Deoxypyrimidine monophosphate bypass therapy for thymidine kinase 2 deficiency

Caterina Garone1,2, Beatriz Garcia-Diaz3, Valentina Emmanuele1,3, Luis C Lopez4, Saba Tadesse1, Hasan O Akman1, Kurenai Tanji5, Catarina M Quinzii1 & Michio Hirano1,*

Abstract

Autosomal recessive mutations in the thymidine kinase 2 gene (TK2) cause mitochondrial DNA depletion, multiple deletions, or both due to loss of TK2 enzyme activity and ensuing unbalanced deoxynucleotide triphosphate (dNTP) pools. To bypass Tk2 deficiency, we administered deoxycytidine and deoxythymidine monophosphates (dCMP+dTMP) to the Tk2 H126N (Tk2−/−) knock-in mouse model from postnatal day 4, when mutant mice are phenotype normally, but biochemically affected. Assessment of 13-day-old Tk2−/− mice treated with dCMP+dTMP 200 mg/kg/day each (Tk2−/−/dCMP+dTMP) demonstrated that in mutant animals, the compounds raise dTTP concentrations, increase levels of mtDNA, ameliorate defects of mitochondrial respiratory chain enzymes, and significantly prolong their lifespan (34 days with treatment versus 13 days untreated). A second trial of dCMP+dTMP each at 400 mg/kg/day showed even greater phenotypic and biochemical improvements. In conclusion, dCMP/dTMP supplementation is the first effective pharmacologic treatment for Tk2 deficiency.

Keywords deoxycytidine monophosphate; deoxythymidine monophosphate; encephalomyopathy; therapy; thymidine kinase

Introduction

Encoded by the nuclear DNA gene TK2, thymidine kinase 2 (TK2) is a mitochondrial matrix protein that phosphorylates thymidine and deoxycytidine pyrimidine nucleosides to generate deoxothymidine monophosphate (dTMP) and deoxycytidine monophosphate (dCMP), which, in turn, are converted to deoxynucleotide triphosphates (dNTPs) required for mitochondrial DNA (mtDNA) synthesis.

Autosomal recessive TK2 mutations cause severe mtDNA depletion and devastating neuromuscular diseases in infants and children, as well as mtDNA multiple deletions and progressive external ophthalmoplegia in adults (Saada et al, 2001; Tyynismaa et al, 2012).

To elucidate the molecular pathogenesis of TK2 deficiency, we generated a homozygous Tk2 H126N knock-in mutant (Tk2−/−) mouse that manifests a phenotype strikingly similar to the human infantile encephalomyopathy (Akman et al, 2008). Between postnatal day 10 and 13, Tk2−/− mice rapidly develop fatal encephalomyopathy beginning with decreased ambulation, unstable gait, coarse tremor, and growth retardation that rapidly progress to early death at age 14–16 days (Dorado et al, 2011). A similar phenotype was observed in the Tk2 knockout mouse (Zhou et al, 2008).

In the Tk2−/− mice, loss of TK2 activity caused dNTP pool imbalances with low dTTP levels in brain and decreased dTTP and dCTP in liver, which, in turn, produce mtDNA depletion and defects of mitochondrial respiratory chain (RC) complexes I, III, IV, and V containing mtDNA-encoded subunits, most prominently in the brain and spinal cord (Dorado et al, 2011).

Based on the understanding of the pathogenesis of Tk2 deficiency, we have assessed a rationale therapeutic strategy to bypass the enzymatic defect with oral dCMP and dTMP supplementation.

Results
dCMP/dTMP delays disease onset, prevents neuromuscular manifestations, and prolongs lifespan of Tk2-deficient mice

Oral treatment with dCMP+dTMP 200 mg/kg/day each in milk (Tk2−/−/dCMP+dTMP) beginning at postnatal day 4 delayed disease onset to 20–25 days (Supplementary Video S1), when the mutant mice developed a mild tremor and stopped gaining weight. In the fourth week, they manifested weakness and reduced movements. In contrast, Tk2−/− mice treated from day 4 with dCMP+dTMP
400 mg/kg/day each in milk (Tk2−/−/400dCMP/dTMP) appeared normal until day 21, when weight gain decelerated and mild head tremor developed (Fig 1A). Untreated Tk2−/− mice had a mean lifespan of 13.2 ± 2.5 days (mean ± SD), whereas Tk2−/−/200dCMP/dTMP survived to 34.6 ± 3.2 days (P = 0.0028; n = 7; Gehan–Breslow–Wilcoxon test) while Tk2−/−/400dCMP/dTMP lived to 44.3 ± 9.1 days (P = 0.0071; n = 7; Gehan–Breslow–Wilcoxon test) (Fig 1B). The cause of death was not evident in postmortem histological studies of major organs in 29-day-old Tk2−/−/200dCMP/dTMP mice. No adverse side effects, including malignancies, were observed in the treated homozygous, and heterozygous wild types (Tk2+; Supplementary Table S1).

Open-field assessment of motor function in 29-day-old Tk2−/−/200dCMP/dTMP, Tk2−/−/400dCMP/dTMP, and wild-type Tk2 mice showed no differences in the distance traveled, horizontal and vertical movements, or resting time (Fig 1C–E). Relative to 29-day-old Tk2−/− mice, age-matched Tk2−/−/200dCMP/dTMP and Tk2−/−/400dCMP/dTMP animals showed decreases in gross muscle mass and muscle fiber diameter that were independent of the treatment dose but paralleled to body weight (Supplementary Fig S1D–F).

Histological and histochemical CNS studies confirmed dCMP/dTMP efficacy

Efficacy of treatment in central nervous system (CNS) was demonstrated in histological studies that showed dramatic reductions in the numbers of vacuoles in neurons of the spinal cord and cerebellar and brain stem nuclei of 13-day-old Tk2−/−/200dCMP/dTMP mice relative to untreated 13-day-old Tk2−/− mice (Fig 2A and B). Furthermore, cytochrome c oxidase (COX, complex IV) histochemistry of cerebellum revealed reduced overall COX activity in 13-day-old untreated Tk2−/− mice (Fig 3A) with normal activities in 13- and 29-day-old Tk2−/−/200dCMP/dTMP (Fig 3C and E) relative to Tk2+ animals (Fig 3B, D and F). No cell-specific immunohistochemical differences in COX protein were detected (Fig 3G and H) while severe reduction in complex I was identified by immunostaining of cerebellum of 29-day-old Tk2−/−/200dCMP/dTMP (Fig 3I and J).

Treatment crosses biological barriers

To confirm that the treatment crosses biological barriers, we assessed dNTP levels in isolated mitochondria. In 13-day-old untreated Tk2−/− mice relative to Tk2+ littermates, isolated brain mitochondria showed decreased levels of dTTP (0.67 ± 0.1 pmol/mg-protein versus 2.52 ± 1.0), while isolated liver mitochondria revealed reduced dCTP levels (1.07 ± 0.8 versus 2.9 ± 1.0)
The treatment crossed the blood–brain barrier increasing the level of dTTP in isolated brain mitochondria of 13-day-old Tk2+/200dCMP/dTMP (3.55 \pm 1) and Tk2+/C0/C0/C0 200dCMP/dTMP (1.5 \pm 0.7) and as a consequence, restored the proportion of dTTP relative to total dNTP in treated mutants. In contrast, levels of dCTP in isolated mitochondria were stable in brain of 13-day-old Tk2+/200dCMP/dTMP (3.07 \pm 2), decreased in brain of Tk2+/C0/C0/C0 200dCMP/dTMP (1.13 \pm 0.5), and decreased in liver of 13-day-old Tk2+/200dCMP/dTMP (1.13 \pm 0.4) and Tk2+/−200dCMP/dTMP (0.56 \pm 0.5) (Supplementary Table S2).

In 29-day-old Tk2+/−200dCMP/dTMP relative to Tk2+ mice, absolute levels of dTTP and dCTP were markedly reduced in isolated mitochondria from brain (dTTP 0.11 \pm 0.05 and dCTP 0.6 \pm 0.2) and from liver (dTTP 0.15 \pm 0.04 and dCTP 0.04 \pm 0.03) (Supplementary Table S2); when these data were expressed as percentage of total dNTPs, there were striking decreases in dTTP/dNTP in brain ($P = 0.0322; n = 7$; Mann–Whitney U-test) and dCTP/dNTP in liver ($P = 0.0338; n = 3$; Mann–Whitney U-test) (Fig 4A and B).

dCMP/dTMP treatment ameliorates biochemical and molecular genetic abnormalities

Treatment with dCMP and dTMP enhanced mtDNA levels in the mutant mice. At pre-treatment baseline, 4-day-old Tk2+/C0/C0/C0 mice did not manifest clinical abnormalities, but showed reductions of mtDNA copy numbers in brain cerebrum (38 \pm 13 % mtDNA relative to wild-type brain, $P = 0.0002; n = 5$; Mann–Whitney U-test), cerebellum (54 \pm 1 %, $P = 0.0228; n = 4$; Mann–Whitney U-test), muscle (28 \pm 12 %), and kidney (62 \pm 11 %) with normal mtDNA levels in heart and liver (Fig 4C). At age 13 days, untreated Tk2+/− animals...
showed marked mtDNA depletion in brain cerebrum (21 ± 3%, P < 0.0025; n = 5; Mann–Whitney U-test), muscle (47 ± 1%, P = 0.0303; n = 7; Mann–Whitney U-test), liver (32 ± 1%, P = 0.0140; n = 5; Mann–Whitney U-test), and kidney (35 ± 9%, P = 0.008; n = 6; Mann–Whitney U-test), but stable mtDNA depletion in the cerebellum (Fig 4C). In contrast, with treatment, 13-day-old Tk2+/C0/C0/200dCMP/dTMP mice manifested moderate mtDNA depletion only in brain cerebrum (66 ± 34%) and normal mtDNA levels in cerebellum, muscle, heart, liver, and kidney (Fig 4C).

At age 29 days, relative to Tk2+, Tk2-/−/200dCMP/dTMP mice showed mtDNA depletion that was severe in cerebellum (23 ± 8%) and brain cerebrum (11 ± 1%) and moderate in muscle (48 ± 23%), liver (70 ± 13%), and kidney (55 ± 6%) (Fig 4D).

Compared with Tk2−/−/200dCMP/dTMP mice, Tk2−/−/400dCMP/dTMP animals had less severe mtDNA depletion in brain cerebrum (22 ± 8%, P = 0.0159; n = 6; Mann–Whitney U-test), but similar mtDNA depletion in muscle (40 ± 8%), liver (71 ± 36%), and kidney (43 ± 11%) and cerebellum (26 ± 12%) (Fig 4D).

To assess the impact of treatment on mitochondrial RC enzymes, their activities and steady-state protein levels in brain cerebrum and cerebellum were measured. In 13-day-old untreated Tk2−/− mice, relative to untreated wild-type, brain cerebrum showed reduced

Figure 4. dCMP/dTMP effects on dNTP pool balance and mtDNA copy number.

A, B Proportions of dNTPs (in percents) in isolated mitochondria of brain (A) and liver (B) of 13 and 29 postnatal day mice (P13 and P29) demonstrate that levels of dTTP (red sections) were increased in treated mutant versus untreated mutant mice at P13, but were severely decreased in P29 Tk2−/−/200dCMP/dTMP versus Tk2+/−/200dCMP/dTMP (***P < 0.005; Mann–Whitney U-test).

C mtDNA copy numbers in mice reveal rescue of mtDNA depletion in all tissues of treated mutants at P13 (Tk2−/− versus Tk2+/−/200dCMP/dTMP, *P < 0.05; **P < 0.005; ***P < 0.0005; Mann–Whitney U-test).

D Dose-related increase in mtDNA copy number in cerebral hemispheres of mutant mice at P29 (expressed as percent of untreated Tk2+ controls; mean ± SD; Tk2−/−/200dCMP/dTMP versus Tk2−/−/400dCMP/dTMP, *P < 0.05; Mann–Whitney U-test) (n = 5 for each group).

Source data is available online for this figure.
COX activity (57 ± 19%, $P = 0.0159$; $n = 5$; Mann–Whitney $U$-test) and significantly increased citrate synthase (CS) activity (148 ± 17%; $P = 0.0317$; $n = 5$; Mann–Whitney $U$-test) (Fig 5A, Supplementary Table S3) and, when normalized to CS, revealed decreased activities of complexes I+III (NADH-cytochrome $c$ reductase) (76 ± 0.06%, $P = 0.0159$; $n = 5$; Mann–Whitney $U$-test) and II+III (succinate-cytochrome $c$ reductase) (72 ± 9%) in addition to IV (41 ± 14%, $P = 0.0079$; $n = 5$; Mann–Whitney $U$-test) (Fig 5B, Supplementary Table S3). The RC defects were more severe in cerebellum with significant reductions in all of the complexes when normalized either to CS (Fig 5C) or to mg-proteins with predominant defect in complex I (29 ± 15%; $P = 0.0087$; $n = 5$; Unpaired $t$-test with Welch’s correction) and increased CS activity (129 ± 34%) (Supplementary Table S4). In contrast, 13-day-old $Tk2_{−/−}-200dCMP/dTMP$ had normal RC enzyme activities in brain cerebrum (Fig 5A and B, Supplementary Table S3) and only a mild defect in complex I (56 ± 21%) in cerebellum compared withagematched treated control mice (Fig 5C, Supplementary Table S4). In 29-day-old $Tk2_{−/−}-200dCMP/dTMP$, activities of RC enzymes were normal in brain cerebrum (Supplementary Table S3). In contrast, cerebellum of $Tk2_{−/−}-200dCMP/dTMP$ manifested a mild defect in complex IV (62 ± 20%) and severe defect in complex I+III (35 ± 24%, $P = 0.0296$; $n = 5$; Mann–Whitney $U$-test), while RC activities were completely rescued in the $Tk2_{−/−}-400dCMP/dTMP$.

**Figure 5.** dCMP/dTMP efficacy on brain hemisphere and cerebellum biochemistry.

A, B (A) Cerebral hemispheres of untreated $Tk2_{−/−}$ mice relative to untreated wild types showed significant increase in CS activity and deficiency of complex IV activity (micromole/min/mg tissue normalized to mg-protein; mean ± SD) as well as (B) defects of complexes IV and I+III activities when referred to CS (mean ± SD). In contrast, with 200 mg/kg/day dCMP/dTMP, activities of citrate synthase and complex IV were normal in cerebrum of 13-day-old $Tk2_{−/−}$. C In cerebellum of mutant mice at ages 13 and 29 days, activities of mitochondrial RC referred to CS (expressed as percent of $Tk2^+$) showed treatment-dose-related increases.

D–E Western blot of OXPHOS protein (MitoProfile® Total OXPHOS Rodent WB Antibody Cocktail, MitoSciences®) in brain (D) and cerebellum (E) of 13-day-old untreated $Tk2_{−/−}$, 13- and 29-day-old $Tk2_{−/−}$. F–H Quantitation of western blot bands demonstrated that treatment normalized levels of complexes I and IV protein in both brain cerebrum and cerebellum tissues at 13 days, but did not correct complexes I and II deficiencies in brain cerebrum and complexes I and IV in cerebellum at 29 days of age. Statistical analyses were performed using $Tk2_{−/−}$ versus $Tk2_{−/−}-200dCMP/dTMP$, with P13 cerebral samples (F); $Tk2_{−/−}$ versus $Tk2_{−/−}-200dCMP/dTMP$, with P29 cerebral samples (G); $Tk2_{−/−}$ versus $Tk2_{−/−}-200dCMP/dTMP$, with P13 cerebellar samples (H); and $Tk2_{−/−}$ versus $Tk2_{−/−}-400dCMP/dTMP$, with P29 cerebellar samples (H) $**P < 0.05$.

Data information: Statistical analyses were performed with Mann–Whitney $U$-test and Unpaired $t$-test with Welch’s correction. CS, citrate synthase; IV, cytochrome $c$ oxidase (COX); I+III, NADH-cytochrome $c$ reductase; I, NADH-dehydrogenase; II, succinate dehydrogenase; III, cytochrome $c$ reductase; V, ATP synthase; P, postnatal day.
Mitochondrial DNA depletion syndrome (MDS) is a frequent cause of severe childhood encephalomyopathy characterized molecularly by reduction of mtDNA copy number in tissues and insufficient mtDNA replication or in the metabolism of deoxynucleotide triphosphate pools utilized as precursors for DNA replication (Copeland, 2012).

Treatment for MDS, like most mitochondrial disorders, has been limited to supportive therapies; however, understanding the pathomechanism of MDS enables the design of treatment strategies targeting either the cause of the disease or the downstream metabolic defects. Enzyme replacement by allogenic hematopoietic stem cell transplantation (HSCT) has shown promising initial results with MNGIE due to mutations in the TTYMP gene encoding thymidine phosphorylase, another enzyme involved in nucleoside metabolism (Nishino et al, 1999; Hirano et al, 2006; Garone et al, 2011) but limited to disorders with toxic metabolites that can be eliminated by circulating cells. In contrast to AHSC, gene therapy holds greater promise for mitochondrial diseases and other genetic disorders, but faces several barriers such as inefficient gene delivery, immune responses, and short-lived effects.

An alternative metabolic bypass approach has been tested in mtDNA-depleted myotubes from patients with DGUOK mutations. Remarkably, supplementation of culture media with deoxypurine nucleoside monophosphates (dAMP and dGMP), the products of dGK activity, partially restored mtDNA levels (Bulst et al, 2009) indicating that extracellular dAMP and dGMP are able to cross plasma and mitochondrial membranes and reach the mitochondrial matrix where they enter the nucleotide salvage pathway after bypassing the dGK defect. Concentrations of dAMP/dGMP up to 200–400 μM increased mtDNA levels in a dose-related fashion, while higher levels up to 1,200 μM (corresponding to 150 mg/kg/ day in mice) did not further increase mtDNA or cause nuclear DNA or mtDNA qualitative defects such as chromosomal rearrangement or mtDNA deletions (Bulst et al, 2009).

We tested dCMP and dTMP supplementation in our Tk2 knock-in mouse model to bypass the Tk2 defect. Deoxypuridine nucleoside monophosphate supplementation delayed the disease onset, reduced the severity of phenotypic manifestations, and prolonged the survival of the mutant mice in a dose-related manner. No adverse side effects, including malignancies, were observed. Oral dTMP/dCMP crossed biological barriers including the blood-brain barrier (BBB) because treatment increased dTTP in brain and liver in 13-day-old Tk2−/− mice and augmented levels of mtDNA restoring the mitochondrial RC activities and protein defects in brain, heart, muscle, liver, and kidney of 13- and 29-day-old mutant mice.

**Discussion**

Mitochondrial DNA depletion syndrome (MDS) is a frequent cause of severe childhood encephalomyopathy characterized molecularly by reduction of mtDNA copy number in tissues and insufficient synthesis of mitochondrial RC complexes (Hirano et al, 2001; Spinazzola & Zeviani, 2009). Mutations in eight nuclear genes have been identified as causes of infantile MDS (Tk2, DGUOK, POLG, MPV17, RRM2B, SUCLA2, SUCLG1, and C10orf2) (Mandel et al, 2001; Saada et al, 2003; Naviaux & Nguyen, 2004; Elpeleg et al, 2005; Spinazzola et al, 2006; Bourdon et al, 2007; Ostergaard et al, 2007; Sarzi et al, 2007); 7 of the genes encode proteins involved in mtDNA replication or in the metabolism of deoxynucleotide triphosphate pools utilized as precursors for DNA replication (Copeland, 2012).
Figure 6.

A. LIVER

B. BRAIN

C. MUSCLE

D. THYMIDINE PHOSPHORYLASE ACTIVITY

E. BRAIN

F. MUSCLE

Figure 6.
Treatment-related marked improvements of mtDNA levels and biochemical defects in muscle, which is the most affected tissue in Tk2 mutant patients, suggest that dCMP/dTMP might be more efficacious in patients with myopathy due to TK2 deficiency than in Tk2<sup>−/−</sup> mice with severe CNS involvement.

Analyses of plasma and tissue levels of dCMP/dTMP and their metabolites revealed increases in deoxythymidine (dT) and deoxyuridine (dU), but not dCMP or dTMP 30 min after oral gavage treatment. Based on these findings, we hypothesize that dCMP/dTMP can be effective either by rapid and transient bypassing of the enzyme defect and/or by increasing the dT and thymine (T) substrates as documented by measurements of dT/T levels in plasma and tissue and by dNTP pool analysis in 13-day-old mice. In contrast, dCMP is catabolized by deoxycytidine deaminase, the enzyme responsible for dCMP conversion to deoxyuridine monophosphate (dUMP) (Heinemann & Plunkett, 1989; Jansen et al., 2011). The dUMP may be converted to dTMP via thymidylate synthase and may contribute to the observed increases in dTTP levels (Fig 7). Because nucleosides are intrinsically unstable, catabolized in vascular and tissue compartments, and ineffectively phosphorylated by mutant TK2, we postulate that treatment with dT and dC may be less effective than dTMP and dCMP in Tk2 mutant mice.

We previously demonstrated that Tk2 (mitochondrial) activity is constant in the second week of life, whereas cytosolic Tk1 activity decreases significantly between postnatal day 8 and 13 (Dorado et al., 2011). The downregulation of Tk1 activity unmasks Tk2 deficiency in Tk2<sup>−/−</sup> mice and coincides with the onset of mtDNA depletion causing inexorable organ failure leading to death. In the present work, we demonstrated that oral dCMP and dTMP delayed the reduction in Tk1 activity. Thus, in addition to providing substrates for dNTP synthesis, dCMP/dTMP supplementation in Tk2<sup>−/−</sup> mice appears to enhance compensatory Tk1 activity. Nucleotides generated by de novo synthesis can enter into mitochondria through carriers that have been previously demonstrated indirectly in the case of dTMP or directly by PNC1 in the case of dTTP (Ferraro et al., 2006; Franzolin et al., 2012). Once in mitochondria, dTMP and dTTP can be incorporated into replicating mtDNA as demonstrated directly through <i>in vitro</i> modulation of Tk1 activity and PNC1 carrier in cells exposed to radiolabeled thymidine (Franzolin et al., 2012) and indirectly by our <i>in vivo</i> results. The reduced efficacy of dCMP/dTTP after age 29 days may be due to decreased Tk1 activity.

Oral dCMP/dTMP failed to improve dCTP and dTTP levels in brain cytoplasm and mitochondria of 29-day-old mutants that manifested a head tremor, growth plateau, and subsequently died. The head tremor is likely due to cerebellar dysfunction as we noted a severe complex I protein defect in this tissue. The inability of dCMP/dTMP to prevent CNS manifestations is likely attributable to the development of the blood–brain barrier in Tk2<sup>−/−</sup> mice after age 13 days. Therefore, intrathecal treatment may be required in the Tk2<sup>−/−</sup> mice and Tk2 patients with encephalopathy (Galbiati et al., 2006; Gotz et al., 2008).

There are several additional factors that contribute to the incomplete efficacy of dCMP/dTMP therapy in the Tk2-deficient mice. First, orally administered dTMP is catabolized by the dramatically increased thymidine phosphorylase activity in the small intestine of

![Graphical summary of the pathways modulated by oral gavage dCMP/dTMP treatment.](image-url)

**Figure 7.** Deoxypyrimidine monophosphates pathways. Graphical summary of the pathways modulated by oral gavage dCMP/dTMP treatment.

**A** dTMP metabolism. dTMP treatment may enter as monophosphate into the mitochondria bypassing the TK2 enzymatic defect as demonstrated by the increased level of dTTP in postnatal day 13 mutant mouse tissues. However, dTMP is also rapidly degraded by 5'-nucleotidase in the small intestine to the nucleoside (dT), which may be processed via three different pathways: (i) phosphorylated by residual TK2 activity to eventually produce dTTP within mitochondria; (ii) converted to dTMP by cytosolic TK2; or (iii) catabolized by thymidine phosphorylase (TP). The combination of reduced TK2 activity in brain and increased thymidine phosphorylase (TP) activity in small intestine after postnatal day 13 may account for the reduced efficacy of the treatment in rescuing the dTTP pool balance after age 13 days.

**B** dCMP metabolism. dCMP/dTMP treatment did not increase dCTP levels in mitochondria of Tk2<sup>−/−</sup> mice suggesting that dCMP administered orally does not enter into mitochondria. Instead, dCMP degraded to nucleoside (dC) may be a source of dTMP as shown in the figure or may be catabolized to uracil by cytosolic TP.
29-day-old mice. Second, supplemental dTMP and dCMP failed to fully correct intra-mitochondrial levels of dTTP and dCTP, indicating that mitochondrial membranes impede dNTP entry; this hypothesis is supported by the low intra-mitochondrial dCTP/dTTP levels with normal cytosolic concentrations of dCTP and dTTP in liver of 29-day-old treated mice. Third, the doses of dTMP/dCMP may have been insufficient, as we have observed dose-related efficacy. Addressing these therapeutic obstacles (e.g. inhibiting TP activity) may lead to enhanced effectiveness of dCMP/dTMP treatment in Tk2–/– mice.

In conclusion, deoxypyrimidine supplementation for Tk2 deficiency is the first effective and safe in vitro treatment option that may be readily translated to patient affected by Tk2 mutations. Furthermore, this approach is potentially applicable to individuals with other mitochondrial disorders due to nucleotide pools imbalance.

Materials and Methods

Mice

Generation and characterization of Tk2 H126N knock-in mice were previously reported (Akman et al., 2008). All experiments were performed according to a protocol approved by the Institutional Animal Care and Use Committee of the Columbia University Medical Center and were consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Mice were housed and bred according to international standard conditions, with a 12-h light, 12-h dark cycle, and sacrificed at 4, 13, and 29 days of age.

Organs (brain, spinal cord, liver, heart, kidney, quadriceps muscle, lung, and gastrointestinal tract) were removed and either frozen in the liquid phase of isopentane, pre-cooled near its freezing point (–160°C) with dry ice or fixed in 10% neutral-buffered formalin and embedded in paraffin using standard procedures. Paraffin-embedded tissue were then stained with hematoxylin and eosi (H&E) for morphological study or processed for immunostaining (H&E) for morphological study or processed for immunostaining with antibodies against GFAP, COX subunit 2, and observed closely for comparison.

Brain histology

Brain and spinal cord samples from 13- to 29-day-old mice were fixed with 10% neutral-buffered formalin and embedded in paraffin using standard procedures. Cerebellum, brainstem, hippocampus, cerebral cortex, and cervical, thoracic and lumbar tracts of the spinal cord were analyzed.

Sections (5 μm thick) were stained with H&E and luxol fast blue to analyze the overall structure of the tissue. Immunostaining with antibodies against GFAP, complex I (NDUFB6), or COX subunit 2 was also performed. Briefly, paraffin-embedded brain and spinal cord slides were deparaffinized, rehydrated, and rinsed in phosphate-buffered saline solution (PBS). To block endogenous peroxidase activity, sections were incubated with 3% hydrogen peroxide in methanol. Slides were then placed in 0.1 M sodium citrate buffer (pH 6.0) and heated in a microwave oven for 15 min, for antigen retrieval. Slides were incubated with mouse anti-GFAP antibody (1:100) (Novocastra. NCL-GFAP-GAS) or mouse monoclonal antibody anti-complex I 17 kDa (NDUFB6) subunit (1:100) (A21359; Molecular Probes) or mouse monoclonal anti-COX subunit 2 (1:100) (clone COX 229, A6404; Molecular Probes) overnight at 4°C. Sections were subsequently rinsed in PBS and incubated with anti-mouse M.O.M. Peroxidase kit, 1:200 dilution for 60 min at room temperature. Immunoreactivity was detected by avidin–biotin complex (ABC) with DAB substrate (Vector Laboratories, Burlingame, CA, USA). Slides were examined by light microscopy using an Olympus BX51 microscope, and images were captured with a QImaging Retiga Exi digital camera, using QCapture software version 2.68.6.

dNTP pool by polymerase extension essay

Tissues were homogenized on ice in 10 volumes (w/v) of cold MTSE buffer (210 mM mannitol, 70 mM sucrose, 10 mM Tris–HCl pH 7.5, 0.2 mM EGTA, 0.5% BSA) and centrifuged at 1,000 g for 5 min at 4°C, followed by three centrifugations at 13,000 g for 2 min at 4°C. Supernatant was precipitated with 60% methanol for the mitochondrial fraction and 100% methanol for the cytosolic fraction, kept 2 h at −80°C, boiled 3 min, stored at −80°C (from 1 h to overnight), and centrifuged at 20,000 g for 10 min at 4°C. Supernatants were evaporated until dry, and pellet was resuspended in 65 μl of water and stored at −80°C until analyzed. To minimize ribonucleotide interference, total dNTP pools were determined as reported (Ferraro et al., 2010; Marti et al., 2012a). Briefly, 20 μl volume reactions was generated by mixing 5 μl of sample or

Phenotype assessment

To define the degree of safety and efficacy of dTMP/dCMP, we compared survival time, age at onset of disease, type and severity of symptoms, occurrence of side effects, and proportion of treatment termination due to adverse events in treated and untreated Tk2 mice. General behavior, survival time, and body weights of the mice were assessed daily beginning at postnatal day 4. Videotaping and open-field test with an Opto-Variametrics-3 sensor system (Columbus Instruments) were performed at 13 and 29 days by counting horizontal and vertical movements, by recording ambulatory and resting time and by measuring the total distance traveled in 10 min.
standard with 15 µl of reaction buffer [0.025 U/ml ThermoSequen-
ase DNA polymerase (GE Healthcare, Piscataway, NJ, USA) or
Taq polymerase (Life Technologies, NY, USA), 0.75 µM 3H-dTTP or
3H-dATP (Moravek Biochemicals), 0.25 µM specific oligonucleotide,
40 mM Tris–HCl, pH 7.5, 10 mM MgCl₂, 5 mM DTT]. After 60 min
at 48°C, 18 ml of reaction were spotted on Whatman DE81 filters,
air dried, and washed three times for 10 min with 5% Na₂HPO₄,
once in distilled water and once in absolute ethanol. The retained
radioactivity was determined by scintillation counting.

Nucleotides measurements by HPLC

Nucleotides concentrations were measured as described (Akman
et al., 2008) with minor modifications in brain, muscle, and liver. 50
mg of tissue was homogenized in 500 ml of ice-cold 0.5 M
perchloric acid and centrifuge at 16,000 g for 10 min at 4°C. The
pellets were stored at −80°C for protein measurement, and nucleo-
tides were measured in the resultant supernatant using the Alliance
HPLC (Waters Corporation, Milford, MA, USA) with an Alltima
C18NUC reverse-phase column (Alltech Associates, Deerfield, IL,
USA) and UV detection. After stabilizing the column with the mobile
phase, samples (50 ml) were injected onto the HPLC system. The
mobile phase consists of 0.2 M ammonium phosphate buffer pH 3.5
(phase A) and 30% methanol in 0.2 M ammonium phosphate
buffer, pH 3.5 (phase B). The time schedule for the binary gradient
was as previously reported (Ferraro et al., 2006). Standard curves
for dCTP, dTTP, dTMP, and dCMP were constructed with 15, 30,
and 60 mM of each nucleotide. Absorbance of the samples was
measured with an UV detector at 260 nm wavelength, and the
concentration of each nucleotide in the samples was calculated
based on the peak area. Nucleotide levels were expressed in nmol/
mg prot.

Nucleosides measurements by HPLC

Deoxythymidine (dT), deoxyuridine (dU), uracil (U), and thymine
(T) levels were assessed by a gradient-elution HPLC method as
described previously (Lopez et al., 2009; Marti et al., 2012b), with
minor modifications. Briefly, deproteinized samples were injected
into an Alliance HPLC system (Waters Corporation) with an Alltima
C18NUC reversed-phase column (Alltech) at a constant flow rate of
1.5 ml/min (except where indicated) using three buffers: eluent A
(20 mM potassium phosphate, pH 5.6), eluent B (water), and eluent
C (methanol). Samples were eluted over 60 min with a gradient as
follows: 0–5 min, 100% eluent A; 5–25 min, 100–71% eluent A;
29% eluent B; 25–26 min, 0–100% eluent C; 26–30 min, 100%
eluent C; 30–31 min, 0–100% eluent B; 31–35 min, 100% eluent B
(1.5 – 2 ml/min); 35 – 45 min, 100% eluent B (2 ml/min); 45 –
46 min, 100% eluent B (2–1.5 ml/min); 46–47 min, 0–100% eluent
C; 47–50 min, 100% eluent C; 50–51 min, 0–100% eluent A; and
51–60 min, 100% eluent A.

Absorbance of the elutes was monitored at 267 nm, and dThd
and dUrd peaks were quantified by comparing their peak areas with
a calibration curve obtained with aqueous standards. For definitive
identification of dT, dU, U, and T peaks for each sample, we used a
second aliquot treated with excess of purified E. coli TP (Sigma) to
specifically eliminate dT and dU. The detection limit of this method
is 0.05 mmol/l for all nucleosides.

RT-qPCR: mitochondrial DNA quantification

Real-time PCR was performed with the primers and probes for
murine COX 1 gene (mtDNA) and mouse glyceraldehyde-3-
phosphate dehydrogenase (GAPDH, nDNA) (Applied Biosystems,
Invitrogen, Foster City, CA, USA) as described using standard curve
quantification, in an ABI PRISM 7,000 Sequence Detection System
(Applied Biosystems) (Dorado et al., 2011). MtDNA values were
normalized to nDNA values and expressed as percent relative to
wild type (100%).

Mitochondrial respiratory chain protein levels

Thirty micrograms of whole brain cerebrum or cerebellum extracts
was electrophoresed in an SDS-12% PAGE gel, transferred to
Immun-Blot PVDF membranes (Bio-Rad, Hercules, CA, USA) and
probed with MitoProfile Total OXPHOS Rodent WB Antibody Cocktail
of antibodies (MitoSciences, Eugene, OR, USA). Protein–anti-
body interaction was detected with peroxidase-conjugated mouse
anti-mouse IgG antibody (Sigma-Aldrich, St Louis, MO, USA), using
Amersham ECL Plus western blotting detection system (GE
Healthcare Life Sciences, UK). Quantification of proteins was carried
out using NIH ImageJ 1.37v software. Average gray value was
calculated within selected areas as the sum of the gray values of all
the pixels in the selection divided by the number of pixels.

Mitochondrial respiratory chain enzyme activities by
spectrophotometer analysis

Mitochondrial RC enzymes analysis was performed in cerebrum and
cerebellum tissues as previously described (DiMauro et al., 1987).

Nucleosides and nucleotides metabolic enzymes

Thymidine phosphorylase and thymidine kinase 1 and 2 activities
were measured as previously described (Marti et al., 2003; Lopez
et al., 2009; Dorado et al., 2011).

Statistical methods

Data are expressed as the mean ± SD of at least three experiments
per group. Gehan-Breslow-Wilcoxon test was used to compare the
survival proportion of each group of mice. Unpaired t-test with
Welch’s correction and Mann-Whitney U-test were used to compare
13-day-old Tk2+/−, 13-day-old untreated
Tk2+/− versus Tk2−/−, 29-day-old wild-type versus
Tk2+/− versus Tk2−/−, and Tk2+/− versus Tk2−/−
for molecular and
biochemical studies. Response to treatment was evaluated comparing
Tk2+/− versus Tk2−/− and Tk2+/− versus Tk2−/− at 13 days and
Tk2−/− versus Tk2+/− and Tk2−/− versus Tk2+/−. A P-value of < 0.05
was considered to be statistically significant.

Supplementary information for this article is available online:
http://embomolmed.embopress.org

Acknowledgements

This work was supported by research grants from the Muscular Dystrophy
Association (MH) and the Associazione Malattie Metaboliche Congenite eredit-
The paper explained

Problem
Mitochondrial DNA depletion syndrome (MDS) is a frequent cause of severe childhood encephalomyopathy characterized molecularly by reduction of mtDNA copy number in tissues and insufficient synthesis of mitochondrial respiratory chain complexes. Mutations in TK2, encoding mitochondrial thymidine kinase 2, frequently cause mtDNA depletion syndrome presenting as fatal myopathy, spinal muscular atrophy (SMA-like) disease, or encephalomyopathy in early childhood. In addition, Tk2 deficiency has been found to cause adult-onset myopathy with progressive external ophthalmoplegia and multiple deletions of mtDNA. We generated and characterized thymidine kinase 2 H126N knock-in mouse model that recapitulates the human infantile encephalomyopathy and further demonstrated that the pathogenesis of the disorder is due to loss of Tk2 enzyme activity and unbalanced deoxynucleotide triphosphate (dNTP) pools with lack of deoxythymidine and deoxycytidine triphosphates.

Results
We have demonstrated that ‘molecular bypass therapy’ with orally administered deoxythymidine monophosphate and deoxycytidine monophosphate produces dramatic clinical, molecular, biochemical, and histological improvements in our Tk2 knock-in mouse model.

Impact
Our results reveal, for the first time, in vivo efficacy and safety of a molecular therapy that improves downstream metabolic defects in TK2 deficiency. The treatment is potentially translatable to human use; therefore, our work may impact the treatment of patients with this rare devastating disorder. Furthermore, our approach can be extended to other mitochondrial disorders with unbalanced nucleotide pools.

Author contributions
CG designed the study, carried out experiments and data analysis, and drafted the manuscript. BGD and LCL contributed to molecular and biochemical experiments. VE contributed to histology experiments. KTS supervised experiments and edited the manuscript. MH designed the study, directed and supervised experimental procedures, and edited the manuscript.

Conflict of interest
The authors declare that they have no conflict of interest.

References
transplantation corrects biochemical derangements in MNGIE. Neurology 67: 1458–1460


License: This is an open access article under the terms of the Creative Commons Attribution 4.0 License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.