8; K_i' = 6.8$</td>
<td>[11]</td>
</tr>
<tr>
<td>Stavudine</td>
<td>$K_i = 0.001; K_i' = 0.008$</td>
<td>[12,13]</td>
</tr>
<tr>
<td>Lamivudine</td>
<td>$K_i = 15.8$</td>
<td>[55]</td>
</tr>
</tbody>
</table>