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Administration of deoxyribonucleosides or inhibition of their catabolism as a pharmacological approach for mitochondrial DNA depletion syndrome

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Mitochondrial DNA (mtDNA) depletion syndrome (MDS) is characterized by a reduction in mtDNA copy number and consequent mitochondrial dysfunction in affected tissues. A subgroup of MDS is caused by mutations in genes that disrupt deoxyribonucleotide metabolism, which ultimately leads to limited availability of one or several deoxyribonucleoside triphosphates (dNTPs), and subsequent mtDNA depletion. Here, using *in vitro* experimental approaches (primary cell culture of deoxyguanosine kinase-deficient cells and thymidine-induced mtDNA depletion in culture as a model of mitochondrial neurogastrointestinal encephalomyopathy, MNGIE), we show that supplements of those deoxyribonucleosides (dNs) involved in each biochemical defect (deoxyguanosine or deoxycytidine, dCtd) prevents mtDNA copy number reduction. Similar effects can be obtained by specific inhibition of dN catabolism using tetrahydrouridine (THU; inhibitor of cytidine deaminase) or immucillin H (inhibitor of purine nucleoside phosphorylase). In addition, using an MNGIE animal model, we provide evidence that mitochondrial dNTP content can be modulated *in vivo* by systemic administration of dCtd or THU. In spite of the severity associated with diseases due to defects in mtDNA replication, there are currently no effective therapeutic options available. Only in the case of MNGIE, allogeneic hematopoietic stem cell transplantation has proven efficient as a long-term therapeutic strategy. We propose increasing cellular availability of the deficient dNTP precursor by direct administration of the dN or inhibition of its catabolism, as a potential treatment for mtDNA depletion syndrome caused by defects in dNTP metabolism.

INTRODUCTION

Mitochondrial DNA (mtDNA) depletion syndrome (MDS) comprises several severe autosomal diseases characterized by a reduction in mtDNA copy number in affected tissues. Most of the MDS causative nuclear genes encode proteins that belong to the mtDNA replication machinery or are involved in deoxyribonucleoside triphosphate (dNTP) metabolism.

The mitochondrial dNTP pool size and composition in non-proliferating tissues depend on a balance between dNTPs produced by anabolic mitochondrial and cytosolic pathways and consumed by incorporation into mtDNA or catabolism (Fig. 1). Some forms of MDS are caused by mutations in genes encoding anabolic enzymes: *TK2* (encoding thymidine kinase 2, TK2), *DGUOK* (encoding deoxyguanosine kinase, dGK) and *RRM2B* (encoding p53R2, the p53-inducible small subunit of ribonucleotide reductase, RNR) (1–3). In contrast, mutations in *TYMP* (encoding thymidine phosphorylase, TP) cause mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) (4).

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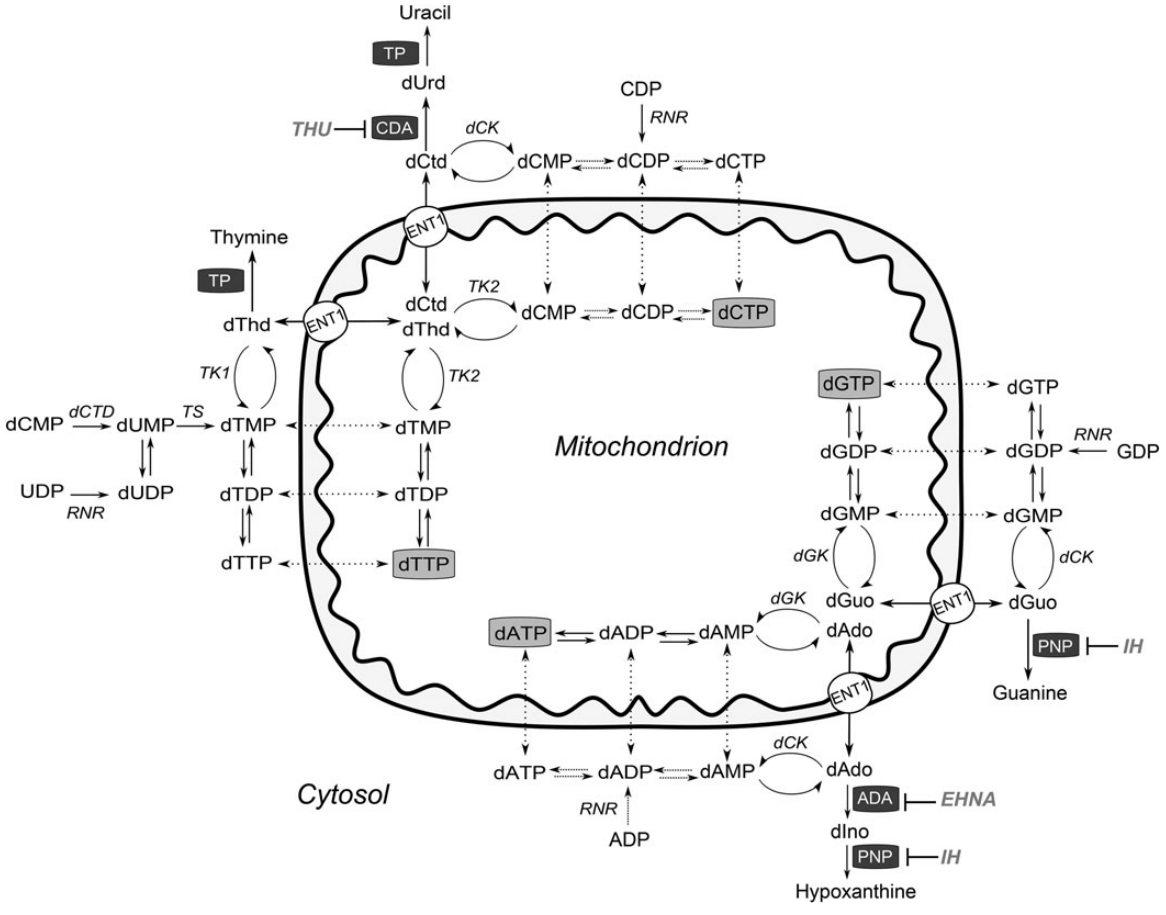

Figure 1. Representation of the main dNTP metabolic pathways. Catabolic enzymes are marked in boxes and inhibitors used in this study are written in gray italics. ADA, adenosine deaminase; CDA, cytidine deaminase; dAdo, deoxyadenosine; dCK, deoxycytidine kinase; dCtd, deoxycytidine; dCTD, deoxycytidylate deaminase; dGK, deoxyguanosine kinase; dGuo, deoxyguanosine; dIno, deoxyinosine; dThd, thymidine; dUrd, deoxyuridine; EHNA, erythro-9-(2-hydroxy-3-nonyl) adenine; ENT1, equilibrative nucleoside transporter 1; IH, Immucillin H; PNP, purine nucleoside phosphorylase; RNR, ribonucleotide reductase; THU, tetrahydrouridine; TK1, thymidine kinase 1; TK2, thymidine kinase 2; TP, thymidine phosphorylase; TS, thymidylate synthase.

TP catalyzes the first reaction in catabolism of the pyrimidine deoxyribonucleosides (dNs), thymidine (dThd) and deoxyuridine (dUrd), and its dysfunction leads to increased deoxythymidine triphosphate (dTTP) (5,6) and a secondary decrease in mitochondrial deoxycytidine triphosphate (dCTP), which results in mtDNA depletion (7). Hence, a limited availability of one or more dNTPs is the common mechanism accounting for mtDNA depletion due to defective mitochondrial dNTP homeostasis.

The therapy options for MDS are mainly symptomatic or invasive (8). Previous experimental studies have shown that bypassing the defective step in deoxyribonucleotide biosynthesis can ameliorate mtDNA depletion. Addition of purine dN monophosphates (dNMPs) to the cell culture medium has been found to rescue mtDNA depletion in dGK-deficient cells (9–12). Other studies suggest that mtDNA depletion can be prevented by supplying an excess of dNs, indicating that the cytosolic salvage pathway (and/or residual mitochondrial salvage activity) may be enhanced by increasing the substrate availability (7,13,14). Here, we provide evidence that the use of dNs and/or specific inhibitors of their catabolism may be an effective pharmacological approach for treating MDS due to defects in dNTP homeostasis.

RESULTS

In a previous study, we found that dThd-induced mtDNA depletion can be prevented or reverted by deoxycytidine (dCtd) in cell culture and proposed that treatment with this nucleoside could prevent mtDNA depletion in MNGIE (7). However, rapid dCtd deamination by the ubiquitous enzyme cytidine deaminase (CDA) would limit dCtd availability *in vivo*. For this reason, we tested the effect of the CDA inhibitor tetrahydrouridine (THU) on the mtDNA copy number in the same cell model. MtDNA depletion was induced in quiescent human skin fibroblasts by exposing them to dThd. Cells were treated with 5 μM THU, alone or in combination with a range of dCtd concentrations (0, 1, 5, 20 μM). As was expected, THU partially prevented catabolism of both endogenous and added dCtd, increasing dCtd levels in the medium and reducing dUrd accumulation (Table 1). As for the effect on mtDNA under dThd treatment, cotreatment with dCtd prevented mtDNA depletion (Fig. 2A), as has been reported (7). An additional experiment using 5 μM [6-^{3}H]-dCtd and THU allowed us to confirm that the added dN is incorporated into DNA (data not shown). In addition, THU alone partially counteracted the effect of dThd on mtDNA copy number, indicating that

Table 1. dCtd and dUrd concentration in cell media of dCtd or THU-treated fibroblasts

Added compound (μM)		Nucleoside levels detected (μM)	
dCtd	THU	dCtd	dUrd
–	–	0.01 ± 0.002	0.02 ± 0.01
–	5	0.03 ± 0.003	0.02 ± 0.002
1	–	0.26 ± 0.02	0.28 ± 0.01
1	5	0.9 ± 0.1	0.021 ± 0.001
5	–	2.0 ± 0.3	1.4 ± 0.1
5	5	4.2 ± 0.9	0.08 ± 0.02
20	–	8.4 ± 1.7	1.7 ± 0.3
20	5	10.4 ± 2.3	0.38 ± 0.04

dCtd and dUrd concentrations in cell culture medium. Quiescent fibroblasts from healthy controls were treated with 30 μM dThd. dCtd and dUrd were analyzed after 3 days of cotreatment with THU (5 μM) and/or the indicated doses of dCtd. Results are expressed as the mean ± standard error of the mean (SEM) of four different cell lines. Minus signs indicate no addition.

the moderate THU-induced increase of endogenous dCtd noticeably influenced mtDNA replication. However, THU addition did not produce any appreciable synergistic-positive effect over only dCtd addition at the concentrations tested (Fig. 2A). These data indicate that, in our experimental conditions, addition of 1 μM dCtd probably exceeds the needs to achieve mitochondrial dCTP concentrations above those that limit the mtDNA replication rate.

We used a double *Tymp/Upp1* knockout murine model of MNGIE (5) to test the *in vivo* effect of dCtd or THU administration on dNTP imbalances. A single dose of 100 mg/kg THU resulted in a 3-fold increase of plasma dCtd 3 h after injection, concomitant with a 50% reduction of circulating dUrd (Fig. 2B), which indicates that a major source of dUrd in double KO mice is dCtd deamination. A similar increase of plasma dCtd concentration was observed after a single injection of 400 mg/kg dCtd; however, most of the dCtd had been rapidly deaminated, as plasma dUrd concentrations were between 9- and 80-fold higher than those of dCtd. When dCtd (400 mg/kg) and THU (100 mg/kg) were co-administered, dCtd deamination was largely prevented. Importantly, mitochondrial dCTP levels increased in response to the elevated systemic dCtd concentrations (Fig. 2C). Significant dCTP increases were observed in liver and brain mitochondria, which correlated with dCtd levels ($r = 0.926$, $P < 0.001$ for liver; $r = 0.853$, $P = 0.001$ for brain). Therefore, dCtd or THU, either individually or coadministered, can be used to increase mitochondrial dCTP. It is noteworthy that mitochondrial dATP, dTTP and dGTP were unaffected or only slightly modified by the treatments. A slight tendency to increase was only observed for dTTP and dGTP (Supplementary Material, Fig. S1).

After dCtd or THU administration, CDA or dCMP deaminase activities may lead to undesired dUTP expansion, but we could not detect dUTP in liver or brain mitochondria in any of the treated animals (<5 pmol/mg prot), probably due to the action of highly efficient dUTPases.

It has been repeatedly reported that treatment with dAMP + dGMP prevents mtDNA depletion in dGK-deficient cultured cells (9–12). Our results demonstrate that complete prevention of mtDNA depletion is achieved by addition of 50 μM dGuo alone to the culture, and that deoxyadenosine (dAdo) addition does not have any detectable effect (Fig. 3A). Addition of 50 μM dGuo was also sufficient to restore mtDNA copy number in already depleted dGK-deficient fibroblasts (Supplementary Material, Fig. S2). The positive effects of 50 μM dGuo or 200 μM dGMP on dGK-deficient cells were comparable, but addition of 200 μM dAMP did not improve mtDNA copy number, similar to the lack of dAdo effect on mtDNA levels (Fig. 3B).

Analysis of intracellular and extracellular levels of dAMP, dAdo, dGMP and dGuo 24 h after their addition to cell culture revealed that (Table 2): (i) dAMP and dGMP were undetectable in cell culture medium and cell homogenates; (ii) addition of dGMP or dGuo to the culture resulted in pronounced, dose-dependent extracellular and intracellular dGuo increases, and the amounts did not depend on the chemical form of the added nucleoside, that is, unphosphorylated (dGuo) or phosphorylated (dGMP); and (iii) intracellular dAdo was undetectable and dramatically reduced in the medium 24 h after dAdo or dAMP addition. Additional experiments showed that high percentages of dAMP (25–85%) and dGMP (15–50%) are degraded over 24 h when added to conditioned medium without cells (Table 2). These results indicate that dNMPs are rapidly dephosphorylated in cell culture medium and strongly suggest that dephosphorylation mainly occurs extracellularly.

Given the positive effects that CDA inhibition had on mtDNA copy number in the MNGIE cell model, we tested whether inhibition of dGuo catabolism with the purine nucleoside phosphorylase (PNP) inhibitor immucillin H (IH) had a similar positive effect in dGK-deficient cells. Addition of a low concentration of dGuo (1 μM) to cell culture media had no detectable effects on mtDNA. However, cotreatment with 1 μM dGuo plus IH partially prevented mtDNA depletion in the cells (Fig. 4A). Addition of dGuo at a higher concentration (50 μM) produced a more pronounced effect that was not improved by cotreatment with IH. Analysis of the culture medium confirmed that IH prevents dGuo catabolism (Table 3), and that addition of 1 μM dGuo is suboptimal in this model, but addition of 50 μM dGuo exceeds the level required to completely prevent mtDNA depletion in dGK-deficient cells. Similarly, in additional experiments in which cell medium was not replaced for 21 days, IH treatment induced a mild accumulation of endogenous dGuo (0.5 ± 0.2 μM) that led to a 153 ± 23% increase in mtDNA copy number relative to untreated cells. Overall, these results indicate that mtDNA copy number responds to dGuo concentration in a dose-dependent manner, and that IH may be helpful in preventing degradation of endogenous or added dGuo.

Our results (Fig. 3A) also demonstrated that dAdo, alone or in combination with dGuo, does not have an effect on mtDNA copy number in dGK-deficient cells. Because dAdo is rapidly degraded by adenosine deaminase (ADA), we studied the effect of ADA inhibition using erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) (Fig. 4B and Table 4). Addition of 50 μM dAdo in combination with EHNA substantially preserved dAdo in the culture medium (Table 4). However, increased dAdo concentration in the medium not only failed to exert a positive effect, but rather slightly reduced mtDNA copy number (Fig. 4B).

We analyzed *DCK* and *DGUOK* expression in the cultured cells used in our experiments. No differences in the relative *DCK* mRNA levels were detected between controls (1.0 ± 0.1, mean ± SD) and dGK-deficient cells (1.2 ± 0.7), nor in

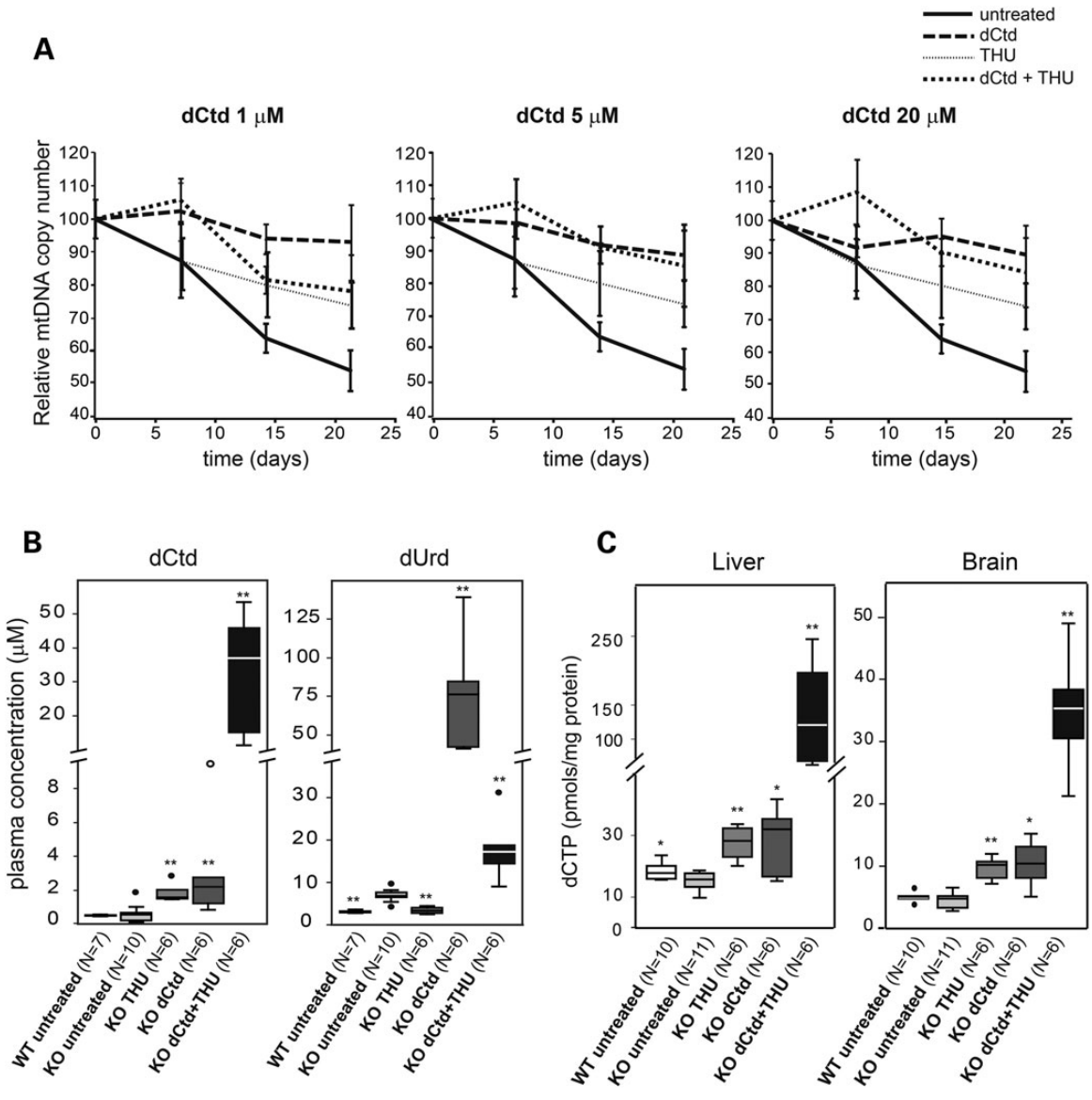

Figure 2. Effect of dCtd and THU administration *in vitro* and *in vivo*. (**A**) Influence of dCtd (1, 5 or 20 μM) and THU (5 μM) treatment on mtDNA copy number in dThd-treated (30 μM) quiescent fibroblasts in culture. Cells were collected at the times indicated and the mtDNA/nDNA ratio was assessed. Results are the mean ± SEM of four different cell lines, plotted as percentages of the ratios obtained for parallel untreated cultures. dCtd and dUrd concentration in plasma (**B**), and deoxycytidine triphosphate in liver and brain mitochondria (**C**) of $Tymp^{-/-}/Upp1^{-/-}$ mice after 3 h of treatment with THU (100 mg/kg), dCtd (400 mg/kg) or THU + dCtd. Boxplots represent the median (horizontal line), interquartile range (box) and minimum and maximum (whiskers), except outliers (full circle) and extreme values (open circles). *P*-values obtained with the Mann–Whitney *U*-test: $^{*}P < 0.05$; $^{**}P < 0.01$, compared to KO untreated.

DGUOK mRNA levels (controls 0.9 ± 0.2 and dGK deficient 1.1 ± 0.4). Treatment with 50 μM dGuo over 7 days did not affect the expression of these genes (treated controls, *DCK* mRNA 0.9 ± 0.2 and *DGUOK* mRNA 0.9 ± 0.3; treated dGK deficient, *DCK* mRNA 1.0 ± 0.1 and *DGUOK* mRNA 1.1 ± 0.1).

DISCUSSION

Previous data from our and other groups indicate that the ultimate cause leading to mtDNA depletion due to defective nucleotide metabolism is the limited availability of one or more of the dNTPs being used as substrates for DNA synthesis (7,14). The administration of the required dN may be sufficient to counteract dNTPs deficiency and thus mtDNA depletion. Once incorporated in the cell through dedicated transporters, increased dN concentration would enhance their phosphorylation by dN kinases in the cytosolic or mitochondrial salvage pathways to generate dNTPs.

This study shows that addition of specific dNs and/or inhibition of their catabolism prevents mtDNA depletion in two different cell models: dThd-induced mtDNA depletion in fibroblasts from healthy controls (MNGIE cell model), and spontaneous mtDNA depletion in dGK-deficient fibroblasts. In MNGIE cells, dCTP deficiency is caused by the inhibition of

TK2-catalyzed dCtd phosphorylation, induced by dThd excess (7). This effect can be counteracted by administration of dCtd, which can be phosphorylated by either dCK (cytosolic) or TK2 (mitochondrial), thus preventing dCTP depletion. In the case of dGK-deficient cells, supply of purine dNs would enhance their phosphorylation by dCK (or dGK residual activity, if any). Although both dGuo and dAdo are phosphorylated by dGK in mitochondria, only addition of dGuo (but not dAdo or dAMP) prevented mtDNA depletion. Preservation of dAdo in the medium by specific ADA inhibition did not alter this outcome. Thus, at least in our *in vitro* design, mtDNA maintenance requirements for dATP must be sustained by sources other than dGK-mediated dAdo salvage, such as cytosolic salvage or *de novo* synthesis. Therefore, mitochondrial dGTP should be the limiting substrate for mtDNA replication in dGK-deficient quiescent fibroblasts, although this situation may be different *in vivo*. To address this issue, a murine model of dGK deficiency would be extremely useful.

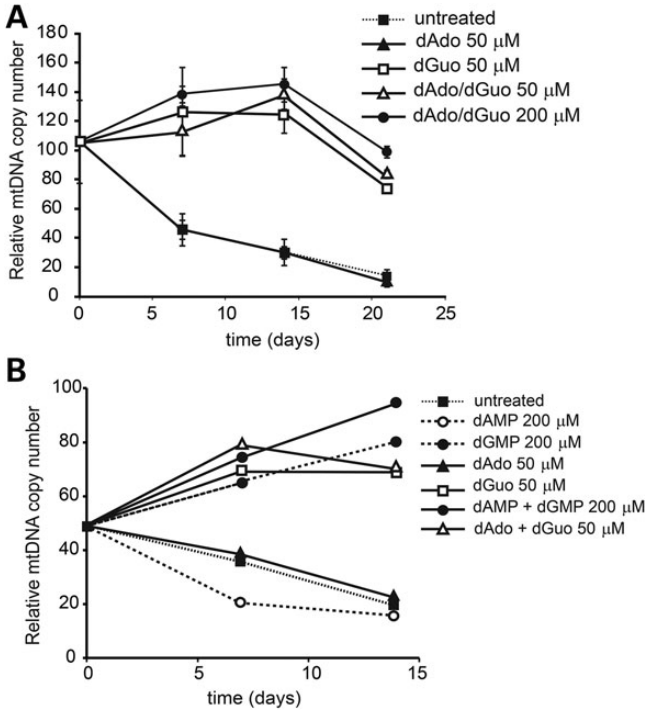

Figure 3. Effect of purine dNs and monophosphates on mtDNA of dGK-deficient cultured cells. (**A**) Progression of mtDNA copy number in response to dAdo/dGuo treatment in quiescent dGK-deficient fibroblasts. Results are expressed as the mean ± SEM of three different cell lines, and plotted as percentages of the ratios obtained for parallel untreated cultures from healthy controls. (**B**) Progression of mtDNA copy number in dGK-deficient fibroblasts after treatment with dAdo/dGuo/dAMP/dGMP. Results are expressed as percentages of the ratios obtained for parallel untreated cultures.

We also observed that dGuo is as effective as dGMP for preventing mtDNA depletion in dGK-deficient cells. Our results strongly support the notion that dGMP, rather than exerting a positive effect on its own, ultimately acts as a source of dGuo, providing substrate for salvage kinases. Charged nucleotides cannot diffuse across biological membranes, and there is no experimental evidence of dNMP transporters in plasma membranes. In fact, nucleoside monophosphate analogs used as antiviral prodrugs require charge masking groups to allow their delivery across plasma membranes (15). We show that dNMPs are rapidly dephosphorylated to dNs in cell culture, probably by factors excreted by the cells although membrane-bound activities, such as ecto-nucleotidases (16), may contribute to this dephosphorylation.

As previously discussed, a potential strategy for the treatment of MDS would be administration of specific dNs based on the patient's genetic defect. However, the bioavailability of these substances could be challenged by ubiquitous dN catabolic enzymes. Enzyme inhibitors such as THU and IH, originally used in chemotherapy, increase plasma concentrations of dCtd and dGuo, respectively (17,18). Hence, inhibition of catabolism stabilizes coadministered dNs, induces a sufficient increase in their endogenous concentrations, or both.

The bioavailability of the administered substance and its impact on the mitochondrial dNTP pool in targeted tissues

Table 2. Purine dNs concentration in cell media of dGK-deficient fribroblasts after administration of dAdo/dGuo/dAMP/dGMP

Added compound (μM)				Nucleoside levels detected					
				In cell medium (μM)		In conditioned cell medium (μM)		Intracellular (nmol/mg prot)	
dAdo	dGuo	dAMP	dGMP	dAdo	dGuo	dAdo	dGuo	dAdo	dGuo
–	–	–	–	und	und			und	und
50	–	–	–	0.03	0.1	0.5	und	und	0.02
–	50	–	–	und	20.8	und	56.0	und	2.3
50	50	–	–	0.18	21.4	3.5	58.3	und	1.7
200	–	–	–	53.0	0.1			und	0.1
–	200	–	–	und	79.6			und	25.5
200	200	–	–	74.0	94.0			und	21.8
–	–	50	–	1.2	0.03			und	0.03
–	–	–	50	und	30.8			und	3.3
–	–	50	50	1.5	32.5			und	3.3
–	–	200	–	0.4	0.2	41.0	und	und	und
–	–	–	200	und	65.9	und	122.0	und	21.2
–	–	–	200	38.0	70.1	23.5	108.8	und	14.7

Intracellular and extracellular dAdo and dGuo concentrations in cultured dGK-deficient fibroblasts from Patient 3, after 24 h with the indicated treatment. Similar results were obtained for fibroblasts from a healthy control. Und, undetectable levels of analyte. Empty cells indicate not determined analytes. Minus signs indicate no addition.

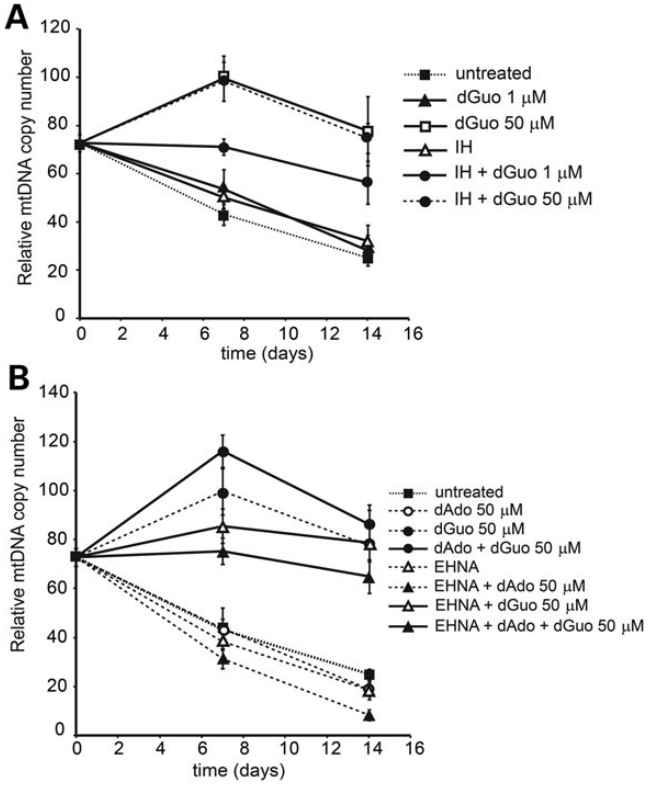

Figure 4. Effect of a combined administration of purine deoxyribonucloeosides and specific inhibitors of their catabolism on mtDNA of dGK-deficient cultured cells. (**A**) Progression of mtDNA copy number in response to dGuo and IH (1 μM) treatment in quiescent dGK-deficient fibroblasts. Results are expressed as the mean ± SEM of three different cell lines, and plotted as percentages of the ratios obtained for parallel untreated cultures from healthy controls. (**B**). Effect of dAdo and 5 μM EHNA on mtDNA copy number progression in dGK-deficient fibroblasts. Results are mean values from three-independent cell lines, expressed as percentages of the ratios obtained for parallel untreated cultures. Error bars indicate SEM.

Table 3. dGuo concentration in cell media of dGK-deficient fibroblasts after treatment with dAdo, dGuo and/or IH

Added compound (μM)			dGuo levels detected (μM)	
dAdo	dGuo	IH	Control	dGK deficient
–	–	–	und	und
–	–	1	0.3 ± 0.01	0.1 ± 0.01
–	1	–	0.1 ± 0.03	0.2 ± 0.1
–	1	1	1.3 ± 0.2	1.3 ± 0.1
–	50	–	20.1 ± 5.3	28.7 ± 4.4
–	50	1	53.5 ± 3.7	54.7 ± 1.5
50	–	–	0.03 ± 0.01	0.04 ± 0.02
50	–	1	und	und
50	50	–	22.2 ± 7.0	28.7 ± 2.3
50	50	1	52.0 ± 1.1	49.5 ± 3.2

dGuo concentration in cell culture media after 3 days of treatment with dAdo, dGuo and/or IH (1 μM). Results are mean ± SEM from three different dGK deficient and four control cell lines. Und, undetectable levels of dGuo. Minus signs indicate no addition.

cannot be investigated in cell culture. For this reason, we studied an MNGIE murine model and found that mitochondrial dNTPs can be manipulated *in vivo* by administration of the corresponding dN and/or inhibition of its catabolism. Based on our results, we propose that a sustained increase in systemic dCtd using this strategy could restore normal dCTP levels in mitochondria, thus preventing mtDNA depletion in MNGIE. Importantly, the increased dCtd concentration did not produce dTTP depletion, a finding consistent with the fact that dCtd is a poor competitor of dThd phosphorylation by TK2 (19). THU would have the additional positive effect of reducing the dUrd accumulation observed in these patients. Therefore, we propose a treatment based on THU, alone or in combination with low doses of dCtd, to prevent further progression of mtDNA depletion in MNGIE patients.

A possible drawback of either MNGIE or dGK-deficient therapies based on dNs supply, is that they both may rely on dCK activity, an enzyme which is poorly expressed in some typically affected tissues (20). However, we have observed correction of mtDNA depletion in fibroblasts, where dCK expression level did not appear to be higher than that of dGK. More importantly, in our *in vivo* model, dCtd administration induces a clear increase in mitochondrial dCTP content in brain and liver, supporting the notion that, even if lowly expressed, dCK activity may be sufficiently enhanced by increased substrate availability.

Careful studies will be needed to ensure an absence of undesirable secondary effects of these therapies, especially regarding inhibitors of dN catabolism. Although CDA deficiency has not been associated with human disease, PNP dysfunction causes immunodeficiency (21). Therefore, the consequences of IH dosage would need to be carefully studied and eventually modulated, as this therapy would have to be administered chronically. It is possible that total inhibition of the catabolic enzyme may not be required, and the combined treatment would likely lower inhibitor requirements.

Taken together, our results indicate that the appropriate dNs and/or inhibitors of their catabolism are good candidates for pharmacological treatment of MDSs due to defective nucleotide metabolism. We provide here direct biochemical and molecular evidence of the positive effects of this strategy in two of these disorders (loss-of-function mutations in *TYMP* and *DGUOK*), but the concept could be extended to other diseases affecting similar pathways, such as those caused by mutations in the *TK2* and *RRM2B* genes.

MATERIALS AND METHODS

Ethics statement

This study was performed in accordance with the rules established by the *Generalitat de Catalunya* for the Care and Use of Laboratory Animals. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Vall d'Hebron Research Institute (Permit Number: 14/13).

Cell culture

Primary human skin fibroblast cultures from three patients with *DGUOK* mutations and four healthy controls were used. Patient 1 had a homozygous c.749T>C mutation; p.L250S (709T>C in the original publication (22)), Patient 2 carried the mutation c.677A>G; p.H226R in homozygosity (cells kindly provided by Dr Antònia Ribes, mutation previously described for a

Table 4. Concentration in cell media of dAdo and derived metabolites after treatment with dAdo alone or in combination with EHNA

Added compound	Compound levels detected (μM)					
	dAdo		dIno		Hypoxanthine	
	Control	dGK deficient	Control	dGK deficient	Control	dGK deficient
untreated	und	und	und	und	0.7 ± 0.1	0.7 ± 0.1
dAdo	und	und	21.3 ± 3.7	26.0 ± 2.4	41.6 ± 8.5	37.5 ± 1.8
EHNA	und	und	und	und	0.8 ± 0.1	0.9 ± 0.1
EHNA + dAdo	16.1 ± 5.3	18.3 ± 3.6	8.9 ± 4.6	9.5 ± 4.6	22.1 ± 4.5	25.7 ± 8.8

Concentrations of dAdo and its metabolites, deoxyinosine (dIno), and hypoxanthine in cell culture media after treatment with 50 μM dAdo and/or 5 μM EHNA over 3 days. Results are the mean ± SEM values from 3 dGK deficient and four independent control cell lines. Und, undetectable levels of analyte.

different patient (23)) and Patient 3 was compound heterozygous for the mutations c.763_766dupGATT and c.494A>T (p.E165V) (24) (reference sequence: NM_080916.2).

Fibroblasts were seeded in 3.5-cm diameter plates (100 000 cells per plate) and expanded in DMEM with 4.5 g/l glucose, supplemented with 2 mM L-glutamine, 100 U/ml penicillin and streptomycin and 10% dialyzed FBS (Invitrogen) in a humidified incubator at 37°C and 5% CO_2. After tight confluence was reached, FBS was reduced to 0.1% to induce quiescence. Four days later (Day 0), the specific nucleoside or drug treatment was added to the cell medium. All treatments were performed in duplicate. The following compounds were added to the cell media at the concentrations indicated in the Results section: THU (BioVision), IH (gift from Prof. Vern Schramm, Albert Einstein College of Medicine, New York, USA), dAdo, dGuo, dAMP, dGMP, dCtd and EHNA (Sigma-Aldrich). During the treatment period, cell media (and respective additives) were replaced every 3–4 days unless otherwise stated. At the times required according to the experimental designs, media were collected and stored at −20°C until further use. For DNA analysis, cells were harvested by trypsinization, washed with phosphate-buffered saline, pelleted and stored at −20°C until DNA isolation. For determination of dNs and dNMPs in medium and cell homogenates, see the specific section below.

dCtd and THU administration *in vivo*

Male *Tymp/Upp1* double-knockout (5) and wild-type mice, aged 10–15 weeks, were administered a single dose of dCtd (400 mg/kg in PBS), THU (100 mg/kg in PBS) or combined dCtd plus THU treatment (400 and 100 mg/kg in PBS, respectively) through the tail vein. Three hours after injection, mice were killed by cervical dislocation and tissues were processed as follows: 200–400 μl of blood was rapidly collected by cardiac puncture in EDTA (Microvette capillary tubes, SARSTEDT), whole anticoagulated blood was 1:3 diluted in PBS, and the diluted plasma was separated by centrifugation and kept at −20°C until further use. Brain and liver were rapidly dissected and immersed in mitochondrial extraction buffer for subsequent immediate mitochondrial isolation.

Mitochondrial isolation and dNTP quantification

Mitochondrial fractions from mouse liver and brain were isolated by differential centrifugation in A and AT buffer, respectively (buffer A: 320 mM sucrose, 1 mM EDTA and 10 mM Tris–HCl, pH 7.4; buffer AT: 75 mM sucrose, 225 mM sorbitol, 1 mM EGTA, 0.1% fatty acid-free BSA and 10 mM Tris–HCl, pH 7.4), as described (7). The isolation procedure and further steps were performed at 4°C. A 0.5-mg amount of protein from the mitochondrial suspensions was treated with trichloroacetic acid 0.5 M (final concentration), and centrifuged at 20 000*g* for 5 min at 4°C. Supernatants were neutralized by vortexing with 1.5 volumes of 0.5 M trioctylamine in Freon (1,1,2-trichlorotrifluoroethane) and centrifuged for 10 min at 10 000*g* at 4°C. Half of the upper aqueous phase was then collected to avoid contamination from the organic phase. The neutralized aqueous extracts were dried under speed-vacuum and dry residues were redissolved in 125 μl of 40 mM Tris–HCl (pH 7.4) and stored at −80°C until analysis. dNTP concentrations were determined using a polymerase-based method on an adequate amount of mitochondrial extract (ranging from the equivalent of 20–60 μg protein), as described (7). For dUTP determination, one aliquot of the extract was treated with 0.25 units of dUTPase (ProsPec) at 48°C for 30 min to eliminate any dUTP present in the sample. dTTP was then measured, and the result was compared with the results obtained for another aliquot without dUTPase treatment, which represented the total dTTP + dUTP content. The dUTP detection limit was 5 pmol/mg of mitochondrial protein.

Quantification of mtDNA copy number and mRNA levels by real-time PCR

Total DNA was isolated from cell pellets (QuiAmpDNA Mini kit, Qiagen), dissolved in 10 mM Tris–HCl (pH 8.0) and quantified by spectrophotometry (NanoDrop Spectrophotometer, Thermo Scientific). Duplicates were assessed for each experimental condition. Real-time PCR was performed using an ABI PRISM® 7900HT sequence detector (Applied Biosystems). Detection of mtDNA and nuclear DNA was performed as a multiplex PCR reaction. TaqMan Universal PCR Master Mix with UNG (Applied Biosystems) and custom-designed sets of TaqMan probes and primers were used for relative mtDNA (12S rRNA gene) versus nuclear DNA (RNase P single copy gene) copy number quantification, as described (7). A standard curve with cloned-12S and RNAse P amplicons was used for absolute quantification of mtDNA and nuclear DNA in the samples. RNA was isolated from quiescent fibroblasts with Trizol Reagent (Invitrogen) and resuspended in RNAase-free water. Two micrograms of RNA were treated with DNase I (DNase I Amplification grade, Invitrogen) and reverse

transcribed to cDNA by using the High-Capacity cDNA Archive kit (Applied Biosystems). Quantitative real-time PCR reactions were carried out in 384-well plates with the TaqMan Universal PCR Master Mix No Amperase UNG (Applied Biosystems). Predesigned-TaqMan Gene Expression Assays (Applied Biosystems) were used for the detection of *DCK* (Hs01040726_m1) and *DGUOK* (Hs00176514_m1) mRNAs. Each sample was analyzed in triplicates, and the average value was used to determine the level of mRNA. The amount of the specific mRNA in each sample was normalized to 18S rRNA (Hs99999901_s1) with the 2-ΔCT method for comparison. All samples included in an independent 384-well plate were referred to an untreated control sample to avoid inter-plate variation.

dN and dNMP determination

dNMPs, dNs and some related metabolites were measured by liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS), using an Acquity UPLC-MS/MS apparatus (Acquity UPLC-Xevo^{TM} TQ Mass Spectrometer, Waters, Milford, MA, USA). Cell medium and diluted mouse plasma samples were prepared as previously described. For intracellular dN and dNMP determinations, 60 cm^2 cell monolayers were washed three times with ice-cold PBS in a cold room. Cells were then scraped from the dishes with 250 μl of ice-cold deionized Milli-Q water (Millipore). Collected cells were homogenized by 10 passes through a 23 G × 1 in. (0.6 × 25 mm) needle fitted to a syringe, homogenates were centrifuged at 13 000*g* at 4°C for 5 min, and supernatants were stored at −80°C until further use. Protein concentration was measured by the Bradford method. Cell medium, diluted plasma and cell homogenates were deproteinized by ultrafiltration (3 kDa Amicon Ultra filters, Millipore) at 14 000*g* and 4°C for 30 min before injection in the LC–MS/MS system.

For all determinations, the stationary phase was an Acquity UPLC BEH C18 column (100 × 2.1 mm, 130 Å pore, 1.7 μm particle, Waters). For dN and metabolite determinations, 5 μl of deproteinized sample was injected into the LC–MS/MS system and resolved at 0.5 ml/min through a binary gradient elution using a saline buffer (20 mM ammonium acetate, pH 5.6) and acetonitrile as follows: time 0–1.1 min, isocratic 100% saline buffer; 1.1–5 min, gradient from 0 to 13.6% acetonitrile; 5–5.1 min, gradient from 13.6 to 100% acetonitrile; 5.1–6.1 min, isocratic 100% acetonitrile; and 6.1–7.2 min, isocratic 100% saline buffer. Detection of the eluate components was performed using multiple reaction monitoring, with positive electrospray mode with the following *m*/*z* transitions: 251.8 > 136.1 (dAdo), 227.9 > 112.1 (dCtd), 242.8 > 127.1 (dThd), 267.9 > 152.1 (dGuo), 136.8 > 110.1 (hypoxanthine), 136.8 > 110.1 (dIno) and 228.8 > 113.1 (dUrd). Identification of dIno and hypoxanthine with equal *m*/*z* transitions was based on different retention times. dNMPs were resolved and determined with the same column and an ion pairing-based elution using *N*,*N*-dimethylhexylamine (DMHA). Briefly, eluent A (5 mM DMHA in water adjusted to pH 7.0 with formic acid) and eluent B (5 mM DMHA in 50:50 water:acetonitrile, not pH adjusted) were combined as follows: constant flow, 0.4 ml/min; time 0–4.7 min, gradient from 100% A to 20% A; 4.7–4.8 min, 20–100% A; and 4.8–6.1 min, isocratic 100% A. Detection of the eluate components was performed using multiple reaction monitoring, with negative electrospray mode with the following *m*/*z* transitions: 329.8 > 195.0 (dAMP) and 345.7 > 79.0 (dGMP). Identification of all compounds was based on retention time and specific ion transitions. Calibration curves made with aqueous standards were processed in parallel, and concentrations were obtained from interpolation of the peak areas using TargetLynx software (Waters).

Statistics

Statistical analysis was performed with SPSS 15.0 software. The tests used are indicated in the Results section or the figure legends. For statistical purposes, undetectable values were considered zero.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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