Role of Pyrimidine Depletion in the Mitochondrial Cardiotoxicity of Nucleoside Analogue Reverse Transcriptase Inhibitors

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Objective: Long-term antiretroviral treatment with nucleoside analogue reverse transcriptase inhibitors (NRTI) may result in a cardiomyopathy due to mitochondrial DNA (mtDNA) depletion. An intact mitochondrial function is required for the synthesis of intramyocellular pyrimidine nucleotides, which in turn are building blocks of mtDNA. We investigated if NRTI-related cardiomyopathy can be prevented with pyrimidine precursors.

Methods: Mice were fed with zidovudine or zalcitabine with or without simultaneous Mitocnol, a dietary supplement with high uridine bioavailability. Myocardia were examined after 9 weeks.

Results: Both NRTI induced a cardiomyopathy with mitochondrial enlargement, a disrupted cristal architecture on electron microscopy and diminished myocardial mtDNA copy numbers. The myocardial mtDNA-encoded cytochrome c-oxidase I subunit was impaired more profoundly than the nucleus-encoded cytochrome c-oxidase IV subunit. The myocardial formation of reactive oxygen species and mtDNA mutations was enhanced in zidovudine and zalcitabine treated animals. Mitocnol attenuated or normalized all myocardial pathology when given with both NRTI, but by itself had no intrinsic effects and no apparent adverse effects.

Conclusions: Zidovudine and zalcitabine induce a mitochondrial cardiomyopathy, which is antagonized with uridine supplementation, implicating pyrimidine pool depletion in its pathogenesis. Pyrimidine pool replenishment may be exploited clinically because uridine is well tolerated.

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indispensable for pyrimidine de novo synthesis\(^{16}\) and critical for cell survival.

As it has been recently shown in vitro that pyrimidine loss sensitizes and contributes to the mitochondrial toxicity of thymidine analogues,\(^{17}\) we aimed to explore the role of pyrimidine depletion in the pathogenesis of the NRTI-induced mitochondrial cardiomyopathy and to study if uridine, a nucleoside which replenishes pyrimidines via the salvage pathway, may be therapeutically employed to antagonize the onset of zidovudine cardiomyopathy. As a source of exogenous uridine, we used Mitocnol, a dietary supplement with a high uridine bioavailability.\(^{18}\)

**METHODS**

**Animals**

Seven-week-old BALB/c mice (Janvier, France) were divided into 6 groups. Group A animals (n = 10) were kept without any treatment and served as controls. Group B mice (n = 10) received 340 mg/kg/d of Mitocnol (Pharma Nord, Vojens, Denmark) in the drinking water. Groups C and D (n = 10 each) received zidovudine (100 mg/kg/d; MP Biomedicals, Strasbourg, France) in the drinking water. Groups E and F (n = 9 each) received 13 mg/kg/d of zalcitabine (Sigma, Taufkirchen, Germany). The dosages of zidovudine and zalcitabine corresponded to human dosage adjusted for body area and the higher drug disposal rate in rodents and was calculated on the basis of a daily liquid consumption of 5 mL.\(^{19}\) Groups D and F were cotreated with 340 mg/kg/d of Mitocnol in the drinking water.

Mice were killed by cervical dislocation at 16 weeks of age. Left ventricle, apex, and septum were snap frozen and cryopreserved. Aliquots were fixed in glutaraldehyde (3\%) for electron microscopy. All work was approved by our animal care protocol. Histopathology and Mitochondrial Ultrastructure

The severity and extent of the myocardial lesions was scored on a qualitative/quantitative morphological grading scale on apical heart sections (5 \(\mu\)m) stained with hematoxylin and eosin.\(^{20}\) Two randomly selected samples from each group were examined with electron microscopy by a person blinded to the treatment status of the animals.

**Myocellular Lipids**

Cardiomyocyte steatosis was assessed in oil red O stains. Lipids were also extracted from the myocardia using methanol/chloroform/water and quantified spectrophotometrically using a sulfo-phospho-vanillin reaction on lipid standards (Sigma, Taufkirchen, Germany). The severity and extent of the myocellular lipid accumulation was assessed quantitatively.

**MitDNA Copy Number**

Total DNA was extracted with the QIAamp DNA isolation kit (Qiagen, Hilden, Germany). MtDNA and nDNA copy numbers were determined on a Roche LightCycler 480 real-time polymerase chain reaction (PCR) system, as described.\(^{22}\) Amplifications of mitochondrial and nuclear products were performed in triplicates. Absolute mtDNA and nDNA copy numbers were calculated using serial dilutions of plasmids with known copy numbers.

**MtDNA-Encoded Respiratory Chain Protein**

The mtDNA-encoded subunit I of cytochrome c-oxidase (COX I) was quantified by immunoblot. The COX I signal was normalized to the expression of the subunit IV of cytochrome c-oxidase (COX IV), which is encoded by nuclear DNA.\(^{23}\)

**Lipid Peroxidation**

Malondialdehyde (MDA) is one of the end products of lipid peroxidation and an indicator of free radical production and oxidative stress. MDA was spectrophotometrically quantified in cardiac tissue using an assay for material reactive with thiobarbituric acid.\(^{24}\)

**Detection of the “Common” mtDNA Deletion**

MtDNA contains direct repeats between which base pairs may be deleted by slipped mispairing during replication.\(^{25}\) In humans, an age-related 4977 base pair (bp) deletion is the most frequent, so-called “common” deletion.\(^{25}\) In mice, a deletion almost identical in size to the human “common” deletion has been described.\(^{26}\) We probed for the murine “common” mtDNA deletion by amplifying 10 ng of genomic DNA with extradeletional primers F8265 (5’-AATTACAGGCTTCCGACACA-3’) and B13703 (5’-GAGATTTGGTTGATGTATGAG-3’) in a PCR. By choosing a short extension time (30 seconds), the deleted molecule was preferentially amplified as a 445 bp product. The identity of the amplicon was confirmed by sequencing (MWG Biotech, Ebersberg, Germany).

The “common” mtDNA deletion was quantified on agarose gels with Scion Image, using defined DNA ladder amounts as standard.

**Statistics**

Group means were compared by unpaired t test or Mann–Whitney analysis, as appropriate. Regressions were computed by exponential regression analysis (Sigma Plot 9.0, SPSS Inc, Chicago, IL).

**RESULTS**

**Macroscopic and Microscopic Pathology**

Daily fluid consumption, body weight, and heart weight were unaffected by zidovudine or zalcitabine treatment (data not shown). On post mortem examination, no clinical signs of cardiomyopathy were detected.

The histopathological cardiomyopathy score was increased after treatment with zidovudine (540% of untreated control values; \(P < 0.001\)) and with zalcitabine (313% of control values; \(P < 0.001\); Table 1). Cotreatment with Mitocnol significantly attenuated the zidovudine-induced and zalcitabine-induced histopathological lesions but did not have an intrinsic effect (Fig. 1).

On ultrastructural investigations, the mitochondria in the Mitocnol-only hearts did not show abnormalities (Fig. 2). The myofibrillar lattice of the cardiomyocytes of animals treated with zidovudine or zalcitabine in the absence of Mitocnol was...
TABLE 1. Effect of Uridine Supplementation With Mitocnol on Cardiac Muscle Histology, mtDNA-Encoded Respiratory Chain Subunits, mtDNA Content, Lipid Peroxidation (MDA Levels) and the “common” mtDNA Deletion in Mice Treated With Zidovudine or Zalcitabine

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
<th>Group D</th>
<th>Group E</th>
<th>Group F</th>
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<tr>
<td>Control</td>
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<tr>
<td>Mitocnol 340 mg/kg/d</td>
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<tr>
<td>Zidovudine 100 mg/kg/d</td>
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<td>9</td>
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<tr>
<td>Zidovudine 100 mg/kg/d + Mitocnol 340 mg/kg/d</td>
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<td>10</td>
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<td>Zalcitabine 13 mg/kg/d</td>
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<td>Zalcitabine 13 mg/kg/d + Mitocnol 340 mg/kg/d</td>
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<th>mtDNA Deletions</th>
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<td>“Common” mtDNA deletion (arbitrary units)</td>
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<th>Values represent group means (±SD).</th>
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<td>*P &lt; 0.001 vs. controls.</td>
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<td>†P &lt; 0.05 vs. controls.</td>
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<td>‡P &lt; 0.001 vs. mice treated without Mitocnol.</td>
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<td>§P &lt; 0.05 vs. mice treated without Mitocnol.</td>
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in disarray, the cardiomyocytes contained enlarged mitochondria in which the cristal architecture was disrupted. Cotreatment with Mitocnol normalized the myocardial ultrastructure.

Myocellular Lipids

Neither zidovudine nor zalcitabine augmented myocellular lipid deposition compared with untreated controls as evidenced by oil red O staining (data not shown) and biochemical quantification (Table 1). Zalcitabine, however, induced a slight, but quantitatively significant decrease of cardiac lipids. Mitocnol treatment per se did not alter myocardial lipids.

MtDNA Copy Number

In mice treated with zidovudine alone, the mean mtDNA copy numbers were diminished by 13% compared with control values (P = 0.02). Zalcitabine caused a similar mtDNA depletion (14%; P = 0.01). Whereas Mitocnol did not have an intrinsic effect on mtDNA copy numbers, this pyrimidine precursor fully normalized mtDNA copy numbers in zidovudine-treated and zalcitabine-treated animals. Among all animals, mtDNA copy numbers were inversely correlated with the cardiomyopathy score (r = −0.67; P < 0.001; Fig. 3A).

MtDNA-Encoded Respiratory Chain Subunits

The mean COX I/COX IV ratio was substantially reduced in the zidovudine group (57% of control values; P < 0.001) and to a lesser extent in the zalcitabine group (82%; P = 0.005) (Table 1). The COX I protein expression relative to COX IV was not altered in mice treated with Mitocnol alone. Mitocnol however attenuated the zidovudine-induced down-regulation of COX I expression relative to COX IV (P = 0.03). Furthermore, Mitocnol normalized the COX I expression in mice cotreated with zalcitabine. Among all mice, the COX I/COX IV ratio was inversely correlated with the cardiomyopathy score (r = −0.51; P < 0.001; Fig. 3B).

MDA Levels and Lipid Peroxidation

MDA levels are an indirect indicator of lipid peroxidation due to reactive oxygen species (ROS). Compared with controls, MDA levels were increased by 70% (P < 0.001) in hearts exposed to zidovudine, and by 106% (P < 0.001) in hearts exposed to zalcitabine (Table 1). Uridine supplementation normalized cellular MDA formation in both zidovudine-treated and zalcitabine-treated animals (both P < 0.05).

The cardiac MDA content was positively correlated with the cardiomyopathy score (r = 0.68; P < 0.001; Fig. 3C); mtDNA copy numbers were inversely correlated with MDA levels (r = −0.70; P < 0.001; Fig. 3D).

MtDNA Deletions

A 445 bp PCR product, corresponding to the “common” mtDNA 4974 bp deletion in mice, was identified in all zidovudine and zalcitabine-treated animals. In contrast, 70% of the myocardia of the control animals and 80% of Mitocnol-only myocardia did not harbor detectable amounts of the deletion. In all cases in which the “common” mtDNA deletion was detectable in the control or Mitocnol-only myocardia, the deletion quantity was lower than the lowest deletion quantity in the zidovudine or zalcitabine hearts.

On average, the quantity of the mtDNA mutation was increased by 433% in the zidovudine group compared with controls (P < 0.001), and by 454% in the zalcitabine group (P < 0.001; Table 1; Fig. 4). With Mitocnol cotreatment, the frequency of the mtDNA deletion relative to controls was 305% in the zidovudine group (P = 0.001) and 313% in the zalcitabine group (P = 0.002). Thus cotreatment with Mitocnol diminished the presence of the mtDNA common deletion significantly compared with NRTI without Mitocnol (both P < 0.001).

Among mice in which the “common” mtDNA deletion was detectable, its intensity was positively correlated with the myocardial MDA content (r = 0.60; P < 0.001; Fig. 3E).
The intensity of the “common” mtDNA deletion was inversely correlated with the COX I/COX IV ratio ($r = -0.52; P < 0.001$) and positively correlated with the cardiomyopathy score ($r = 0.70; P < 0.001$).

**DISCUSSION**

The present study analyzed the effects of uridine supplementation on NRTI-mediated mitochondrial toxicity in cardiac muscle. Our findings demonstrate that zidovudine and zalcitabine induce a cardiomyopathy in which mtDNA-encoded respiratory chain deficiency plays a significant role. The reduction of COX subunit I expression suggests that quantitative and qualitative mtDNA lesions are important if not pivotal in this form of respiratory dysfunction and does not support mitochondrial toxicity secondary to other mechanisms such as increased ROS formation.

Our results also exhibit that the marked mitochondrial cardiotoxicity can be antagonized by oral uridine supplementation and support a central role of intracellular pyrimidine pools in its pathogenesis. As discussed above, mtDNA depletion by zidovudine may result from the inhibition of mitochondrial TK2, with consecutive depletion of normal deoxypyrimidine pools as mtDNA building blocks.\(^{12,13}\) This mechanism operates predominantly in postmitotic tissues such as the cardiac muscle in which thymidine kinase 1 activity as an alternate pathway of thymidine phosphorylation is down-regulated.\(^{12,27}\)

Thymidine triphosphate concentrations are critical and rate limiting for mitochondrial DNA replication\(^{28}\) and reduced pyrimidine pools promote mutagenesis by reducing replication fidelity.\(^{28}\) Thus, the diminished thymidine triphosphate pools resulting from the inhibition of TK2 can explain the reduced wild-type mtDNA content and the increased prevalence of mtDNA deletions in zidovudine-treated myocardia.

The murine “common” mtDNA deletion\(^{26}\) was also present in the hearts of mice treated with zalcitabine, a polymerase gamma inhibitor not known to antagonize TK2 activity. MtDNA mutations with zalcitabine may arise from ROS which are produced by the defective respiratory chain.\(^{29,30}\) Mutagenic pyrimidine nucleotide depletion may also arise from defective de novo synthesis by dihydroorotate dehydrogenase secondary to the dysfunctional oxidative phosphorylation.\(^{28}\) Both mechanisms can explain our observation that ROS formation as assessed by myocardial MDA content was fully normalized with uridine supplementation.

Most importantly our study demonstrates that uridine supplementation attenuates all aspects of NRTI-related cardiomyopathy. The exact mechanism of the beneficial effect of uridine is not fully delineated, but it is likely that uridine

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**FIGURE 1.** Hematoxylin and eosin stains of representative apical heart muscle sections. Size bars 20 μm.
itself or its metabolites disinhibit mtDNA replication by competing with zidovudine and zalcitabine as pyrimidine nucleotides either at TK2 or at polymerase gamma. Alternatively, uridine may compete with pyrimidine NRTI at other steps of intracellular NRTI transport or phosphorylation. Uridine may in addition correct an intracellular pyrimidine deficit which in itself is sufficient to induce apoptosis. The dose of the uridine supplement used in this study deserves some consideration. The dosage of Mitocnol administered to mice in our study corresponded on a per body weight basis to a human dose of 24 g/70 kg, and on a per body surface area basis to 13 g/m² of Mitocnol. Mitocnol consists of 0.6 g of uridine plus 5.4 g of triacetyl uridine, a uridine precursor with several-fold higher uridine bioavailability over conventional uridine. Mitocnol constitutes the active ingredient of a commercially available dietary supplement named NucleomaxX. NucleomaxX is delivered in sachets (36 g), each of which contain 6 g of Mitocnol plus other substances to camouflage the bitter taste of uridine. Continuous administration of 3 sachets of NucleomaxX per day to men yields mean uridine plasma levels of 163 µM about 1 hour after administration. These uridine plasma levels are in a similar order of magnitude as those found to be protective against the mitochondrial toxicity of pyrimidine nucleoside analogues in vitro. This was the rationale for the dose administered to the mice in this study. In vitro studies have also indicated that it took considerably longer for mitochondrial toxicity to develop in the absence of uridine (weeks), than it took for uridine to abrogate established mitochondrial toxicity (days). This relatively quick therapeutic effect of uridine relative to the more prolonged development of mitochondrial toxicities in these in vitro studies may allow for intermittent dosing to “reset the mitochondrial clock”, an approach which has been tested in clinical trials of NucleomaxX for NRTI-related hepatotoxicity and lipoatrophy. A number of studies in indications not related to myocardial disease or HIV infection have demonstrated the safety and good tolerability of uridine and triacetyl uridine. High doses of uridine were also shown to not compete with NRTI at HIV reverse transcriptase. Thus our work may not only open a new avenue for the therapy of NRTI-related cardiotoxicity in HIV patients and potentially also for the cardiomyopathies associated with inherited mtDNA mutations.

ACKNOWLEDGMENT

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FIGURE 3. Correlations between the severity of the cardiomyopathy, markers of mitochondrial function, and myocardial ROS production. Of note, Mitocnol-only, zidovudine + Mitocnol, and zalcitabine + Mitocnol, and control animals are placed in similar areas of the graphs, whereas zidovudine-only and zalcitabine-only animals are clustered in different areas, indicating that Mitocnol abrogates or attenuates the effects of zidovudine and zalcitabine.
Balcarek et al J Acquir Immune Defic Syndr • Volume 55, Number 5, December 15, 2010

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30. Larsson NG, Holme E, Kristiansson B, et al. Progressive increase of the mitochondrial DNA ladder standard. Compared with untreated control (lane 2) or Mitocnol-only–exposed hearts (lane 3), PCR products from zidovudine (lane 4), zidovudine + Mitocnol (lane 5), zalcitabine (lane 6) and zalcitabine + Mitocnol (lane 7) hearts were more abundant. Mitocnol cotreatment, however, diminishes the prevalence of the “common” mtDNA deletion. Lane 8: PCR without DNA template.


