Longitudinal effects of thymidine analogues on mtDNA, mtRNA and multidrug resistance (MDR-1) induction in cultured cells

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Objectives: HIV nucleoside reverse transcriptase inhibitors (NRTIs) can cause mitochondrial toxicity. In spite of several studies performed on cells, little is known about their long-term effects on mitochondrial DNA (mtDNA), mitochondrial gene expression (mtRNA) and cellular protective mechanisms that such exposure may trigger. Our aim was to investigate the longitudinal effects of two thymidine analogue NRTIs, zidovudine and stavudine, on human cells, measuring their effects on the levels of mtDNA, mtRNA and on induction of the multidrug resistance (MDR) gene MDR-1.

Methods: K562 lymphoblastoid cells were treated for 74 days with zidovudine or stavudine concentrations corresponding to ~1, 20 x and 400 x those measured in plasma. Samples were collected longitudinally and assayed for mtDNA, mtRNA and MDR-1 mRNA levels by real-time quantitative PCR. mtDNA deletions were investigated by long PCR.

Results: Upon exposure to both zidovudine and stavudine, an early dose-dependent and transient increase in mtDNA content was observed. This was followed by a concurrent and transient elevation in both mtRNA and MDR-1 mRNA levels. Interestingly, the increase in mtRNA was most pronounced at low concentrations, whereas that of MDR-1 expression occurred at the highest concentrations only. No mtDNA deletions were detected under any conditions.

Conclusions: Cellular response to thymidine analogue NRTI exposure showed a complex, time- and dose-dependent pattern over time. We report for the first time that NRTIs can induce MDR-1 expression; however, this effect is delayed, possibly in response to oxidative damage or mitochondrial dysfunction. Our results indicate that longitudinal experiments may refine our knowledge about NRTI toxicity.

Keywords: stavudine, zidovudine, toxicity, in vitro, lymphoblastoid cells, long-term

Introduction

Nucleoside reverse transcriptase inhibitors (NRTIs) are common components of highly active antiretroviral therapy (HAART) regimens. Although targeted towards the viral polymerase, NRTIs can also affect human polymerase-γ, which can result in mitochondrial DNA (mtDNA) depletion. The mitotoxic effects of NRTIs have been investigated in cell culture models, predominantly in cross-sectional short-term experiments. Under these experimental conditions, NRTIs are toxic in most cell lines, causing mtDNA depletion and cytotoxicity after a few days of exposure. One study on the longitudinal long-term effect of exposing ovarian epithelial HeLa cells to high concentration (800 µM) zidovudine showed an initial mtDNA increase followed by depletion. Results on NRTI-induced cytotoxicity and changes in mtDNA and mtRNA content tend to vary depending on the type of cell or the tissue investigated. Induction of multidrug resistance (MDR) proteins plays an important role in modulating drug efficacy and toxicity. In cell culture models, HIV infection protease inhibitors and non-NRTIs were shown to induce MDR-1 expression, although NRTIs inhibited MDR-1 function. Surprisingly, little is known about MDR-1 expression in relation to NRTI exposure and mitochondrial toxicity.

The goal of this study was to longitudinally evaluate the effects of zidovudine and stavudine, two thymidine analogues commonly used in HAART in the developing world, on cultured cells’ mtDNA, gene expression (mtRNA) levels and MDR-1 induction.
**Materials and methods**

Tissue culture materials were purchased from Gibco (Invitrogen, Carlsbad, CA, USA), and zidovudine, stavudine and DMSO were purchased from Sigma Chemicals (St Louis, MO, USA). The lymphoblastoid cell line K562 (ATCC, CCL-243) was chosen because it is a peripheral blood mononuclear cell (PBMC) line representing the most commonly investigated cells in clinical studies. The cells were cultured in 25 mL T-flasks containing 6 mL of medium, in RPMI supplemented with 10% fetal bovine serum, and were maintained in the range of $5 \times 10^5$ to $2 \times 10^6$ cells/mL for 74 days. The medium was changed every 3 days and fresh NRTI-containing medium was prepared weekly. The cells were treated with zidovudine at final concentrations of 2.0, 40 and 800 μM, whereas stavudine was used at final concentrations of 0.5, 10 and 200 μM. We chose these concentrations such that the lowest of the three would fall at the low end of the physiologically relevant concentrations found in adult HAART patients, pregnant women and umbilical cord plasma. On the basis of recent literature, these range from 2.2 to 5.0 μM for zidovudine and from 0.5 to 5.0 μM for stavudine.19–21

The drugs were dissolved in DMSO (final concentration 0.5–2.0%), whereas stavudine was assayed to verify reproducibility of the results.

mtDNA deletions were investigated by PCR of two long fragments using MT16535F+MT8388R and MT7988F+MT708R at baseline and at days 3, 23, 44 and 74, using an Expand Long Template PCR System (Roche), and were analysed by agarose gel electrophoresis. Reaction conditions were 93°C/3 s, 58°C/30 s and 68°C/6 min for 45 cycles.

**Results**

K562 lymphoblastoid cells were exposed for 74 days to 2.0, 40 and 800 μM zidovudine or 0.5, 10 and 200 μM stavudine. The cells remained >95% viable throughout the entire experiment under all culture conditions. Exposure to both zidovudine (Figure 1a) and stavudine (Figure 1d) caused an early increase in the mtDNA/nDNA ratio, peaking at day 3, returning to the control level after ~10 days of exposure and remaining stable thereafter. This early mtDNA increase appeared to be dose-dependent, with the highest concentrations causing the highest increase, 6- to 8-fold compared with its respective control. Of note, a slow gradual loss of mtDNA was observed over time in both the treated and untreated cells, such that the longitudinal control culture was used to normalize each measurement. Longitudinal mitochondrial (CCOI) and MDR-1 gene expression showed a similar pattern of transient induction for both zidovudine and stavudine. mtRNA and MDR-1 levels remained stable over the early period during which mtDNA levels increased. For

<table>
<thead>
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<th>Oligonucleotide</th>
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<td>CCOI-R</td>
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<td>AAGATTATACAAAAATCTAGGGC</td>
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<td>fluorescein probe</td>
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<td>LC Red640 probe</td>
<td>LCre640-AAGACACACATCTACAACGTATTACGC-P</td>
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<tr>
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<td>forward primer</td>
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<td>LCre640-GGCATTTCTAAGTGGAAGCAAGCA-P</td>
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<tr>
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<td>MT16535F</td>
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FL, fluorescein; P, phosphate.

**Table 1. Study primers and probes**
both zidovudine and stavudine, mtRNA levels increased starting around day 16 and were most elevated (5- to 10-fold) in cells exposed to the lowest drug concentrations (Figure 1b and e). MDR-1 gene expression followed a similar pattern, but in contrast to mtRNA, induction of up to 14-fold maximal increase in zidovudine and 5-fold increase in stavudine-treated cells occurred only at the highest drug concentrations (Figure 1c and f). These increases in gene expression were transient and returned to the control level at around day 54 with MDR-1 appearing slightly delayed when compared with mtRNA. No mtDNA deletions were detected in any of the samples (data not shown).

**Discussion**

In this study, K562 cells tolerated exposure to zidovudine and stavudine well, in agreement with the results obtained by others, which enabled this long-term experiment. An early dose-dependent increase in the mtDNA levels was observed in all drug-exposed cells. mtDNA returned to control levels after 16 days. A similar observation was made recently in ovarian epithelial (HeLa) cells exposed to high concentrations of zidovudine. However, the latter study reported greater (~30%) mtDNA depletion after ~6 months in culture relative to an early culture time point than what was seen here. This difference might be because our results were expressed relative to longitudinal control untreated cells (0.1% DMSO).

As had been seen in HeLa cells, K562 cells showed increased mitochondrial gene expression in response to NRTI exposure. This increase was transient, and interestingly, its initial rise coincided with the time at which mtDNA returned to

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**Figure 1.** Effect of thymidine analogues on mtDNA, mitochondrial (COI) mRNA and MDR-1 mRNA levels. Cells were treated with 2.0 μM (open circles), 40 μM (grey triangles) and 800 μM (black squares) zidovudine, or 0.5 μM (open circles), 10 μM (grey triangles) and 200 μM (black squares) stavudine. All concentrations of zidovudine (a) and stavudine (d) caused a transient increase in the mtDNA level during the first week of treatment. For mtRNA levels, a transient increase was seen between days 16 and 54 of treatment. This effect was most pronounced at the lowest concentrations (b and e). Only the highest concentrations of zidovudine (c) or stavudine (f) significantly induced the expression of the multidrug-resistance protein gene MDR-1, starting after day 23. Each condition was cultured in duplicate flasks. Samples from one of the flasks were assayed in duplicate. For data points that showed changes compared with control cells, the second flask was also assayed in duplicate. Error bars represent the standard deviation of those assays. The y-axes depict the fold difference between the treated and control cells (treated/control) for each measurement.
control levels. Induction of mtRNA expression started after 16 days of exposure and was most pronounced under physiologically relevant thymidine analogue concentrations (2.0 μM zidovudine or 0.5 μM stavudine). Intermediate and high doses induced a muted response. The fact that mtDNA increase was greatest at highest drug concentration, whereas mtRNA expression showed the reverse, with highest mtRNA levels seen at lower drug concentrations, suggests that different drug concentrations trigger different compensating mechanisms, even within the same cell type. Low concentrations might be well tolerated, intermediate concentrations might cause changes in the expression pattern of relevant genes and high doses might trigger alternative routes such as the induction of drug-resistance genes, as seen here.

The non-simultaneous changes of mtDNA and RNA could represent a biphasic response to the same pressure: the early rise in mtDNA may reflect a slowing in cell growth along with some mitochondrial proliferation in response to drug-induced stress. The later increase in mitochondrial gene expression (mtRNA) may, however, reflect cellular adaptation to pressure on the mitochondrial transcriptional machinery through nucleotide pool imbalance or oxidative damage. As no definition or markers are known to differentiate between acute and chronic toxicities, longitudinal studies are useful to establish tools to distinguish between these two phases and study toxicity versus adaptation responses.

MDR-1 gene expression was induced after 23 days of drug exposure and only at the highest concentrations of zidovudine (800 μM) and stavudine (200 μM). In vitro studies suggest that free radicals trigger MDR-1 expression.23,24 Because damaged mitochondria are the major source of free radicals,25 mitochondrial toxicity caused by NRTIs may lead to increased oxidative stress in the form of free radicals, which, in turn, may explain the induction of MDR-1 seen here.26 However,cultured cells often rely on mitochondria-independent pathways such as glycolysis for their energy production,2 possibly leading to less mitochondrial transcriptional activity, as well as lower or delayed production of free radicals. This may partially explain why some cells can tolerate exposure to very high concentrations of drug. However, at very high drug levels such as the highest concentrations used here, increased mitochondrial gene expression may not be sufficient to protect the cells, requiring MDR-1 expression to lower drug exposure. Induction of MDR-1 expression has been reported in PBMCs following exposure to HIV protease inhibitors and non-NRTIs,17 as well as in the placental tissue of HAART-treated HIV pregnancy.27 To the best of our knowledge, this is the first report of MDR-1 induction by NRTIs and it would be of interest to determine whether NRTIs that typically show lower clinical toxicity such as lamivudine or abacavir would elicit a similar response.

In conclusion, our results show that physiologically relevant concentrations of zidovudine and stavudine can cause changes in mtDNA and mtRNA levels, even in this relatively tolerant lymphocyte-like cell line. The time-dependent pattern of the changes stresses the relevance of longitudinal studies to better understand the effects of NRTIs and the protective mechanisms that may modulate HIV therapy toxicity. Future studies of oxidative DNA and RNA damage and mitochondrial function in various cell types may further enhance our understanding of the long-term effects of nucleoside analogues.

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Transparency declarations
The University of British Columbia has a patent on the use of the mtDNA/nDNA real-time PCR assay used in this study on blood cells, of which H. C. F. C. is one of the inventors.

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