Impact of Nucleoside Reverse Transcriptase Inhibitors (NRTIs) on Mitochondrial DNA and RNA in Human Skeletal Muscle Cells

Running head: NRTIs and Mitochondrial Toxicity in Muscle Cells

Akihiko Saitoh\textsuperscript{1,2}, Richard Haas\textsuperscript{2,3}, Robert Naviaux\textsuperscript{2,4}, Neurita G. Salva\textsuperscript{1,2}, Justine K. Wong\textsuperscript{1,2}, Stephen A. Spector\textsuperscript{1,2}

\textsuperscript{1}Division of Infectious Diseases, \textsuperscript{2}Department of Pediatrics, \textsuperscript{3}Department of Neuroscience, \textsuperscript{4}Department of Medicine, University of California San Diego, La Jolla, CA.

Words, abstract: 247 words

Words, text: 3,546 words

1) Each author certifies that he or she has no commercial associations that might pose a conflict of interests in connection with the submitted article.

3) Financial Support: Supported by the Pediatric AIDS Clinical Trials Group and by Grants from the National Institute of Allergy and Infectious Diseases [5K23AI-56931 to AS and AI-39004, AI-27563, AI-33835, AI-41110; AI-36214 (Virology Core UCSD Center for AIDS Research), AI-32921].

4) Corresponding Author: Akihiko Saitoh, M.D.
Division of Infectious Diseases
Department of Pediatrics
University of California, San Diego
9500 Gilman Drive
La Jolla, CA 92093-0672
Telephone: (858) 534-7258
Fax: (858) 534-7411
Email: asaitoh@ucsd.edu

5) Key words: Didanosine, Nucleoside reverse transcriptase inhibitors (NRTI); Mitochondrial toxicity; Mitochondrial DNA; Mitochondrial RNA; Human skeletal muscle myoblasts; Myotubes
ABSTRACT

Background: We previously reported that 2',3'-dideoxyinosine (didanosine, ddI) significantly altered mitochondrial DNA (mtDNA) in peripheral blood mononuclear cells (PBMC) in HIV-1 infected children who had undetectable plasma HIV-1 RNA more than 2 years while receiving highly active antiretroviral therapy (HAART). This research examines the in vitro effects of nucleoside reverse transcriptase inhibitors (NRTIs) on mitochondria of human skeletal muscle cells (HSMCs) including myoblasts and differentiated myotubes.

Methods: Mitochondrial DNA, mitochondrial RNA (mtRNA), and messenger RNA (mRNA) levels for nuclear mitochondrial regulatory factors were quantified in vitro using HSMCs including myoblasts and differentiated myotubes treated with NRTIs singly and in combination.

Results: After five days of treatment, mitochondrial DNA was significantly decreased in myoblasts and myotubes treated with ddI (P < 0.001 and P = 0.01, respectively) and ddI-containing regimens (P < 0.001 and P < 0.001, respectively) compared to untreated cells. Mitochondrial RNA (MTCYB) was also significantly decreased in the myoblasts and myotubes treated with ddI (P = 0.004) and ddI-containing regimens (P < 0.001). Regardless of the NRTI regimens examined, NRTI combinations significantly decreased mtRNA (MTCO3) in myoblasts and myotubes (P = 0.02, P = 0.01, respectively). No significant differences were observed for nuclear mitochondrial regulatory factor mRNA in myoblasts or myotubes when treated with NRTIs (P > 0.07).
**Conclusion:** Didanosine and ddI-containing regimens significantly decrease mtDNA and mtRNA in HSMCs, most notably in myoblasts. These findings may be of particular importance in developing countries where ddI is widely used for first-line treatment of HIV-infected children.
INTRODUCTION

Nucleoside reverse transcriptase inhibitors (NRTIs) remain the backbone for the majority of highly active antiretroviral therapy (HAART) regimens in combination with protease inhibitors (PIs) or non-nucleoside reverse transcriptase inhibitors (NNRTIs) in HIV-1 infected patients (13). Mitochondrial toxicity, which is believed to result from depletion of mitochondrial DNA (mtDNA) by NRTIs interacting selectively with the DNA polymerase γ (POLG) (21, 25), is a major concern for HIV-infected patients receiving antiretroviral therapy (20). Mitochondrial dysfunction due to the depletion of mtDNA is at least partly responsible for various NRTI-associated adverse effects including myopathy, cardiomyopathy, peripheral neuropathy, pancreatitis, hepatic steatosis, lipodystrophy, and in severe cases, lactic acidosis (11).

Several studies have evaluated the impact of NRTIs on mtDNA using different in vitro models (14). Studies of human skeletal muscle cells (HSMCs) are generally considered one of the most clinically relevant in vitro indicators of potential mitochondrial damage in patients because mitochondrial toxicity often presents with muscular symptoms including muscle atrophy, weakness, fatigue, and cardiomyopathy.

Human skeletal muscle myoblasts are precursors of HSMCs and are committed to become differentiated muscle cells (32). Once myoblasts have migrated, differentiated into multi-nucleated skeletal muscle cells and fused into parallel arrays, they are referred to as myotubes. A few studies have evaluated the impact of NRTIs on mtDNA in differentiated myotubes (1, 2); however, no data are currently available regarding the
impact of NRTIs on mitochondria in myoblasts. The impact of NRTIs on myoblasts is of particular importance in infants and children who are actively differentiating myotubes from myoblasts, and carry more myoblasts in muscle than adults (8, 12).

Beyond the evaluation of mtDNA, levels of mtRNA and mitochondrial regulatory genes can provide additional information regarding the risk of clinically important mitochondrial damage. Although decreases in mtRNA have been found to coincide with the simultaneous upregulation of nuclear genes involved in transcriptional regulation of messenger RNA (mRNA) (24), such as mitochondrial transcriptional factor A (Tfam), no studies to date have assessed the impact of NRTIs singly or in combination on these indicators of mitochondrial function (14).

We previously reported that 2',3'-dideoxyinosine (didanosine, ddI) significantly altered mtDNA in PBMC of HIV-1 infected children with undetectable plasma HIV-1 RNA for more than 2 years while receiving HAART (30). This is particularly important for children who are potentially more vulnerable than adults to the adverse effects of antiretrovirals because of their long-term exposure and the possible negative impact on growth and development (26). The objective of this study was to investigate the effects of NRTIs used singly and in combination on mtDNA, mtRNA and mRNA expression for nuclear mitochondrial regulatory factors in human skeletal muscle myoblasts and myotubes. We have specifically focused on ddI because our previous data suggested that it may be particularly deleterious to mitochondria (30).
MATERIALS AND METHODS

In vitro assays for mitochondrial toxicity using human skeletal muscle myoblasts.

Proliferating human skeletal muscle myoblasts in a 24-well plate (Lonza, Walkersville, MD) were used to investigate the impact of NRTIs on mitochondria. The cells were isolated from post-gestational quadriceps or psoas muscle. Didanosine and 3’-deoxy-2’,3’-didehydrothymidine ( stavudine, d4T) were purchased from Sigma-Aldrich (St. Louis, MO) and 3’-azido-3’-deoxythymidine (zidovudine, AZT), 2’,3’-dideoxy 3’-thiacytidine (lamivudine, 3TC), and abacavir sulfate (ABV) were purchased from Moravek Biochemicals (Brea, CA). The concentrations of NRTIs used for this assay were based on the mean peak steady-state levels in human plasma during antiretroviral therapy (Cmax): ddI 11.8µmol, d4T 3.6µmol, AZT 7.1µmol, 3TC 8.3µmol and ABV 3.0µmol (31, 35). The myoblasts were treated with each NRTI and clinically relevant NRTI combinations including ddI + d4T, d4T + 3TC, AZT + 3TC and AZT + 3TC + ABV. All NRTIs were suspended in sterile water and diluted with the human skeletal muscle growth medium (Lonza) before being added into the wells to reach a total volume of 1.0 mL. As a control, myoblasts were incubated with the medium alone in triplicate. Myoblasts were incubated at 37ºC in 5% CO2; the medium with/without NRTIs was replaced every 2 days. The NRTIs were added to the cells at 20-30% confluence and cells were harvested on days 2 and 5 before reaching 70-80% confluence. Each evaluation of myoblasts was repeated five times.
In vitro assays for mitochondrial toxicity using human skeletal muscle myotubes. To derive differentiated myotubes, myoblasts at approximately 70-80% confluence had the media changed to differentiation media containing alpha-MEM (Gibco, Carlsbad, CA) with 10% fetal calf serum (Gibco), Penicillin/Streptomycin (100 U/mL and 100 µg/mL, respectively, Gibco) and incubated for 72 hr to achieve complete differentiation. The NRTIs were suspended in sterile water, diluted with the differentiation media and added into the wells to a total volume of 1.0 mL. The myotubes were incubated at 37°C in 5% CO₂ and the medium with/without NRTIs was replaced every 2 days. The cells were harvested on day 5. The assays for myotubes were repeated five times.

Evaluation of the cell proliferation. The cell counts in each well were estimated using Cell Proliferation Reagent WST-1 (Roche Diagnostics, Indianapolis, IN). After the cells were treated with NRTIs, 100 µL of WST-1 was added to each well and incubated at 37°C in 5% CO₂ for 1 hr. The supernatants were collected and transferred into 96-well plates in duplicate, and quantified by spectrophotometer at 450 nm. The ratios were calculated based on the absorbance values in the cells treated with NRTIs or NRTI combinations to the values in the cells without treatment (control); all assays were performed in triplicate.

Extraction of genomic DNA and RNA from myoblasts and myotubes. The cells were washed twice with phosphate saline buffer (PBS, Gibco) and trypsinized using Trypsin/EDTA solution (Lonza) and trypsin neutralized solution (Lonza) according to the manufacture’s protocol. Trypsinized cells were washed, resuspended with PBS, and
divided into two aliquots. Genomic DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA) and the genomic RNA was extracted using the QIAamp RNeasy kit (Qiagen).

**Quantitation of mitochondrial DNA and nuclear DNA using real-time PCR.** The mtDNA and the nuclear DNA (nDNA) were quantified by real-time PCR using a LightCycler (Roche Applied Science, Indianapolis, IN) as described previously (4). The results are expressed as a ratio of the mean mtDNA level to the mean nDNA level for a given extract in duplicate (mtDNA: nDNA, unit: copies/cell). The mean mtDNA ratios were calculated based on the mtDNA levels in the cells treated with NRTIs and NRTI combinations, compared to untreated controls.

**Quantitation of mitochondrial RNA and messenger RNA for DNA polymerase γ and mitochondrial transcriptional factor A using real-time PCR.** We developed novel assays to quantify the expression of mtRNA, evaluating functionally important mtRNA genes; *MTCYB* (the genes for cytochrome B of complex III) and *MTCO3* (the genes for cytochrome c oxidase subunits III of complex IV) (24). We also developed novel assays for messenger RNA (mRNA) for *POLG* and *Tfam*, which are critical factors to determine the levels of mtDNA (7, 17, 21, 25). The mRNA was quantified by a LightCycler (Roche Applied Science) using specific primers and probes (IT Biochem, Salt Lake City, UT) (Table 1) and LightCycler RNA Master Hybridization Probes (Roche Applied Science). For quantitation, standard curves for *MTCYB*, *MTCO3*, *POLG*, *Tfam* genes were generated using serially diluted DNA extracted from PBMC of HIV-negative donors.
Glucose-6-phosphate dehydrogenase (G6PDH) was selected as a housekeeping gene using the LightCycler h-Housekeeping Gene Selection Set (Roche Applied Science) because no difference was observed in the G6PDH mRNA levels in myoblasts or myotubes treated with NRTIs and NRTI combinations compared to those without treatment (P > 0.82). For quantitation, a standard curve for G6PDH was generated using LightCycler h-G6PDH Housekeeping Gene Set (Roche Applied Science).

Each assay included standards with predetermined copies of G6PDH and MTCYB, MTCO3, POLG and Tbam genes used as a reference. The samples of different NRTI treatments were assayed at the same time in the same run. Data were analyzed by RelQuant software (Roche Diagnostics) and expressed as a ratio of copies of MTCYB, MTCO3, POLG, Tbam and copies of G6PDH. The mean RNA ratios were calculated as the targeted RNA: G6PDH ratios in the cells treated with treatment and those in the cells without treatment. All assays including different NRTIs and NRTI combinations were run at the same time.

Statistical analyses. Statistical analyses were performed using SPSS 13.0 software (Chicago, IL). The Student T-test was used for comparison of numerical variables in two independent groups. The analysis of variance (ANOVA) test was used for the comparison of numerical variables in ≥3 independent groups. All P-values calculated were two-sided and a P-value of <0.05 was considered to be statistically significant.
RESULTS

**NRTIs did not affect myoblasts and myotubes cell numbers.** The myoblast and myotube cell counts with or without treatment estimated by the cell proliferating reagent (WST-1) did not differ between untreated cells and the cells treated with different NRTIs and NRTI combinations during the five day incubation period (range: 0.84 – 1.08, P = 1.00).

**Mitochondrial DNA changes between human skeletal muscle myoblasts and myotubes treated with NRTIs and NRTI combinations.** Mitochondrial DNA ratios were evaluated in myoblasts treated with NRTIs and NRTI combinations (Table 2). On day 2, the mtDNA ratios in myoblasts treated with ddI (P < 0.001) and ddI + d4T (P < 0.001) were significantly decreased compared to the myoblasts without treatment. In contrast, there was an increase in mtDNA ratios in myoblasts treated with AZT (P = 0.03) and AZT + 3TC + ABV (P = 0.02). Other NRTIs and NRTI combination did not change mtDNA ratios significantly (P = 0.18–0.39).

On day 5, the decline in mtDNA ratios in myoblasts treated with ddI and ddI + d4T were significantly lower compared to those in myoblasts without treatment (P < 0.001, P < 0.001, respectively) (Figure 1, Table 2). Similarly, significant increases in mtDNA ratios were observed in myoblasts treated with AZT containing regimens including AZT + 3TC (P = 0.003) and AZT + 3TC + ABV (P = 0.02) compared to those in myoblasts without treatment. Other treatments did not change the mtDNA levels significantly (P = 0.30–0.66) (Figure 1, Table 2).

Next, the impact of NRTIs on mtDNA in differentiated myotubes was investigated. The mtDNA ratios in myotubes treated with ddI (0.50 ± 0.19) and ddI +
d4T (0.38 ± 0.11) for five days were significantly decreased compared to untreated cells (1.00) (P = 0.01, P = 0.001, respectively). Of note, the impact of NRTIs was not as significant as those in myoblasts (P = 0.03, P = 0.16, respectively) (Figure 1). There were significant differences in mtDNA ratios in myotubes treated with AZT (P = 0.03), d4T + 3TC (P = 0.003), and AZT + 3TC (P = 0.01), but not with the other treatment (P = 0.21–0.56).

**Mitochondrial RNA changes in human skeletal muscle myoblasts treated with NRTIs.**

The mtRNA ratios including *MTCYB* and *MTCO3* in myoblasts treated with NRTIs and NRTI combinations did not change significantly on day 2 (P = 0.37, P = 0.94, respectively). However on day 5, the mtRNA ratios for *MTCYB* in myoblasts treated with ddI (0.51 ± 0.14, P < 0.001), ddI + d4T (0.38 ± 0.13, P < 0.001), d4T + 3TC (0.63 ± 0.17, P = 0.002), AZT + 3TC (0.74 ± 0.25, P = 0.05) were lower compared to those without treatment (1.00) (Figure 2A). Other treatment combinations did not change mtRNA expression for *MTCYB* in myoblasts significantly (P = 0.10-0.40). Myoblasts treated with ddI + d4T (0.61 ± 0.05, P < 0.001) and d4T + 3TC (0.71 ± 0.13, P = 0.001) showed significantly decreased mtRNA ratios for *MTCO3* compared to untreated cells (1.00) (Figure 2B). The decline of mtRNA ratios for *MTCO3* was not statistically significant in myoblasts treated with other drug combinations (P = 0.12-0.60).

Next, we evaluated the impact of overall NRTI combinations on mtRNA in myoblasts. The mtRNA ratios for *MTCYB* in myoblasts treated with NRTI combinations (0.65 ± 0.26) and those in myoblasts treated with single NRTI (0.81 ± 0.27) did not differ significantly (P = 0.08). However, for the mtRNA ratios for *MTCO3*, myoblasts treated
with NRTI combinations both including and excluding ddI were significantly lower than those in myoblasts treated with single NRTI (0.93 ± 0.20) (P = 0.02). When we compared the mtRNA ratios in MTCYB and MTCO3 between ddI or d4T alone and ddI + d4T combination in myoblasts and myotubes, significant additive results were observed in MTCO3 expression between ddI and ddI + d4T in myoblasts (P = 0.004) and myotubes (P = 0.05) as well as MTCTB expression between d4T and ddI + d4T in myoblasts (P = 0.004) and myotubes (P = 0.002).

**Mitochondrial RNA changes in human skeletal muscle myotubes treated with NRTIs.**
Similar effects of NRTIs and NRTI combinations on mtRNA were also observed in myotubes; however, the patterns of decline in mtRNA were different. On day 5, the mtRNA levels for MTCYB were significantly decreased in myotubes with different treatments (P = 0.03) (Figure 2A). The myotubes treated with ddI (0.66 ± 0.18, P = 0.004), d4T (0.80 ± 0.06, P < 0.001), ddI + d4T (0.45 ± 0.05, P < 0.001), and AZT + 3TC + ABV (0.74 ± 0.19, P = 0.02) demonstrated significant declines in mtRNA ratios for MTCYB compared to those without treatment. Similarly, the decline in mtRNA ratios for MTCO3 was different among myotubes treated with different treatments (P = 0.02) (Figure 2B). Significant declines were observed in myotubes treated with d4T (0.69 ± 0.11, P = 0.001), ddI + d4T (0.78 ± 0.12, P = 0.005), d4T + 3TC (0.75 ± 0.12, P = 0.002), AZT + 3TC (0.72 ± 0.16, P = 0.006), and AZT + 3TC + ABV (0.64 ± 0.17, P = 0.003). Similarly, the mtRNA ratios for MTCO3 in myotubes treated with NRTI combinations were consistently lower than the ratios for myoblasts treated with single NRTI (0.72 ± 0.13 vs. 0.91 ± 0.20, P = 0.01). No difference was observed for the mRNA ratios for
*MTCYB* between myotubes treated with a single NRTI compared to those treated with NRTI combinations (0.77 ± 0.26 vs. 0.85 ± 0.16, P = 0.20).

**Messenger RNA expression for POLG and Tfam in myoblasts and myotubes treated with NRTIs.** We evaluated the mRNA expression for *POLG* and *Tfam* in myoblasts and myotubes treated with NRTIs singly and in combinations. The treatment did not alter the mRNA ratios for *POLG* and *Tfam* on day 2 (P = 0.62, P = 0.62, respectively) and day 5 (P = 0.79, P = 0.70, respectively) in myoblasts. Similarly, no differences were observed in the mRNA ratios for *POLG* and *Tfam* on day 5 in myotubes (P = 0.44 and P = 0.07, respectively).
DISCUSSION

In the current research, we have shown that ddI and ddI-containing regimens are associated with the greatest degree of mtDNA suppression in HSMCs, most notably in myoblasts. The patterns of mtRNA decrease were different from those of mtDNA, particularly for cells treated with NRTI combinations. These findings suggest that evaluating markers beyond mtDNA can provide additional information on the potential adverse effects of these drugs on mitochondrial function.

To our knowledge, this is the first study that has investigated the impact of NRTIs and NRTI combinations on mitochondria in human skeletal muscle myoblasts, a cell type likely to be susceptible to mitochondrial damage in vivo. Myoblasts can differentiate into myotubes (32) and are more abundant in skeletal muscle of infants and children compared to adults (8, 12). Moreover, age is known to alter the potential of myoblasts to differentiate into myotubes (22) and to affect myoblast metabolism and proliferation (3). These differences are of particular interest because our data indicate that the decline in mtDNA resulting from ddI exposure was greater for myoblasts than myotubes. Thus, it is likely that children are more vulnerable than adults to the mitochondrial toxicity of ddI which could negatively impact growth and development. Recent in vivo data also demonstrated that mitochondrial damage at birth in monkey offspring exposed perinatally to AZT + ddI was severe and there was no improvement during the first year of life with significant reduction of mtDNA in muscle compared to other NRTI regimens (6). Of note, ddI is the only purine analog that is commonly used in developing countries in
contrast to other NRTIs such as AZT, and d4T, which are pyrimidine analogs. This is of
particular concern in developing countries where ddI is widely used as part of first-line
treatment of HIV-infected children (28, 37).

The decrease in mtDNA abundance is determined by either a decrease in
replication or an increase in degradation of mtDNA. Important factors for the replication
of mtDNA include the nuclear genes encoding the mtDNA-specific replication and
transcription factors such as POLG and Tfam. In our current study, the mRNA
expression for \textit{POLG} and \textit{Tfam} did not demonstrate any significant changes in HSMCs
treated with ddI. It is possible that a longer observation beyond five days used for our
study might have altered mRNA expression of \textit{POLG} or \textit{Tfam}. It is also possible that the
decline in mtDNA was partially due to other pathways including reactive oxygen species
production, uncoupling proteins, and depletion of deoxyribonucleotide triphosphate
(dNTP) pools in mitochondria (29). In contrast, the degradation of mitochondria by
autophagy, specifically called mitophagy (19), plays a central role in the degradation of
whole mitochondria and their contents (15). Therefore, the effects of each NRTI on
mitophagy may, in part, determine the degree of mtDNA and mtRNA degradation.

Interestingly, NRTIs were found to induce different patterns of decline between
mtDNA and mtRNA levels in HSMCs. Didanosine and ddI-containing regimens showed
a rapid decline in mtDNA levels, but slower and smaller declines in mtRNA. Moreover,
NRTI combinations seemed to lower the mtRNA levels in HSMCs significantly greater
than single NRTIs, demonstrating the cumulative negative impact of NRTI combinations
on mtRNA levels; a finding not observed in mtDNA levels. Recent data obtained using HepG2 cells also showed the similar negative effects of NRTI combinations on mitochondria (34). These findings combined with our current data suggest the importance of evaluating NRTI combinations and not relying on single drug studies if the impact of antiretrovirals on mitochondria is to be assessed in in vitro models (14) because patients take NRTI combinations as a part of HAART.

The decline in mtRNA levels in MTCYB by ddI and ddI-containing regimens was more pronounced than the decline in MTCO3 mtRNA levels, suggesting a differential impact of ddI on mitochondrial oxygen phosphorylation (OXPHOS) complexes. Importantly, MTCYB encoded subunit of respiratory complex III, and MTCO3 encoded subunits of respiratory Complex IV, both transcriptions are initiated by the same heavy strand promoter (P_H). Therefore, the difference may be explained by the instability of each mtRNA due to the different length of poly(A) tails at the 3’end of mtRNA in MTCYB and MTCO3 (9, 33). Our current data combined with a previous study using lymphoblast lines (10) suggests the importance of investigating additional markers beyond mtDNA, such as mtRNA levels, for the evaluation of mitochondrial toxicity. Further investigations are needed, however, to fully understand the long-term consequences of these mtDNA and mtRNA changes.

In agreement with our previous in vivo data (30), mtDNA significantly increased in myoblasts treated with AZT containing regimens. Similar findings are also observed in other in vitro models including human hepatoblastoma (HepG2) (5) and human skeletal muscle cells (2). The mechanisms of increase in mtDNA by AZT are still
unknown; however, these results strongly suggest that AZT upregulates genes encoding mtDNA. Because AZT causes significant mitochondrial damage in different in vitro models (14), the increase in mtDNA may reflect a compensatory response to mitochondrial dysfunction resulting from different causes including mtDNA polymerase γ (23), oxidative stress (16), and increases in mtDNA and mitochondrial mass by oxidative stress (18, 36).

There are limitations of this study. First, we do not know the actual concentrations of NRTIs in HSMCs, although the drug levels in culture were designed to approximate those used clinically. Even so, the intracellular drug concentrations used for this study may not reflect the concentrations in vivo. One study using an in vivo rat model demonstrated that intracellular concentration in rat hepatocytes of ddI was almost half the extracellular concentration, whereas the intracellular and extracellular concentrations of AZT were similar (27). Evaluating the assays using concentrations higher than 1X Cmax may be more informative for ddI. Second, although we demonstrated changes in mtDNA and mtRNA abundance, but the actual function of mitochondria was not evaluated in the current study. These include lactate production, mitochondrial membrane potential, and other relevant mitochondrial parameters. Finally, as noted earlier, the duration of treatment of two to five days may not reflect the drug effect on mitochondria in patients receiving long-term antiretroviral therapy.

In conclusion, we have shown that ddI and ddI-containing regimens in clinically relevant concentrations have a significant impact on mtDNA and mtRNA levels in human
skeletal muscle cells, most notably myoblasts. The results are consistent with our previously published \textit{in vivo} data demonstrating that mtDNA levels in PBMC were significantly affected by ddI. These data suggest that the use of ddI in children should include provider awareness of the potential of mitochondrial toxicity, especially in developing countries where ddI has been used widely as a first-line antiretroviral therapy.
ACKNOWLEDGEMENT

The authors acknowledge Carol Mundy, Joseph Sanding, and Mary Strauss at the University of California, San Diego for the help and assistance collecting blood samples from donors and Dr. Happy Araneta at the University of California, San Diego for the help performing statistical analyses.
REFERENCES


FIGURE LEGENDS

Figure 1. Mitochondrial DNA (mtDNA) levels in human skeletal muscle myoblasts and myotubes treated with different nucleoside reverse transcriptase inhibitors (NRTIs) and NRTI combinations. The white rectangles indicate the mean values of mtDNA ratios in human skeletal muscle myoblasts treated with NRTIs and NRTI combinations with treatment and those without treatment for five days. The black rectangles indicate the mean values of mtDNA ratios in human skeletal muscle myotubes treated with NRTIs and NRTI combinations with treatment and those without treatment for five days. For each experiment, the mean mtDNA ratios were calculated based on the values in the cells treated with NRTIs and NRTI combinations, compared to untreated controls, which were always set as 1.00. Each control or treatment in myoblasts and myotubes was repeated five times. No Tx: no treatment, ddI: didanosine, d4T: stavudine, AZT: zidovudine, 3TC: lamivudine, ABV: abacavir. The bar indicates ± 1 standard deviation (S.D.). * indicates P < 0.05 and ** indicates P < 0.01.

Figure 2. Mitochondrial RNA (mtRNA) levels in human skeletal muscle myoblasts treated with different nucleoside reverse transcriptase inhibitors (NRTIs) and NRTI combinations on day 5. (A) MTCYB: the gene for cytochrome B of complex III, (B) MTCO3: the gene for cytochrome c oxidase subunits III of complex IV. The white rectangles indicate the mean values of mtRNA ratios in human skeletal muscle myoblasts treated with NRTIs and NRTI combinations with treatment and those without treatment for five days. The black rectangles indicate the mean values of mtRNA ratios in human...
skeletal muscle myotubes treated with NRTIs and NRTI combinations with treatment and those without treatment for five days. For each experiment, the mean mtRNA ratios were calculated based on the values in the cells treated with NRTIs and NRTI combinations, compared to untreated controls, which were always set as 1.00. Each control or treatment in myoblasts and myotubes was repeated five times. No Tx: no treatment, ddl: didanosine, d4T: stavudine, AZT: zidovudine, 3TC: lamivudine, ABV: abacavir. The bar indicates ± 1 standard deviation (S.D.). * indicates P < 0.05 and ** indicates P < 0.01.
Table 1. Summary of primers, probes and PCR conditions for the analysis of mitochondrial RNA, messenger RNA for mitochondrial transcription factor A (Tfam) and DNA polymerase γ (POLG)

<table>
<thead>
<tr>
<th></th>
<th>MTCYB mRNA</th>
<th>MTCO3 mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Forward Primer</strong></td>
<td>5'-TCAGTAGACAGTCCCCACC-3'</td>
<td>5'-CGCTAAATCCCTAGAAAG-3'</td>
</tr>
<tr>
<td><strong>Reverse Primer</strong></td>
<td>5'-GCGTCTTTGATTGTGAT-3'</td>
<td>5'-CAATAATGACGTGAAGTG-3'</td>
</tr>
<tr>
<td><strong>Anchor Probe</strong></td>
<td>5'- TCATCTAACCCTCTATTGAGCC –FITC-3'</td>
<td>5'-CTCGCATCAGGAGTATCCACC-FITC-3'</td>
</tr>
<tr>
<td><strong>Sensor Probe</strong></td>
<td>5'-LCR640-AGCAGCCTCCACCTCC-Phos-3'</td>
<td>5'-LCR640-AGCTACCATTAGTAATAGAACAAAC-Phos-3'</td>
</tr>
<tr>
<td><strong>PCR Condition</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MgCl₂ concentration</td>
<td>5µM</td>
<td>7µM</td>
</tr>
<tr>
<td>Reverse transcription</td>
<td>55ºC for 600s</td>
<td>55ºC for 600s</td>
</tr>
<tr>
<td>Preincubation</td>
<td>95ºC for 30s</td>
<td>95ºC for 30s</td>
</tr>
<tr>
<td>Amplification</td>
<td>45 cycles</td>
<td>45 cycles</td>
</tr>
<tr>
<td></td>
<td>95ºC for 0s, 55ºC for 15s, 72ºC for 10s</td>
<td>95ºC for 0s, 55ºC for 15s, 72ºC for 10s</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Tfam mRNA</th>
<th>POLG mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Forward Primer</strong></td>
<td>5'-GGCTCTCCGGATTTG-3'</td>
<td>5'-CTGACATACCACGTACCC-3'</td>
</tr>
<tr>
<td><strong>Reverse Primer</strong></td>
<td>5'-CAACGCTGGGCAATTTC-3'</td>
<td>5'-GTAACGCAACCTGCAT-3'</td>
</tr>
<tr>
<td><strong>Anchor Probe</strong></td>
<td>5'-GACTGCCTCTCCTTTACAGCT-3' –FITC-3'</td>
<td>5'-TACTTACCCATGCTGTTGGCC-FITC-3'</td>
</tr>
<tr>
<td><strong>Sensor Probe</strong></td>
<td>5'-LCR640-TTGTGTATTTACCGAGGTGTCTTC-TCA-Phos-3'</td>
<td>5'-LCR705-AGAGTGCTGTAGAGTTTGC-Phos-3'</td>
</tr>
<tr>
<td><strong>PCR Condition</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MgCl₂ concentration</td>
<td>6µM</td>
<td>4µM</td>
</tr>
<tr>
<td>Reverse transcription</td>
<td>55ºC for 600s</td>
<td>55ºC for 600s</td>
</tr>
<tr>
<td>Preincubation</td>
<td>95ºC for 30s</td>
<td>95ºC for 30s</td>
</tr>
<tr>
<td>Amplification</td>
<td>45 cycles</td>
<td>45 cycles</td>
</tr>
<tr>
<td></td>
<td>95ºC for 0s, 55ºC for 15s, 72ºC for 10s</td>
<td>95ºC for 0s, 55ºC for 15s, 72ºC for 10s</td>
</tr>
</tbody>
</table>
### Table 2. Mitochondrial DNA ratios in myoblasts treated with NRTIs and NRTI combinations

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mitochondrial DNA (ratios to untreated cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 2</td>
</tr>
<tr>
<td>No Treatment</td>
<td>1.00</td>
</tr>
<tr>
<td>ddI</td>
<td>$0.51 \pm 0.15^{**}$</td>
</tr>
<tr>
<td>d4T</td>
<td>$1.20 \pm 0.27$</td>
</tr>
<tr>
<td>AZT</td>
<td>$1.45 \pm 0.30^{*}$</td>
</tr>
<tr>
<td>3TC</td>
<td>$1.16 \pm 0.07$</td>
</tr>
<tr>
<td>ABV</td>
<td>$1.19 \pm 0.34$</td>
</tr>
<tr>
<td>ddI + d4T</td>
<td>$0.42 \pm 0.10^{**}$</td>
</tr>
<tr>
<td>d4T + 3TC</td>
<td>$1.17 \pm 0.27$</td>
</tr>
<tr>
<td>AZT + 3TC</td>
<td>$1.05 \pm 0.15$</td>
</tr>
<tr>
<td>AZT + 3TC + ABV</td>
<td>$1.25 \pm 0.13^{*}$</td>
</tr>
</tbody>
</table>

* P < 0.05, **P < 0.01

ddI: didanosine, d4T: stavudine, AZT: zidovudine, 3TC: lamivudine, ABV: abacavir

For each experiment, the mean mitochondrial DNA ratios were calculated based on the values in the cells treated with NRTIs and NRTI combinations, compared to untreated controls, which were always set as 1.00.
Figure 1

* P < 0.05, ** P < 0.01

Mitochondrial DNA (ratios to untreated cells)

NRTIs

Myoblasts

Myotubes

No Tx  ddl  d4T  AZT  3TC  ABV  ddl + d4T  d4T + 3TC  AZT + 3TC  AZT + ABV
Figure 2

A. **MTCYB**

B. **MTCO3**