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In vitro supplementation with deoxynucleoside monophosphates rescues mitochondrial DNA depletion

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Abstract

Mitochondrial DNA depletion syndromes are a genetically heterogeneous group of often severe diseases, characterized by reduced cellular mitochondrial DNA content. Investigation of potential therapeutic strategies for mitochondrial DNA depletion syndromes will be dependent on good model systems. We have previously suggested that myotubes may be the optimal model system for such studies. Here we firstly validate this technique in a diverse range of cells of patients with mitochondrial DNA depletion syndromes, showing contrasting effects in cell lines from genetically and phenotypically differing patients.

Secondly, we developed a putative therapeutic approach using variable combinations of deoxynucleoside monophosphates in different types of mitochondrial DNA depletion syndromes, showing near normalization of mitochondrial DNA content in many cases. Furthermore, we used nucleoside reverse transcriptase inhibitors to precisely titrate mtDNA depletion in vitro. In this manner we can unmask a physiological defect in mitochondrial depletion syndrome cell lines which is also ameliorated by deoxynucleoside monophosphate supplementation. Finally, we have extended this model to study fibroblasts after myogenic transdifferentiation by MyoD transfection, which similar to primary myotubes also showed deoxynucleoside monophosphate responsive mitochondrial DNA depletion in vitro, thus providing a more convenient method for deriving future models of mitochondrial DNA depletion.

Our results suggest that using different combinations of deoxynucleoside monophosphates depending on the primary gene defect and molecular mechanism may be a possible therapeutic approach for many patients with mitochondrial DNA depletion syndromes and is worthy of further clinical investigation.

Keywords

Mitochondrial disease; Mitochondrial DNA depletion; In vitro supplementation

1. Introduction

Mitochondrial DNA depletion syndrome (MDS) is defined as a profound reduction of mitochondrial DNA (mtDNA) in different tissues, leading to a heterogeneous group of severe and usually lethal diseases in infancy and childhood [1,2]. So far, mutations of nine nuclear genes (*DGUOK*, *MPV17*, *POLG*, *TYMP*, *TK2*, *SUCLA2*, *SUCLG1*, *RRM2B*, *PEO1*) coding for enzymes and proteins involved directly or indirectly in mtDNA replication or in nucleotide metabolism were identified in ~70% of all MDS cases implying further genetic heterogeneity [3–7]. MDS may initially affect single organs, typically skeletal muscle or liver, and later spread to other tissues causing severe hepatoencephalopathy or encephalomyopathy [4,8].

Three main clinical presentations of MDS are known: myopathic, encephalomyopathic and hepatocerebral. However, the clinical phenotypes are heterogeneous, overlapping and expanding [7]. Although there are different disease genes leading to MDS with variable clinical presentations, the common pathway in all types of MDS is either the impairment of the balanced supply of nucleotides for mtDNA synthesis or a defect in mtDNA replication machinery [8].

Mutations in deoxyguanosine kinase (*DGUOK*, *dGK*) [9], polymerase gamma (*POLG*) [10], *MPV17* [11] and Twinkle (*PEO1*) [12] lead to the hepatocerebral form of MDS. It is probably the most common variant of MDS (OMIM#251880) and the onset of symptoms is often in the first year of life. The mitochondrial DNA polymerase gamma (pol γ) is essential for mitochondrial DNA replication and repair [13,14]. More than 150 *POLG* mutations have been reported to date (<http://tools.niehs.nih.gov/polg/>) with both autosomal recessive and dominant inheritance and many of these mutations may also cause different neurological phenotypes, such as progressive external ophthalmoplegia (PEO), sensory-axonal neuropathy, ataxia and parkinsonism [15]. Patients with *POLG* mutations comprise the largest single group of individuals with MDS and the prevalence of Alpers–Huttenlocher syndrome, one of the major *POLG* phenotypes, is around 1 in 100,000 [16]. It was first demonstrated for mutations in *POLG*, that multiple mtDNA deletions and mtDNA depletion can both be the manifestation of mutations in the same gene, resulting in defective mtDNA maintenance.

Recently it became evident, that in addition to *POLG*, other genes (*PEO1*, *RRM2B*, *TK2*) previously thought to result in either mtDNA depletion or multiple mtDNA deletions, may also lead to both, and the clinical phenotype can be more variable than suggested previously [17–19].

RRM2B was demonstrated to be the disease gene for profound muscle mtDNA depletion [20] and leads to the myopathic form of MDS (OMIM#609560). Children carrying mutations in the *RRM2B* gene show myopathy in various combinations with hypotonia,

tubulopathy, seizures, respiratory distress, diarrhea, and lactic acidosis and a severe depletion of mtDNA [20,21]. Some patients may have Kearns–Sayre Syndrome with milder course and longer survival [18] or a myoneurogastrointestinal encephalomyopathy (MNGIE)-like phenotype [22]. Heterozygous *RRM2B* mutations were reported in patients with PEO and multiple mtDNA deletions [19,22]. The nature of the individual pathogenic mutations (nonsense, missense) and the residual amount of functional enzymes may play a major role for the disease variability [23].

We have shown earlier that myotubes represent an excellent model system for studying post-mitotic tissues to investigate the effect of different therapeutic approaches in mtDNA depletion [24]. Myotubes of patients with *DGUOK* and *POLG* deficiency showed a highly significant decrease of mtDNA copy number if compared to control myotubes [24]. MtDNA depletion in *DGUOK* deficient patient myotubes was rescued by dAMP/dGMP supplementation in contrast to *POLG* deficient cells where this supplementation led to a mild, and not significant increase of mtDNA copy number [24], suggesting that the underlying mechanism leading to mtDNA depletion is different in these conditions. The improvement of supplementation with dAMP/dGMP in *DGUOK* deficiency can be explained by the fact, that the synthesis of these two dNMPs is impaired due to the genetic defect of the *DGUOK* enzyme. In *POLG* deficiency there is an overall decrease in the synthesis of mtDNA and supplementation with only two nucleotides could impair the balanced supply, however a mild improvement was observed in mtDNA copy numbers in differentiated myotubes. We decided to further explore the possibility of a therapeutic effect of nucleotide supplementation in different types of mtDNA depletion and we extended our studies for mtDNA depletion due to *POLG* and *RRM2B* mutations.

Our previously published cellular model showed some limitations. Although in myotubes of patients carrying *DGUOK* and *POLG* mutations we detected a significant mtDNA depletion and there was no biochemical deficiency of the respiratory chain enzymes, suggesting that the severity of mtDNA depletion did not reach a biochemical threshold [25]. To further decrease mtDNA copy numbers in myotubes leading to a biochemical defect, we used ethidium bromide in vitro [24]. Ethidium bromide resulted in very severe, irreversible mtDNA depletion both in patients and control cells and because of the deleterious effect of severe mtDNA depletion the number of surviving cells was too low to perform biochemical measurements. To reduce the limitations and improve our cellular model to study mtDNA depletion, we explored whether i) MyoD transfected fibroblasts can be used to study mtDNA depletion, and ii) addressed the applicability of nucleoside reverse transcriptase inhibitor (NRTI) supplementation in further inducing mtDNA depletion.

NRTIs induce a less severe mtDNA depletion compared to ethidium bromide. They represent one of the primary options for the treatment of human immunodeficiency virus (HIV) infection, despite their known side effect of impairing mitochondrial function [26]. In patients suffering from HIV infection or malignant hematological diseases, several NRTIs are being used for therapeutical purposes and many of these drugs have been used in different cellular models to investigate or modify mitochondrial function [26–28]. As previously described, time- and dose-dependent mtDNA depletion was observed with didanosine and stavudine in skeletal muscle cells [26], and mtDNA depletion preceded or

coincided with decreased expression of mtDNA-encoded cytochrome *c* oxidase subunit II (COX II) [27]. We therefore explored the use of different NRTIs as a modifier of mtDNA copy numbers and respiratory chain function in muscle cells of patients with different types of mtDNA depletion.

2. Materials and methods

2.1. Cell cultures and growth conditions

Cell culture was performed as previously described [24]. For supplementation studies the cells were incubated with serum-reduced fusion medium which was supplemented with dAMP/dGMP or with a mixture of all dNMPs (dAMP/dCMP/dGMP/dTMP) in two different concentrations (200 μ M and 400 μ M) during fusion (dNMP mix). The concentrations were calculated for each of the nucleotides separately in the mixture.

To induce mtDNA depletion, myoblasts were grown in skeletal muscle growth medium (SGM) in the presence of didanosine (2′3′-dideoxyinosine; ddI; Sigma-Aldrich, Munich, Germany), stavudine (2′3′-didehydro-3′deoxythymidine; d4T; Sigma-Aldrich, Munich, Germany) and abacavir (Ziagen® 20 mg/ml, GlaxoSmithKline GmbH & Ko, Munich, Germany) for 3 days. Cells were kept between 30% and 80% confluence with excess fresh medium to assure exponential growth. Afterwards differentiation and fusion into multinucleated myotubes were induced by replacing SGM with serum-reduced fusion medium supplemented with didanosine, stavudine and abacavir, respectively.

2.2. Differentiation studies

After 48 h of incubation with abacavir, 0.3×10^6 myoblasts were seeded in 6-well plates containing laminin-coated glass cover slips. Differentiation and fusion into multinucleated myotubes were induced after 24 h with serum-reduced fusion medium for 6 days.

Subsequently beta-dystroglycan staining was performed as previously described [24].

The number of nuclei was determined by DAPI staining. At least 1000 nuclei of control cells were considered in 5–10 randomly selected fields and compared with the number of nuclei of treated cells at the same area. The fusion index was calculated using the formula:

$$FI = [(\text{no. of nuclei within myotube structures}) / (\text{total no. of nuclei in the field}) \times 100]$$

Cell line	Number of nuclei	Number of fields	Area [mm ²]
cx (163)	1106	9	3.4 mm ²
cy (363)	1000	6	2.3 mm ²
p6 (16)	1162	5	1.9 mm ²

After 6 days of fusion (9 days of total incubation) cells were harvested for DNA extraction using High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany).

2.3. MyoD transfection

Since muscle cells from further patients with *POLG* deficiency were not available, we applied an adenoviral vector (MyoD Recombinant Adenovirus, Biocat GmbH, Heidelberg, Germany) to deliver the MyoD gene to both control and patient-derived fibroblasts. Therefore fibroblasts were grown in proliferation medium (Dulbecco's modified Eagle's medium supplemented with 15% fetal calf serum and 2 mM glutamine). At a confluence of 75%, cells were infected with the adenoviral vector [29]. The virus was diluted with infection medium (Dulbecco's modified Eagle's medium supplemented with 2% horse serum and 2 mM glutamine) to a concentration of 75 plaque-forming unit (pfu) per cell. Cells were grown in the presence of virus solution for 12 h, rinsed once with PBS and afterwards incubated with differentiation medium (Dulbecco's modified Eagle's medium supplemented with 2% fetal calf serum and 2 mM glutamine) for 6 days. All cell culture studies were done in triplicates.

2.4. Immunofluorescence

Myoblasts were differentiated into multinucleated myotubes by replacing virus solution with serum-reduced fusion medium (Dulbecco's modified Eagle's medium supplemented with 2% horse serum and 2 mM glutamine) on laminin-coated glass cover slips. After 6 days, coverslips were briefly washed in phosphate-buffered saline (PBS), fixed in 3.7% formaldehyde (freshly prepared from paraformaldehyde) in PBS for 15 min at room temperature and permeabilized with 0.1% Triton X-100 in PBS for 10 min.

After three washes in PBS, blocking of unspecific binding sites with 5% horse serum in PBS for 1 h was followed by incubation with the primary antibody (1:25; desmin; rabbit polyclonal; CellSignaling, Danvers, USA and 1:50; alpha-actinin; mouse monoclonal; Sigma, Saint Louis, USA) diluted in PBS containing 5% horse serum over night at 4 °C. After three washes in PBS, cells were incubated with the secondary antibody conjugated to Texas Red (1:200; anti-rabbit IgG; Dianova, Hamburg, Germany) and FITC-conjugated AffiniPure F(ab')₂ (1:100, anti-mouse IgG; Jackson ImmunoResearch, USA) for 1 h at room temperature.

The nuclei were visualized using bisbenzimidazole H 33258 (40 µg/ml; Sigma, Saint Louis, USA) and finally, after three washes in PBS, the coverslips were mounted in DAKO fluorescent mounting medium (DAKO, Carpinteria, CA, USA) and sealed with nail polish.

Digital images were captured using a Zeiss Axiovert 200 M fluorescence microscope and a Zeiss AxioCam HR photo camera.

2.5. Biochemical measurement for cytochrome c oxidase (COX)

Biochemical measurement was performed as described [30].

2.6. Immunoblotting

Ten micrograms of protein from cell homogenates was loaded on 10% polyacrylamide gels. The following antibodies were used according to the manufacturer's recommendations: anti complex IV subunit II (1:1000, mouse monoclonal, Moleculares Probes, Darmstadt,

Germany), anti complex II 70 kDa Fp subunit (1:10,000, mouse monoclonal, Invitrogen, Darmstadt, Germany), anti alpha-tubulin (1:10,000, rabbit monoclonal, CellSignaling, Danvers, USA) and secondary anti-mouse IgG coupled to horseradish peroxidase (HRP) (1:5000, Dako, Carpinteria, CA, USA) and anti-rabbit IgG coupled to HRP (1:5000, CellSignaling, Danvers, USA). Signals were visualized with enhanced chemiluminescence (ECL plus; Amersham Pharmacia Biotech) according to the manufacturer's recommendations.

2.7. DNA analysis

MtDNA and nuclear DNA copy numbers and mtDNA/nDNA ratios in DNA samples extracted from the different cells were determined by real-time PCR using a fluorescent temperature cycler (Light Cycler, Roche Molecular Biochemicals, Mannheim, Germany) as previously described [24].

3. Results

3.1. dNMP supplementation in different types of mtDNA depletion

3.1.1. dNMP supplementation leads to an increase of mtDNA copy number in *POLG* deficient myotubes—We performed supplementation studies with a combination of all four deoxynucleoside monophosphates (dNMP) in two patient cell lines (p1, p2) with a very different clinical presentation carrying pathogenic autosomal recessive mutations in the *POLG* gene and in controls (Table 1). We added a mixture of dNMPs (dAMP/dCMP/dGMP/dTMP) for 6 days of differentiation. This supplementation led in both *POLG* deficient cells (p1, p2) to a significant ($p < 0.05$) increase of mtDNA copy number (Fig. 1). No effect was detected in control myotubes. We note that p2, who had a very mild clinical phenotype (late-onset PEO) had a higher original mtDNA copy number, than p1 with a severe childhood onset fatal Alpers syndrome. The supplementation studies were performed with two different concentrations of nucleotides respectively (200 μ M and 400 μ M for each dNMP), which both had nearly the same effect (Fig. 1).

To investigate more cell lines carrying different *POLG* mutations, we applied an adenoviral vector to deliver the MyoD gene to 3 additional fibroblast lines of patients with *POLG* deficiency (p6, p7, p8, Table 1) and controls. This method has been successfully used to derive myogenic cells from fibroblasts [31–35]. After infection of the target cells, fusion into multinucleated myotubes was started by serum-deprivation. Six days after infection, the myogenic transdifferentiated cell cultures were screened for mtDNA depletion. Myogenic transdifferentiation after adenovirus-mediated MyoD gene transfer was confirmed by immunofluorescence of the two myoblast markers desmin and actinin (Fig. 1C). After differentiation (6 days), we detected an increase of mtDNA copy number in both patients (p7, p8) and in a control (c5). Due to mutations in the *POLG* gene, the increase in patient cells was lower compared to control myotubes. Supplementation with 400 μ M dNMP mix led to a significant ($p < 0.05$) increase of mtDNA/nDNA ratio in all patient cells (p6, p7, p8) if compared to untreated cells, but not in the control cell line (Fig. 1B).

3.1.2. Human primary myoblasts and myotubes carrying mutations in *RRM2B* showed mtDNA depletion—Myoblasts derived from a patient (p5) with *RRM2B* deficiency had a significantly lower mtDNA copy number compared to control cells, and differentiation to myotubes did not lead to a further decrease of the mtDNA copy number if compared to myoblasts. There were also no changes in the mtDNA copy number observed after supplementation with 400 μ M dNMP mix during differentiation and fusion into myotubes (Fig. 2).

3.2. Triggering mtDNA depletion with nucleoside reverse transcriptase inhibitors (NRTIs)

3.2.1. Supplementation with abacavir resulted in increased mtDNA copy numbers in *POLG* and *DGUOK* deficient myotubes—Myoblasts from two patients with *DGUOK* deficiency (p3, p4) and one control cell line (c1) were supplemented with abacavir, an analog of guanosine. We used this NRTI in *DGUOK* deficiency, because of the direct involvement in the guanosine nucleotide metabolism. We used different concentrations of abacavir (10 μ M, 100 μ M, 300 μ M) for 9 days (3 days before differentiation and 6 days during differentiation and fusion into myotubes). The treatment with abacavir resulted unexpectedly in a dose dependent, but not statistically significant trend to increased mtDNA copy numbers in both patient cells and controls (Fig. 3A). However, abacavir treated cells showed different cellular morphology compared to untreated myotubes, more similar to myoblasts. To investigate whether differentiation and fusion into multinucleated myotubes were complete, a beta-dystroglycan staining was performed and showed impaired differentiation (Fig. 3B). Supplementation with 300 μ M abacavir for 9 days resulted in a decreased cell division and a decreased fusion index, both control and patient myotubes contained lower number of myonuclei than untreated cells (Table 2).

The myoblast fusion index was calculated 6 days after inducing differentiation. Both control cell lines (c3, c4) showed a smaller fusion index after incubation with abacavir, and the average number of nuclei in myotubes was much lower in both controls (c3, c4) and in patient myotubes (p3) (Table 2). Furthermore cell division was impaired in all cell lines after 9 days of treatment, which was detected by counting nuclei in randomly selected fields (Table 2). Incubation with abacavir led to a severe impairment of fusion capacity in both control and patient cell lines. We conclude that the increased mtDNA/nDNA ratio caused by abacavir is not a functional improvement, rather a consequence of impaired differentiation. However, why abacavir affects differentiation remains unclear.

3.2.2. Augmentation of mtDNA depletion with didanosine and stavudine—In order to enhance mtDNA depletion we performed supplementation with two other NRTIs affecting the metabolism of different nucleotides, didanosine (2′3′-dideoxyinosine; ddI) and stavudine (2′3′-didehydro-3′ deoxythymidine; d4T). After 9 days (3 days before, 6 days during differentiation) of incubation with didanosine (300 μ M) and stavudine (300 μ M) significant mtDNA depletion was observed in controls and myotubes of patients with *POLG* (p1, p2) and *DGUOK* deficiency (p3, p4). Stavudine caused a milder decrease of the mtDNA copy number compared to didanosine (Fig. 4A). No decrease of the mtDNA copy number was detected after supplementation with different concentrations of azidothymidine (data not shown). To fine-tune the effect of didanosine we performed supplementation with

two different lower concentrations (5 μ M and 50 μ M). Myoblasts from patients with *DGUOK* deficiency (p3, p4) and a control cell line (c1) were incubated with didanosine for 9 days (3 days before and 6 days during differentiation and fusion into myotubes). After supplementation with 50 μ M didanosine, a significant ($p < 0.05$) decrease of mtDNA copy number was detected in both patient myotubes (p3, p4) and controls (c1), compared to untreated cells. When in addition to didanosine (both 5 μ M and 50 μ M) dAMP/dGMP (400 μ M) was added to the cell culture medium, mtDNA copy numbers were restored ($p < 0.05$) in all cell lines (Fig. 4C).

3.2.3. Didanosine supplementation triggers COX deficiency on biochemical and immunoblotting studies—

To investigate whether the more severe mtDNA depletion observed during didanosine treatment was associated with a functional defect in *DGUOK* deficient cells, we performed biochemical studies. Incubation with 50 μ M didanosine for 9 days (3 days before and 6 days during differentiation and fusion into myotubes) led to a severe decrease of COX activity in patient myotubes and to a lesser extent in controls. Supplementation with 400 μ M dAMP/dGMP resulted in a partial rescue of enzyme activity (Fig. 5A).

On immunoblotting, primary human myoblasts (lane 1) from a patient with *DGUOK* deficiency (p3) and one control cell line (c3) showed a low specific band for cytochrome c oxidase (COX, complex IV) subunit II (~20 kDa) compared to myotubes. Treatment with didanosine (50 μ M) led to a decreased intensity of COX II in patient myotubes, but remained normal in control cells. Supplementation with 400 μ M dAMP/dGMP resulted in a partial COX II rescue (Fig. 5B).

4. Discussion

The maintenance of mitochondrial DNA (mtDNA) requires the concerted activity of several nuclear encoded factors that participate in its replication, either as part of the mitochondrial replisome or by ensuring the balanced supply of dNTPs to mitochondria (Spinazzola and Zeviani 2005). MtDNA depletion leads to clinical symptoms in a tissue specific manner in post-mitotic tissues. As previously described [24], myotubes represent an excellent model system for post-mitotic tissues offering the possibility to investigate the pathomechanisms of mtDNA depletion and although with some limitations, can be used for supplementation studies in order to develop possible future therapies.

We investigated the effect of modifying the nucleotide pools by dNMP supplementation. The nucleotide pools for the nuclear genome are synthesized primarily in the cytosol and presumably pass into the nucleus passively through the nuclear pore complex [36,37]. The supply of nucleotides to the mitochondrial replisome is a more complex process and regulated in concert with the cell cycle. There is no de novo deoxynucleoside triphosphate (dNTP) synthesis in mitochondria and nucleotides are either imported from the cytosol or result from the mitochondrial salvage pathway [38]. The mitochondrial dNTP pool highly depends on cytosolic dNTPs and an unbalanced cytosolic pool can be deleterious for mtDNA [5]. Cytosolic dNTPs are predominantly synthesized de novo by the ribonucleotide reductase (RNR) which catalyzes deoxy-nucleotide triphosphate (dNDP) synthesis from

ribonucleotide diphosphates (rNDPs) [39]. It was previously described that the expression of p53R2 encoded by the *RRM2B* gene is cell-cycle independent [40]. De novo synthesis of ribonucleotide diphosphates (rNDPs) takes place in the cytosol and rNDPs are reduced to dNDPs by the R1–p53R2 complex. Therefore p53R2 plays an additional role in supplying dNDPs for mtDNA replication and possibly enables dNDP synthesis for DNA repair after DNA damage as *RRM2B* is a target of the p53 transcription factor induced by DNA damage [41].

It was previously shown that nucleotides can enter mitochondria from the cytosol in dividing cells, while non-dividing cells rely on mitochondrially synthesized nucleotide supply [42,43]. Our results indicate that nucleotides in a monophosphate form may enter mitochondria, and can therefore be used for supplementation. We added deoxynucleoside monophosphates for several reasons. First, the cell membrane is less permeable for highly charged molecules such as nucleoside triphosphates [37]. Second, the final dNTP concentration is a result of synthesis and degradation pathways [44,45], and the supplementation with dNMPs underlies this complex regulation more than their triphosphates which allows the cellular dNTP supply to remain adequate and balanced during both replication and quiescence. Finally, we did not detect a toxic effect, neither chromosomal nor mitochondrial rearrangements in cells treated with high doses of dAMP/dGMP, implying that it may not have deleterious effects on DNA structure [24].

In vitro studies in mtDNA depletion syndromes were reported in different cell types [24,36,46]. Due to the different pathomechanisms of other types of MDS, we tested the effect of supplementation with all four dNMPs in *POLG* and *RRM2B* deficiencies.

Although no experimental evidence suggested mtDNA depletion or an imbalance of nucleotide pools in fibroblasts of patients with *POLG* deficiency [46], earlier studies in myotubes detected mtDNA depletion and a mild, not significant increase of mtDNA copy number on supplementation with dAMP/dGMP [24]. Here we show that the low mtDNA copy number in *POLG* defect myotubes with impaired mtDNA replication, but without imbalance between nucleotides improves after supplementation with all four dNMPs and mtDNA copy numbers retained to normal. We applied an adenoviral vector to deliver the *MyoD* gene to both control and 3 patient-derived fibroblast lines.

After initiating transdifferentiation and cell fusion, similar to primary myotubes, *MyoD* treated *POLG* deficient fibroblasts showed mtDNA depletion, which was rescued by dNMP supplementation. No significant effect was observed in control cells. Our results show for the first time that myotubes formed from muscle cells derived after incubation of human primary fibroblasts with *MyoD* recombinant adenovirus undergo an increase of the mtDNA copy number in both control and patient cell lines, probably suggesting higher metabolic activity of muscle cells if compared to fibroblasts. After initiating fusion and differentiation, similar to primary myotubes, *MyoD* expressing *POLG* deficient fibroblasts showed reversible mtDNA depletion. Our results indicate that *MyoD* transfection is an effective way to derive myotubes for further mitochondrial studies, especially investigating mtDNA copy numbers, and for supplementation experiments. Interestingly, mtDNA copy numbers in five patient cell lines showed slight correlation with disease severity, since the highest copy

number was observed in the late-onset patient (p2), and the lowest in the most severe Alpers syndrome case (p6). The localization and type of the mutation may affect the response on dNMP supplementation, since cell lines carrying mutations in the polymerase domain showed a better response to treatment compared to cells with 2 linker region mutations. Further studies in animal models are needed to define, whether dNMP supplementation may become a feasible approach for therapy of *POLG* deficiency.

Myoblasts of a patient, carrying autosomal recessive *RRM2B* mutations showed different results in vitro studies. *RRM2B* (p53R2) affects dNDP synthesis, by transforming rNDP to dNDP, which is supported by the fact that mutations in the *RRM2B* gene lead to decreased cytosolic reduction of rNDP to dNDP. As previously described, the two homodimers R1 and R2 of the ribonucleotide reductase (RNR) are essential for dNDP synthesis during S-phase while the R1–p53R2 complex (p53R2 encoded by *RRM2B* gene) enables dNDP synthesis on a cell cycle independent manner [40]. Therefore p53R2 supplies dNDPs for mtDNA replication [41] and possibly enables dNDP synthesis for DNA repair after DNA damage. Unlike *DGUOK* or *POLG* deficiency, *RRM2B* deficient myoblasts had significantly lower mtDNA copy number, and no further decrease was observed in myotubes. Supplementation with dNMPs did not result in any change of mtDNA copy number. We can draw two conclusions from these results. First we suggest that not only myotubes (post-mitotic cells), but also myoblasts and possibly other dividing cells can show mtDNA depletion in *RRM2B* deficiency. Second, supplementation with dNMPs, as expected, had no beneficial effect in *RRM2B* deficiency. Based on the function of this protein supplementation with dNDPs could be tried as an alternative strategy in *RRM2B* deficiency.

We had two difficulties in our previous study in *DGUOK* deficiency. The level of mtDNA depletion in untreated patient myotubes did not reach the biochemical threshold to result in a biochemical respiratory chain defect therefore we could not show a functional evidence for a rescue. On the other hand if ethidium bromide was supplemented in the cell culture medium, it resulted in very severe mtDNA depletion in both patients and controls and no biochemical measurement was possible. The severe mtDNA depletion was not reversible by supplementation with nucleotides. Here we overcome these problems by using NRTIs. In patients suffering from HIV infection or malignant hematological diseases several NRTIs are routinely used for therapy [47–49]. We studied the effect of different NRTIs in our cellular model to trigger and rescue mtDNA depletion.

Abacavir, a guanosine analog [47] was selected first for cells with *DGUOK* deficiency. Supplementation with different concentrations of abacavir (10 μ M, 100 μ M and 300 μ M) resulted in both patients (*DGUOK* deficiency) and control myotubes in a mild, dose-dependent but nonsignificant increase in mtDNA copy number (Fig. 3A) with normal biochemical COX activity (data not shown). The different morphology of the cells during differentiation prompted us to further investigate the effect of abacavir in vitro. Beta-dystroglycan staining showed an impaired fusion and differentiation implying that the effect of abacavir on the mtDNA copy number may be in fact a result of its negative effect on cell growth and differentiation and does not imply a positive effect on mitochondrial biogenesis, suggesting that abacavir is not well suited for our in vitro studies. We did not observe this effect with other NRTIs (data not shown).

Didanosine (adenosine analog) or stavudine (thymidine analog) led to a moderate, but significant ($p < 0.05$) decrease of mtDNA copy number. Similar to previously published data [48,49] the effect of stavudine on mtDNA copy number was much milder compared to didanosine. By supplementing didanosine to the culture media, a significant biochemical defect (COX deficiency) was detected in both control and patient myotubes, which was confirmed by biochemical measurement and by immunoblotting for the mitochondrial COX II (Fig. 5). Myotubes from a patient with *DGUOK* deficiency showed a more prominent decrease compared to controls, suggesting that a deficient function of DGUOK may enhance the negative effect of didanosine on mtDNA copy number. Supplementation with dAMP/dGMP resulted in a functional rescue. Due to the increase of the mtDNA copy number, both the activity of COX and the level of COX II protein increased, implying that nucleotide supplementation can improve cellular function. We conclude, that didanosine was successfully used to trigger mtDNA depletion in our in vitro assay.

In summary, we show that supplementation with all four dNMPs in *POLG* deficient myotubes resulted in a significant rescue of mtDNA copy number and supplementation with dAMP/dGMP in *DGUOK* deficient patient myotubes had a beneficial effect on COX activity through an increase of mtDNA copy numbers. Based on these results, substitution of different forms of nucleosides should be further studied as a possible therapeutic approach for mitochondrial DNA depletion.

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Abbreviations

COX	cytochrome <i>c</i> oxidase
COX II	cytochrome <i>c</i> oxidase subunit II
DGUOK	deoxyguanosine kinase
dNMP	deoxy-nucleoside monophosphate
dNDP	deoxy-nucleoside diphosphate
dNTP	deoxy-nucleoside triphosphate
HIV	human immunodeficiency virus
HRP	horseradish peroxidase
MDS	mitochondrial depletion syndromes
MNGIE	myoneurogastrointestinal encephalomyopathy

mtDNA	mitochondrial DNA
NRTI	nucleoside reverse transcriptase inhibitor
PBS	phosphate-buffered saline
PEO	progressive external ophthalmoplegia
POLG	polymerase gamma
rNDP	ribonucleoside diphosphate
RNR	ribonucleotide reductase
SGM	skeletal muscle growth medium.

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Fig. 1.

(A) Human myotubes (MT) with *POLG* (p1, p2) deficiency showed a significant ($p < 0.05$) increase of the mtDNA copy number reaching normal levels when supplemented with 200 μ M dNMP mix or with 400 μ M dNMP mix compared with unsupplemented myotubes of the same genotype. Remarkably, myotubes derived from a patient (p2) with a very mild clinical phenotype showed a higher original mtDNA copy number, than myotubes derived from a patient (p1) with a severe childhood onset fatal Alpers syndrome. No effect of dNMP supplementation was detected in control myotubes. A significant difference ($p < 0.05$) between unsupplemented and supplemented myotubes of the same genotype is indicated by a star. Error bars indicate standard deviation ($n=3$). MB (myoblasts). (B) Myogenic transdifferentiation of four fibroblast lines by MyoD gene transfer resulted in 6 days in

multinucleated myotubes and an increase of mtDNA copy number in both patients (p7, p8) and in a control (c5) cell line. Supplementation with 400 μ M dNMP mix led to a significant ($p<0.05$) increase of mtDNA/nDNA ratio in all patient cells (p6, p7, p8) compared to untreated cells, but not in the control cell line. A significant difference ($p<0.05$) between unsupplemented and supplemented myotubes of the same genotype is indicated by a star. Error bars indicate standard deviation ($n=3$). (C) Myogenic transdifferentiation after adenovirus-mediated MyoD gene transfer was confirmed by immunofluorescence of the two myoblast markers desmin and actinin.

Fig. 2.

Myoblasts (MB) derived from a patient (p5) with *RRM2B* deficiency had significantly lower mtDNA copy number compared to control cells, and differentiation to myotubes (MT) did not lead to a further decrease of mtDNA copy number as compared to myoblasts. There were no changes in mtDNA copy number observed after supplementation with 400 µM dNMP mix during differentiation and fusion into myotubes. A significant difference ($p<0.05$) between patient and control cells is indicated by a star. Error bars indicate standard deviation.

Fig. 3.

(A) Treatment with abacavir resulted in apparent increase in relative mtDNA copy number. Error bars indicate standard deviation. MB myoblasts, MT myotubes. (B) Beta-dystroglycan staining revealed a seriously reduced fusion capacity and decreased cell division in cells treated with abacavir, indicating that abacavir treated cells showed different morphology compared to untreated myotubes due to decreased cell division and a decreased fusion, thus explaining the apparent increase in cellular mtDNA copy number.

Fig. 4.

(A) The incubation with didanosine (300 μ M) and stavudine (300 μ M) respectively led to a significant ($p < 0.05$) mtDNA depletion in controls and myotubes (MT) of patients with *POLG* (p1, p2) and *DGUOK* deficiency (p3, p4). Stavudine caused a milder decrease of the mtDNA copy number compared to didanosine. A significant difference ($p < 0.05$) between untreated and treated myotubes of the same genotype is indicated by a star. Error bars indicate standard deviation. (B) Incubation with lower concentrations (5 μ M and 50 μ M) of didanosine after 3 days led to a decrease of the mtDNA copy number in myoblasts from patients with *DGUOK* deficiency (p3, p4) and a control cell line (c1). A significant difference ($p < 0.05$) between untreated and treated myoblasts of the same genotype is indicated by a star. Error bars indicate standard deviation. MB myoblasts. (C) When dAMP/dGMP (400 μ M) was added to didanosine-supplemented media (5 μ M and 50 μ M), mtDNA copy numbers were restored ($p < 0.05$) in all cell lines. A significant difference ($p < 0.05$) between unsupplemented and supplemented myotubes of the same genotype is indicated by a star. Error bars indicate standard deviation.

Fig. 5.

(A) Incubation with 50 μ M didanosine for 9 days led to a severe decrease of COX activity in myotubes (p3) derived from a patient with *DGUOK* deficiency and to a lesser extent in control cells (c3). (B) Lane 1: myoblasts; lane 2: myotubes; lane 3: myotubes + didanosine; lane 4: myotubes + didanosine + dAMP/dGMP. Primary human myoblasts (lane 1) from a patient with *DGUOK* deficiency (p3) and one control cell line (c3) showed a low specific band for cytochrome *c* oxidase (COX, complex IV) subunit II (COX II) (~20 kDa) compared to myotubes. Treatment with didanosine (50 μ M) led to a decreased intensity of COX II in patient myotubes, but remained normal in control cells. Supplementation with 400 μ M dAMP/dGMP resulted in a partial COX II rescue.

Table 1

Clinical presentation and primary gene defect of the patients. The clinical presentation of patients 1, 2, 4 and 6 was previously reported (p1, p2 and p6 in Horvath et al., 2006 [49], p4 in Freisinger et al., 2006 [50]).

Patient	Clinical phenotypes	Gene defect	Pathogenic mutations
p1	Alpers–Huttenlocher syndrome	<i>POLG</i>	p.Ala467Thr/p.Lys1191Asn
p2	progressive external ophthalmoplegia (PEO)	<i>POLG</i>	p.Thr251Ile/p.Pro587Leu
p3	hepatoencephalopathy, muscular hypotonia	<i>DGUOK</i>	Homozygous c.705+1_delGTAA
p4	hepatoencephalopathy, muscular hypotonia	<i>DGUOK</i>	Homozygous p.Ser52Phe
p5	mitochondrial encephalomyopathy	<i>RRM2B</i>	p.Asn70Asp/p.Asp104Asn
p6	Alpers–Huttenlocher syndrome	<i>POLG</i>	p.Gly737Arg/p.Ala767Asp
p7	Alpers–Huttenlocher syndrome	<i>POLG</i>	p.Trp748Ser/c.3600delT
p8	Alpers–Huttenlocher syndrome	<i>POLG</i>	p.Ala467Thr/p.Ser1104Phe

Table 2

Fusion index, average number of nuclei within myotubes and total number of nuclei.

Cell lines	Fusion index	Average number of nuclei/myotube	Total number of nuclei
c3			
Untreated 300 μ M	27%	6	1106
Abacavir	5%	2	299
c4			
Untreated	93%	14	1000
300 μ M abacavir	74%	4	508
p3			
Untreated	71%	22	1162
300 μ M abacavir	75%	4	400