Deoxycytidine and Deoxythymidine Treatment for Thymidine Kinase 2 Deficiency

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Deoxycytidine and deoxythymidine treatment for thymidine kinase 2 deficiency

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Abstract

Objective—Thymidine kinase 2 (TK2), a critical enzyme in the mitochondrial pyrimidine salvage pathway, is essential for mitochondrial DNA (mtDNA) maintenance. Mutations in the nuclear gene *TK2* cause TK2 deficiency, which manifests predominantly in children as myopathy with mtDNA depletion. Molecular bypass therapy with the TK2 products, dCMP and dTMP, prolongs the lifespan of Tk2-deficient ($Tk2^{-/-}$) mice by 2-3 fold. Because we observed rapid catabolism of the deoxynucleoside monophosphates to deoxythymidine (dT) and deoxycytidine (dC), we hypothesized that: 1) deoxynucleosides might be the major active agents and 2) inhibition of deoxycytidine deamination might enhance dTMP+dCMP therapy.

Methods—To test these hypotheses, we assessed two therapies in $Tk2^{-/-}$ mice: 1) dT+dC and 2) co-administration of the deaminase inhibitor, tetrahydrouridine (THU), with dTMP+dCMP.

Results—We observed that dC+dT delayed disease onset, prolonged lifespan of Tk2-deficient mice, and restored mtDNA copy number as well as respiratory chain enzyme activities and levels. In contrast, dCMP+dTMP+THU therapy decreased lifespan of $Tk2^{-/-}$ animals compared to dCMP +dTMP.

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Potential Conflict of Interest: Dr. Hirano and Columbia University Medical Center (CUMC) have filed patent applications covering the potential use of deoxynucleoside treatment for TK2 deficiency in humans. CUMC has licensed pending patent applications related to the technology to Meves Pharmaceuticals, Inc. and CUMC may be eligible to receive payments related to the development and commercialization of the technology. Any potential licensing fees earned will be paid to CUMC and are shared with Dr. Hirano through CUMC distribution policy. Dr. Hirano will serve as a paid consultant to Meves Pharmaceutical, Inc.

Interpretation—Our studies demonstrate that deoxynucleoside substrate enhancement is a novel therapy, which may ameliorate TK2 deficiency in patients.

Introduction

Encoded by the nuclear DNA gene *TK2*, thymidine kinase 2 (TK2) is a mitochondrial matrix enzyme, which phosphorylates the nucleosides deoxycytidine (dC) and deoxythymidine (dT) to generate deoxythymidine monophosphate (dTMP) and deoxycytidine monophosphate (dCMP). These pyrimidine nucleoside monophosphates are subsequently converted to deoxynucleotide triphosphates (dNTPs) required for mitochondrial DNA (mtDNA) replication and maintenance. *TK2* mutations were originally identified in four children with myopathic weakness and severe depletion of muscle mitochondrial DNA (mtDNA) that began in the first two years of life and progressed rapidly to complete paralysis with respiratory insufficiency requiring mechanical ventilation or causing death by age 4 years [1]. Subsequent clinical reports have expanded the clinical and molecular genetic spectrum of TK2 deficiency leading to the recognition of three disease subtypes: i) infantile-onset myopathy with rapid progression to early death [2]; ii) childhood-onset myopathy, which resembles spinal muscular atrophy (SMA) type III, begins between ages 1 and 12 years with progression to loss of ambulation within few years [3] (Garone C, Taylor RW, Nascimento A, et al. Phenotypic spectrum and retrospective natural history of thymidine kinase 2 deficiency, submitted 2016); and iii) late-onset myopathy starting at age 12 year or later with moderate to severe myopathy manifesting as either isolated chronic progressive external ophthalmoplegia (CPEO) or a generalized myopathy with CPEO plus facial and limb weakness, gradual progression, and, in some cases, respiratory failure and loss of ability to walk in adulthood [4, 5]. Molecular genetics defects in muscle include severe depletion of mtDNA in the infantile- and childhood-onset forms as well as multiple deletions with or without depletion of mtDNA in late-onset patients.

We previously reported that molecular bypass therapy using oral administration of the TK2 products, dCMP and dTMP, in our mouse model of TK2 deficiency (*Tk2* H126N knockin [$Tk2^{-/-}$] mice) [6] delays onset of molecular and biochemical abnormalities, ameliorates the symptoms, and prolongs the lifespan of the animals by 2-3 fold [7]. We also observed that after administration, dCMP and dTMP were rapidly catabolized to dC and dT suggesting that nucleosides rather than nucleotides are the major active therapeutic agents. Thus, we hypothesize that the increase of the nucleoside substrates enhances residual activity of TK1, TK2, or both leading to correction of dNTP pool imbalances. Furthermore, dCMP+dTMP treatment increased levels of deoxyuridine in liver indicating deamination of dC.

To further enhance this therapy, in this study, we have assessed two different approaches. First, because we have hypothesized that nucleosides are the active therapeutic compounds, we have tested the effects of oral dC+dT on the *Tk2* H126N knockin mouse model. Second, we have assessed the effects of co-administration of tetrahydrouridine (THU), an inhibitor of the cytidine deaminase, with dCMP+dTMP to attempt to increase levels of dC. We have observed that oral dC+dT prolongs the lifespan of mutant animals by restoring mtDNA copy number and respiratory chain enzyme (RCE) activities and levels. In contrast, addition of THU unexpectedly reduces the lifespan of animals comparing to oral dCMP+dTMP therapy

alone. These results reveal a novel nucleoside substrate enhancement therapy, which significantly ameliorates Tk2 deficiency in our mouse model.

Materials and Methods

Mice

Generation and characterization of *Tk2 H126N* knock-in mice were previously reported [6]. 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 13, and 29 days of age.

Organs (brain, liver, heart, kidney, intestine and quadriceps muscle) were removed and either frozen in the liquid phase of isopentane, pre-cooled near its freezing point (-160 °C) with dry ice. All the experiments were performed in at least 3 mice per group. Both heterozygous and homozygous wild type mice were considered as control group ($Tk2^{+}$) since no clinical and biochemical difference were previously described [6, 8].

Treatment administration and experimental plan

Deoxycytidine monophosphate (dCMP), deoxythymidine monophosphate (dTMP), tetrahydrouridine (THU), deoxycytidine (dC) and deoxythymidine (dT) (Hongene Biotechnology, Inc.) were administered in 50 μl of Esbilac milk formula for small pets (Pet-Ag) by daily oral gavage to Tk2 H126N knockin mice ($Tk2^{-/-}$) and aged matched controls ($Tk2^{+}$). dCMP and dTMP at a dose of 400mg/kg/day (3.2mM) were co-administered with a dose of 100mg/kg/day of THU, while dC and dT were administered using 2 doses, 260 mg/kg/day and 520 mg/kg/day (3.2 and 6.4mM). All treatments were administered from post-natal day 4 to 29 days. At age 21 days, mice were separated from the mother and the treatment was continued by oral administration. Mutant and control $Tk2^{+}$ mice were weighted and observed closely for comparison.

Phenotype assessment

Body weight was assessed daily, since it has been previously observed that incapacity of gaining weight is the first sign of disease [6]. To define the degree of safety and efficacy of each therapy, we compared survival time, age-at-onset of disease, type and severity of symptoms, occurrence of side effects, proportion of treatment termination due to adverse events in treated and untreated Tk2 mice. Behavior, survival time, and body weights of the mice were assessed daily beginning at postnatal day 4.

dNTP pool by polymerase extension assay

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 1000g for 5 min at 4°C, followed by three centrifugations at 13,000g for 2 min at 4 °C. Supernatant was precipitated with 60% methanol, kept 2 h at -80 °C, boiled 3 min, stored at -80 °C (from 1 h to overnight) and centrifuged at 20,800g 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 analysed. To minimize ribonucleotide interference, total dNTP pools were determined as reported [9, 10]. Briefly, 20 μl volume reactions was generated by mixing 5 μl of sample or standard dNTP with 15 μl of reaction buffer [0.025 U/ml ThermoSequenase DNA polymerase (GE Healthcare, Piscataway, NJ, USA) or Taq polymerase (Life Technologies, NY, USA), 0.75 μM ^{3}H-dTTP or ^{3}H-dATP (Moravek Biochemicals), 0.25 μM specific oligonucleotide, 40 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 5mM 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% Na_2HPO_4, once in distilled water and once in absolute ethanol. The retained radioactivity was determined by measuring scintillation counts.

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 [11, 12], 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 four 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 were monitored at 267 nm and dT and dU peaks were quantified by comparing their peak areas with a calibration curve obtained with aqueous standards. For definitive identification of dT, dU, uracil, and thymine 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. Results were expressed as nmol/mg of protein.

RT-qPCR: mitochondrial DNA quantification

Real-time PCR was performed with the primers and probes for murine COX I gene (mtDNA) and mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH, nuclear DNA [nDNA]) (Applied Biosystems, Invitrogen, Foster City, CA, USA) as described using ddCt method in a Step One Plus Real Time PCR System (Applied Biosystems) [8]. mtDNA values were normalized to nDNA values and expressed as percentage relative to wild-type (100%).

Mitochondrial respiratory chain protein levels

Thirty micrograms of whole brain cerebrum or cerebellum extracts were electrophoresed in an SDS-12% PAGE gel, transferred to Immun-Blot™ PVDF membranes (Biorad, Hercules, CA, USA) and probed with MitoProfile® Total OXPHOS Rodent WB Antibody Cocktail of antibodies (MitoSciences, Eugene, OR, USA) against CI subunit Ndufb8, CII-30kDa, CIII-Core protein 2, CIV subunit I, and CV alpha subunit. Protein–antibody 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 tissue as previously described [13, 14].

Statistical methods

Data are expressed as the mean ± SD of at least 3 experiments per group. For data grouped in columns, Mann-Whitney test was used to compare each group. A *p*-value of <0.05 was considered to be statistically significant. For survival curves, Mantel-Cox test was used to compare groups.

Results

Oral dC/dT delays disease onset and prolongs mice lifespan

Mice treated with oral dC+dT (260 or 520 mg/kg/day, which are molar equivalents of 400 and 800mg/kg/day of dCMP+dTMP, from age 4 days) appeared normal until postnatal day 21. After age 21 days, mutant mice treated with 260 mg/kg/day dose ($Tk2^{-/-260\ dC+dT}$) stopped gaining weight and developed mild head tremor and weakness that led to death at postnatal day 31 ± 4 (Fig 1A and B). Mutant mice treated with the 520 mg/kg/day dC+dT ($Tk2^{-/-520\ dC+dT}$) continued to gain weight for one additional week, but after age 28 days deteriorated, similar to $Tk2^{-/-260\ dC+dT}$ after age 21 days, and died at postnatal day 43 ± 10. The extended lifespans of Tk2 mutant mice treated with dC+dT at 260 and 520mg/kg/day are comparable to those of $Tk2^{-/-}$ mice treated with 200 or 400 mg/kg/day of oral dCMP +dTMP treatment [7]. Treated control mice, $Tk2^{+\ 260\ dC+dT}$ and $Tk2^{+\ 520\ dC+dT}$, followed until postnatal day 60 showed no significant changes in growth or other side-effects (Fig 1C and D).

In contrast, co-administration of oral dCMP+dTMP (400 mg/kg/day) with THU (100 mg/kg/day) significantly reduced the mean lifespan of mutant animals ($Tk2^{-/-400\ dCMP+dTMP+THU}$) to 30 ± 1 days, compared to 44 ± 7 days survival of $Tk2^{-/-400\ dCMP+dTMP}$ mice (p=0.007) (Fig 1A).

Oral dC+dT ameliorates molecular abnormalities in brain and liver

In both $Tk2^{-/-260\ dC+dT}$ and $Tk2^{-/-520\ dC+dT}$ mice, mitochondrial dNTP pool imbalances at postnatal day 13 were partially corrected in a tissue-specific manner with a trend towards elevation of diminished dTTP levels in $Tk2^{-/-}$ liver (23 ± 0.5% in $Tk2^{-/-260\ dC+dT}$ compared to 9 ± 5% in untreated $Tk2^{-/-}$ [p=0.13]), and significant lowering of elevated dCTP in $Tk2^{-/-}$ brain (23 ± 10% in $Tk2^{-/-260\ dC+dT}$ [p=0.02] and 30 ± 4% in $Tk2^{-/-520\ dC+dT}$ [p=0.004] compared to 49 ± 8% in untreated $Tk2^{-/-}$). In contrast, deficiencies of dTTP in brain and dCTP in liver remained severe despite nucleoside supplementation (Fig 2A). At postnatal day 29, dNTP pool deficiencies persisted, but showed trends towards less severity in

$Tk2^{-/-520\ dC+dT}$ compared to $Tk2^{-/-260\ dC+dT}$ (dTTP in brain: 2 ± 2% in $Tk2^{-/-260\ dC+dT}$ *vs* 8 ± 6% in $Tk2^{-/-520\ dC+dT}$ [p=0.17] and 47 ± 6% in untreated $Tk2^{+}$; dCTP in liver: 1 ± 0.8% in $Tk2^{-/-260\ dC+dT}$ *vs* 9 ± 10% in $Tk2^{-/-520\ dC+dT}$ [p>0.9] and 20 ± 16% in untreated $Tk2^{+}$). Similarly, $Tk2^{-/-400\ dCMP+dTMP+THU}$ at postnatal day 13 showed deficiencies of dTTP in brain (5 ± 1% compared to 38 ± 6% in $Tk2^{+untreated}$ [p=0.004]) and dCTP in liver (5 ± 3% compared to 25 ± 17% in $Tk2^{+untreated}$ [p=0.033]).

In $Tk2^{-/-400\ dCMP+dTMP+THU}$, $Tk2^{-/-260\ dC+dT}$, and $Tk2^{-/-520\ dC+dT}$ mice at postnatal day 13, treatments prevented mtDNA depletion in heart, liver, kidney, intestine, and muscle (Fig 2B). In contrast, mtDNA copy numbers in brain were only partially ameliorated by these treatments at postnatal day 13 with mtDNA/nDNA ratio reaching only 39 ± 0.3% in $Tk2^{-/-400\ dCMP+dTMP+THU}$ (p<0.057), 50 ± 7% in $Tk2^{-/-260\ dC+dT}$ (p=0.009) and 52 ± 9% in $Tk2^{-/-520\ dC+dT}$ p<0.0001, in contrast to 15 ± 7% in untreated $Tk2^{-/-}$ (p=0.030). Measurements of deoxythymidine and the base uracil in brain at postnatal day 13 by HPLC showed higher levels in animals treated with dC+dT and dCMP+dTMP (Fig 3A), further indicating that dT and probably dC cross the blood brain barrier. At postnatal day 29, mtDNA depletion was severe in brain of animals treated with dC+dT therapy (16 ± 4% in $Tk2^{-/-260\ dC+dT}$ [p=0.003] and 25 ± 6% in $Tk2^{-/-520\ dC+dT}$ [p=0.029]). In contrast, mtDNA depletion was partially rescued in both $Tk2^{-/-260\ dC+dT}$ and $Tk2^{-/-520\ dC+dT}$ in heart (40 ± 3% and 35 ± 9%), liver (46 ± 7% and 45 ± 12%), kidney (38 ± 8% and 42% 7 %) and muscle (24 ± 6% and 35 ± 6%), but was more strikingly rescued in intestine (82 ± 3% and 84 ± 10%) (Fig 2B). Furthermore, mtDNA copy number measured in near-terminal $Tk2^{-/-520\ dC+dT}$ animals showed that mtDNA levels were decreased in all tissues (ranging from 12 ± 5% in muscle to 31 ± 7% in kidneys) except intestine (86 ± 8%) (Fig 3B).

Oral dC+dT ameliorates biochemical abnormalities in brain

To assess the effects of partially rescued mtDNA levels in brain on mitochondrial function, we measured respiratory chain enzyme (RCE) activities by biochemical assays as well as RCE protein levels by western blot. At postnatal day 13, we observed that both $Tk2^{-/-260\ dC+dT}$ and $Tk2^{-/-400\ dCMP+dTMP+THU}$ significantly increased complex IV activity to levels similar to those showed by $Tk2^{+}$ mice (86 ± 23% [p=0.19] and 114 ± 5% [p=0.036] respectively compared to 58 ± 19% in $Tk2^{-/-untreated}$ normalized to protein concentration; 92 ± 16% [p=0.016] and 94 ± 26% [p=0.036] compared to 41 ± 15% in $Tk2^{-/-untreated}$ normalized by CS activity) (Fig 4). With both treatments, CS activities normalized to protein concentration decreased at postnatal day 13 (122 ± 26% in $Tk2^{-/-260\ dC+dT}$ [p=0.36] and 84 ± 29% in $Tk2^{-/-400\ dCMP+dTMP+THU}$ [p=0.032] compared to 140 ± 24% in $Tk2^{-/-untreated}$), suggesting reductions of compensatory increases in mitochondrial mass in mutant brains. Nevertheless, both treatments produced non-significant decreases in complex I activity normalized to protein concentration (73 ± 27% in $Tk2^{-/-400\ dCMP+dTMP+THU}$ and 79 ± 56% in $Tk2^{-/-260\ dC+dT}$, compared to 114 ± 61% in $Tk2^{-/-untreated}$), which may contribute to the subsequent deterioration of the mice. Additionally, $Tk2^{-/-400\ dCMP+dTMP+THU}$ showed significant decreased activities of complex II and complexes I+III when normalized to protein concentration (73 ± 25% [p=0.016] and 62 ± 27% [p=0.032] respectively, compared to 129 ± 27% and 111 ± 16% showed by $Tk2^{-/-untreated}$) (Fig 5). Similarly, at postnatal day 29, CS activity normalized to protein remained reduced in both $Tk2^{-/-260\ dC+dT}$ and

$Tk2^{-/-520\ dC+dT}$, compared to untreated mutants (95 ± 14% [p=0.016] and 97 ± 6% [p=0.008] respectively, *vs* 140 ± 24%) and complex IV activity was rescued (110 ± 16% [p=0.016] and 93 ± 6% [p=0.008] normalized to protein concentration; 117 ± 19% [p=0.016] and 93 ± 8% [p=0.008] normalized to CS activity) but defects of complex I activity were also observed (67 ± 27% in $Tk2^{-/-260\ dC+dT}$ [p=0.29] and 44 ± 15% in $Tk2^{-/-520\ dC+dT}$ [p=0.048], normalized to protein concentration; 69 ± 22% [p=0.68] and 42 ± 13% [p=0.030] normalized to CS activity) (Fig 4).

By western blot, RCE protein levels in brain of and $Tk2^{-/-400\ dCMP+dTMP+THU}$ at postnatal day 13 were also enhanced (in $Tk2^{-/-260\ dC+dT}$, ranging from 91 ± 4% of complex IV to 115 ± 13% of complex I and in $Tk2^{-/-400\ dCMP+dTMP+THU}$ ranging from 86 ± 4% of complex I to 103 ± 7% of complex V, normalized to complex II, relative to $Tk2^{+}$ controls) (Fig 6 and 7). However, at postnatal day 29, RCE proteins were less enhanced in $Tk2^{-/-260\ dC+dT}$ compared to postnatal day 13, with pronounced deficiency of complex I (43 ± 13% [p=0.001]) and milder decrease of complexes III (72 ± 19% [p=0.058]), IV (62 ± 22% [p=0.013]), and V (80 ± 11% [p=0.058]) (Fig 6). In contrast, with the higher dose $Tk2^{-/-520\ dC+dT}$ at postnatal day 29, deficiencies of RCE proteins were less severe (complex I, 58 ± 9% [p<0.0001]; complex III, 70 ± 23% [p=0.003]; complex IV, 78 ± 16% [p=0.018]; and complex V, 87 ± 26% [p=0.78]) (Fig 3). The most prominent RCE protein increases in brains of $Tk2^{-/-520\ dC+/dT}$ compared to $Tk2^{-/-260\ dC+dT}$ at postnatal day 29 were in complexes I [p=0.004] and IV [p=0.046]. The increases in RCE levels and activities in brain likely accounts for the prolonged survival observed with the higher dose of deoxynucleosides.

Discussion

Mitochondrial depletion syndrome (MDS) is defined by a reduction of mtDNA copy number causing secondary deficiencies of respiratory chain complex proteins and activities in target tissues. MDS was first described in 1991 [15] and encompasses a phenotypically heterogeneous group of disorders that includes myopathic, encephalomyopathic, and hepatocerebral forms. Interestingly, there are general correlations between the genotypes and phenotype. The myopathic form of the MDS is caused by mutations in *TK2*, hepatocerebral diseases are due to mutations in *DGUOK*, *POLG*, *C10orf2* or *MPV17*, muscle and nephropathy due to *RRM2B*, encephalomyopathy due to *SUCLA2* or *SUCLG1*, and severe infantile encephalomyopathies due to mutations in *OPA1* or *MFN2* [16, 17]. The majority of these genes are required for mtDNA replication or in deoxynucleotides metabolism [17, 18], which demonstrates the critical role of dNTP pool balance in mtDNA maintenance. While manifesting predominantly as skeletal myopathy, *TK2* mutations have presented with other phenotypes including encephalomyopathy and chronic progressive external ophthalmoplegia [1-5]. The reason for the phenotypic diversity is not understood and appears not to be due to severity of TK2 deficiency[19].

To characterize the pathogenesis and develop therapies for TK2 deficiency, we previously generated a mouse model of TK2 deficiency (*Tk2* H126N knockin mice)[6], which recapitulates the encephalomyopathic form of TK2 deficiency. We reported success of a molecular bypass therapy, oral dCMP and dTMP, in our Tk2 mouse model. Doses of 200 and 400mg/kg/day of each dNMP delayed the disease onset and prolonged the lifespan of

animals [7]. Despite the clear benefits of this therapy, we observed that most of the dCMP and dTMP were rapidly converted to their respective nucleosides, dC and dT, suggesting that these nucleosides were the active therapeutic agents. In support of our hypothesis, a study of mitochondria isolated from hearts of rats treated with radiolabeled dT and dTMP, demonstrated that most of the mitochondrial TTP was generated from dT rather than dTMP, and that exogenously administered dTMP was compartmentalized from dTMP synthesized from dT [20].

In the present study, we initially tested a dose of 260 mg/kg/day of dC+dT, which is the equimolar concentration of 400mg of dCMP+dTMP, and subsequently 520 mg/kg/day of dC/dT. Interestingly, we observed that $Tk2^{-/-260\ dC/dT}$ mice had a lifespan equivalent to that of $Tk2^{-/-200\ dCMP/dTMP}$, while $Tk2^{-/-520\ dC/dT}$ had a lifespan equivalent to that showed by $Tk2^{-/-400\ dCMP/dTMP}$. Thus, double the molar amounts of nucleoside relative to deoxynucleoside monophosphates were required in order to achieve the same prolongation of lifespan. The reason for the different dose responses to deoxynucleoside monophosphates relative to deoxynucleosides is unclear. One potential explanation is that, while dC+dT functions as the active therapeutic agents as substrates enhancing Tk1, Dck and Tk2 activities, monophosphates may partially bypass the enzyme activity and partially function as pro-drugs with conversion to nucleosides as indicted by Kamat *et al* (2015). Furthermore, 5′-nucleotidase activity might delay the release of dC/dT in tissues, improving deoxynucleoside monophosphate efficacy relative to deoxynucleosides. Another possibility, based on HPLC data, is that further degradation of dC and dT to their bases limits their availability in tissues.

We subsequently tested the effect of dCMP+dTMP therapy co-administered with THU, to decrease the catalytic conversion of dC to dU, thus providing Tk2 with higher levels of dC. An *in vitro* study showed that THU partially prevented catabolism of dC and mtDNA depletion in a cell culture model of MNGIE while *in vivo* a single dose of 100 mg/kg increased levels of dC in blood in a double knockout MNGIE mouse model [21]. Unexpectedly, in our mouse model of TK2 deficiency, median lifespan of animals on dCMP +dTMP+THU therapy was significantly shorter than that of $Tk2^{-/-}$ mice receiving dCMP +dTMP therapy alone. This result is likely due to the lowering of dTTP levels and more severe mtDNA depletion in brain mitochondria when THU is co-administered with dCMP +dTMP in contrast to dCMP+dTMP treatment. By comparison, addition of THU did not significantly alter levels of dTTP, dCTP, or mtDNA copy number in liver. From these observations, we may conclude that THU exacerbates the dNTP pool imbalance in brain, which, in turn, shortens lifespan. We hypothesize that in brain conversion of dC to dU by CDA increases levels of intra-mitochondrial dTTP through TK1 and TS enzyme activities (Fig 8) while inhibition of CDA by THU decreases intramitochondrial dTTP levels and accelerates demise of the mice.

Our results with dC+dT therapy demonstrated tissue specific-effects of this therapy; intra-mitochondrial levels of dCTP in brain and dTTP in liver showed early corrections at postnatal day 13 (reduced dCTP in brain and increased dTTP in liver) that were sustained until postnatal day 29; however, levels of dCTP in liver and dTTP in brain remained low at both time-points. Hypothetically, TP activity in brain and deaminase activity in liver could

account for these tissue-specific effects; however, TP activity is high in liver and low in brain [16]. Furthermore, co-administration of dCMP+dTMP and THU, which inhibits cytidine deaminase, did not ameliorate dCTP defects in liver at postnatal day 13, despite published reports of THU efficacy *in vivo* [22, 23]. Taken together, these observations indicate the existence of tissue-specific alternative pathways of nucleosides catabolism and the necessity of further studies to better define nucleotide metabolism.

Measurement of mtDNA levels revealed additional tissue-specific effect of the substrate enhancement therapy. Both nucleoside monophosphate and nucleoside treatments failed to fully rescue the mtDNA copy number in brain at postnatal day 13, although both therapies showed higher levels of dT and uracil (a catabolic product of dC) in brain at this time, measured by HPLC. We had previously speculated that formation of the blood-brain barrier might compromise bioavailability of nucleoside monophosphates in brain [7]; however, equilibrative nucleoside transporters 1 and 2 (ENT1 and ENT2), expressed in blood-brain barrier endothelial cells and blood-cerebrospinal fluid barrier epithelial cells can transport both dT and dC from blood to the brain [24, 25]. Thus, increased enzymatic catabolism during brain maturation, rather than blood-brain barrier formation, may impair efficacy of deoxynucleoside and deoxynucleotide therapies. Similarly, in muscle and liver, deoxynucleosides prevented mtDNA depletion at postnatal day 13, but not at day 29. In contrast, 520 mg/kg/day of deoxynucleosides maintained normal mtDNA levels in intestine to the terminal stage indicating oral treatment provides higher intestinal bioavailability of dC +dT relative to other tissues. Intraperitoneal administration of the drugs may increase their bioavailability and beneficial effects; however, further studies are required in order to assess this possibility.

Because pyrimidine metabolism involves multiple pathways, other mechanisms may be contributing to tissue-specific efficacy, including nucleoside transport and activities of other catabolic enzymes. In this context, it is noteworthy that TK2 is a complex enzyme and exhibits different kinetics for each substrate (Michaelis-Menten for dC phosphorylation and negative cooperativity for dT phosphorylation) [26]. These factors may also influence tissue-specific and long term efficacy of dC+dT therapy.

This is the first demonstration of *in vivo* effectiveness of nucleosides treatment for mtDNA depletion syndrome. Manipulation of mitochondrial dNTP pool by addition of nucleosides may be an effective therapy not only in cases of TK2 deficiency, but also in other mtDNA depletion syndrome due to mitochondrial dNTP pool imbalance.

In conclusion, our results reveal a novel nucleoside therapy and refute the hypothesized therapeutic effect of THU for TK2 deficiency. Oral administration of dC+dT demonstrated benefits previously observed with dCMP+dTMP treatment and appears to be well-tolerated and easily translated to patients indicating that this substrate enhancement therapy may retard progression of this devastating disease in patients.

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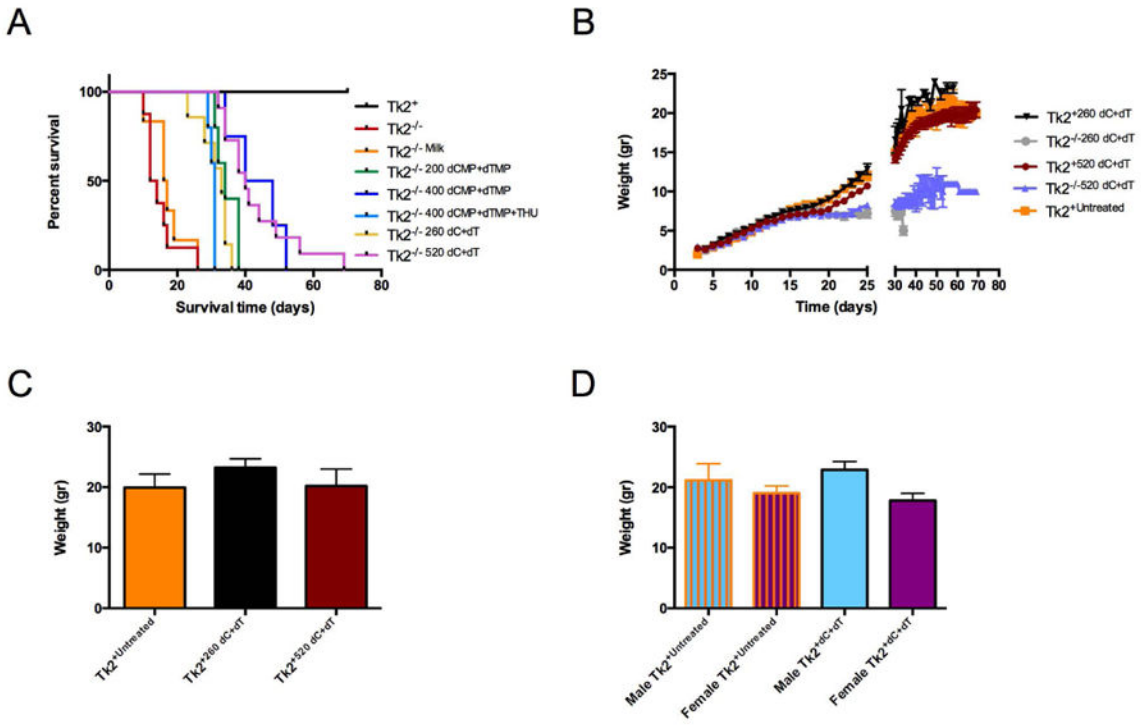

Figure 1. Survival and growth curves of the Tk2 H126N knockin mice receiving different treatments

A. Survival of Tk2 homozygous knock-in mice with the following treatments: $Tk2^{-/-milk}$ (N=6) *vs* $Tk2^{-/-200\ dCMP+dTMP+THU}$ (N=5), p=0.0013; $Tk2^{-/-milk}$ *vs* $Tk2^{-/-260\ dC+dT}$ (N=7), p=0.0006; $Tk2^{-/-milk}$ *vs* $Tk2^{-/-520\ dC+dT}$ (N=11), p<0.0001; $Tk2^{-/-260\ dC+dT}$ *vs* $Tk2^{-/-520\ dC+dT}$, p=0.0009. $Tk2^{-/-200\ dCMP+dTMP}$ (N=5), $Tk2^{-/-400\ dCMP+dTMP}$ (N=4), $Tk2^{-/-400\ dCMP+dTMP+THU}$ (N=5). p-values determined by Mantel-Cox tests.

B. Effects of dC+dT therapy on mouse growth. Each symbol represents mean weight at each time-point ± SEM. Tk2+untreated N=10; $Tk2^{+\ 260\ dC+dT}$ N=18; $Tk2^{-/-260\ dC+dT}$ N=6; $Tk2^{+\ 520\ dC+dT}$ N=21; $Tk2^{-/-\ 520\ dC+dT}$ N=11. C-D. Weight of wild type mice at postnatal day 60 (mean ± SD).

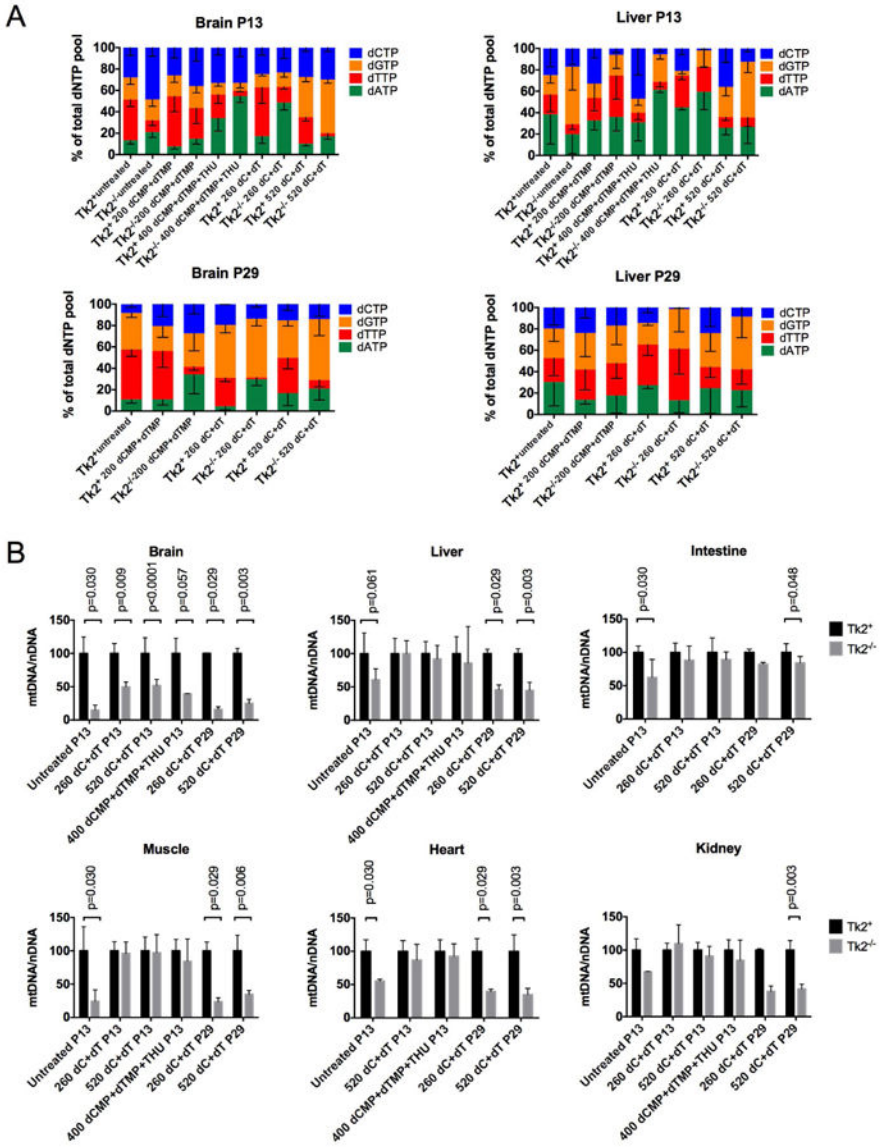

Figure 2. Effect of therapies on dNTP pools and mtDNA levels

A. Proportions of dNTPs in isolated mitochondria from brain (left) and liver (right) at postnatal days 13 (upper) and 29 (lower). Untreated $Tk2^{+}$ P13 N=8; Untreated $Tk2^{-/-}$ P13 N=5; $TK2^{+}$ 200 dCMP+dTMP P13 N= 6; $Tk2^{-/-}$ 200 dCMP+dTMP P13 N=5; $TK2^{+}$ 400 dCMP+dTMP+THU P13 N= 5; $Tk2^{-/-}$ 400 dCMP+dTMP+THU P13 N=4; $Tk2^{+}$260 dC+dT P13 N=5; $Tk2^{-/-}$260 dC+dT P13 N=4; $Tk2^{+}$520 dC+dT P13 N=7; $Tk2^{-/-}$520 dC+dT N=6; Untreated $Tk2^{+}$ P29 N=6; $TK2^{+}$ 200 dCMP+dTMP P29 N= 7; $Tk2^{-/-}$ 200 dCMP+dTMP P29 N=9; $Tk2^{+}$260 dC+dT P29 N=2; $Tk2^{-/-}$260 dC+dT P29 N=4; $Tk2^{+}$520 dC+dT P29 N=7; $Tk2^{-/-}$520 dC+dT P29 N=5.

B. mtDNA levels in brain, liver, intestine, muscle, kidney and heart in Tk2-/- mice. Data are represented as mean ± standard deviation (SD) of the percent of mtDNA copies relative to $Tk2^{+}$. Untreated $Tk2^{+}$ P13 N=10; Untreated $Tk2^{-/-}$ P13 N=2; $Tk2^{+}$260 dC+dT P13 N=9; $Tk2^{-/-}$260 dC+dT P13 N=3; $Tk2^{+}$520 dC+dT P13 N=26; $Tk2^{-/-}$520 dC+dT N=14;

$TK2^{+}$ 400 dCMP+dTMP+THU P13 N= 3; $Tk2^{-/-}$ 400 dCMP+dTMP+THU P13 N=4; $Tk2^{+}$260 dC+dT P29 N=2; $Tk2^{-/-}$260 dC+dT P29 N=4; $Tk2^{+}$520 dC+dT P29 N=7; $Tk2^{-/-}$520 dC+dT P29 N=5. P-values were assessed by Mann-Whitney U tests.

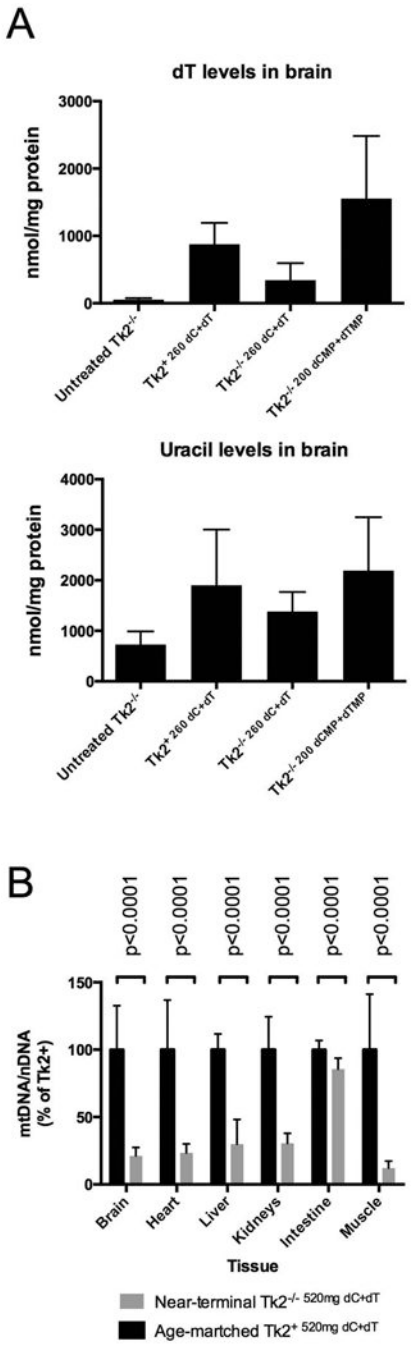

Figure 3. dT and uracil levels in brain at age 13 days and mtDNA levels in near-terminal mice under dC+dT therapy

A. dT and uracil levels in brain were measured by HPLC before and after 30 minutes of each treatment. Data are expressed as mean ± SD. Untreated $Tk2^{-/-}$ N=3; $Tk2^{+}$ 260 dC+dT N=6; $Tk2^{-/-}$ 260 dC+dT N=3; $Tk2^{-/-}$ 200 dCMP+dTMP N=9.

B. mtDNA levels are represented as mean ± SD of the percent mtDNA copies relative to aged-matched $Tk2^{+520\ dC+dT}$ mice. $Tk2^{+520\ dC+dT}$ N=12; $Tk2^{-/-520\ dC+dT}$ N=6.

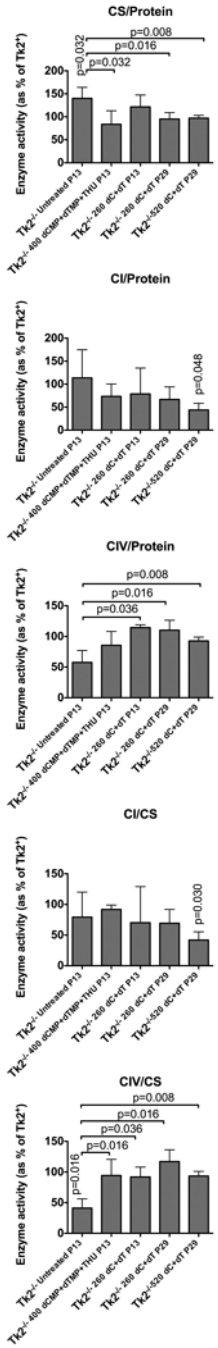

Figure 4. Activities of Respiratory Chain Enzyme (RCE) complexes I and IV normalized to citrate synthase activity and CS, RCE complexes I and IV normalized to protein concentration

Data are represented as percent of RCE activities **normalized to citrate synthase activity or protein concentration** in $Tk2^{-/-}$ mouse tissues relative to $Tk2^{+}$ for each treatment. $TK2^{+400\ dCMP+dTMP+THU}$ P13 N= 5; $Tk2^{-/-400\ dCMP+dTMP+THU}$ P13 N=4; $Tk2^{+260\ dC+dT}$ P13 N=8; $Tk2^{-/-260\ dC+dT}$ P13 N=3; $Tk2^{+260\ dC+dT}$ P29 N=2; $Tk2^{-/-260\ dC+dT}$ P29 N=4; $Tk2^{+520\ dC+dT}$ P29 N=7; $Tk2^{-/-520\ dC+dT}$ P29 N=5.

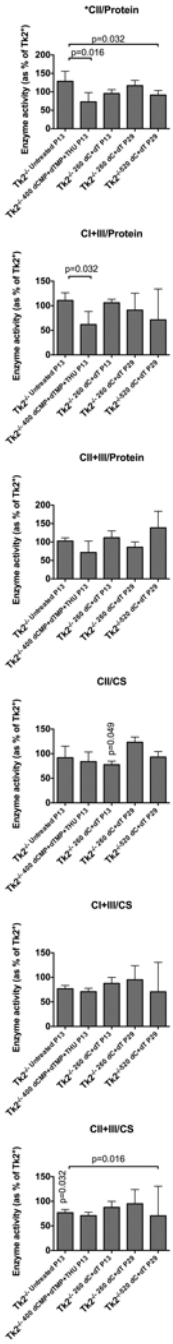

Figure 5. Activities of Respiratory Chain Enzyme (RCE) complexes II, I+III and II+III, normalized to CS activity and to protein concentration

Data are represented as percent of RCE activities **normalized to citrate synthase activity or protein concentration** in $Tk2^{-/-}$ mouse tissues relative to $Tk2^{+}$ for each treatment. $TK2^{+400\ dCMP+dTMP+THU}$ P13 N= 5; $Tk2^{-/-400\ dCMP+dTMP+THU}$ P13 N=4; $Tk2^{+260\ dC+dT}$ P13 N=8; $Tk2^{-/-260\ dC+dT}$ P13 N=3; $Tk2^{+260\ dC+dT}$ P29 N=2; $Tk2^{-/-260\ dC+dT}$ P29 N=4; $Tk2^{+520\ dC+dT}$ P29 N=7; $Tk2^{-/-520\ dC+dT}$ P29 N=5.

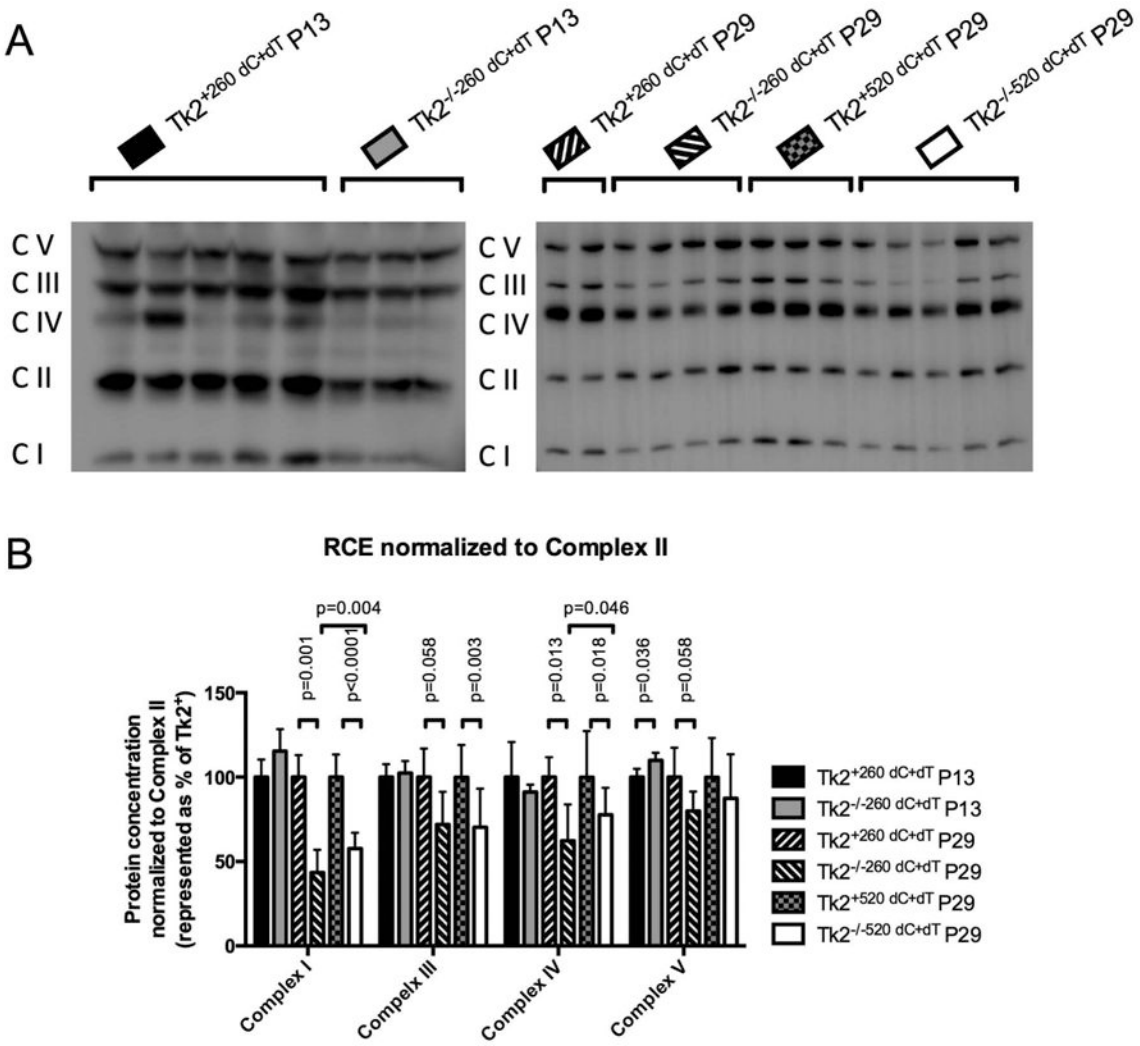

Figure 6. Effect of dC+dT therapy on respiratory chain enzyme (RCE) protein levels in brain, normalized to complex II

A. Two representative western blots showing bands for the five RCE complexes.

B. RCE levels normalized to complex II, represented as percent of the RCE levels in $Tk2^{+}$ mice.

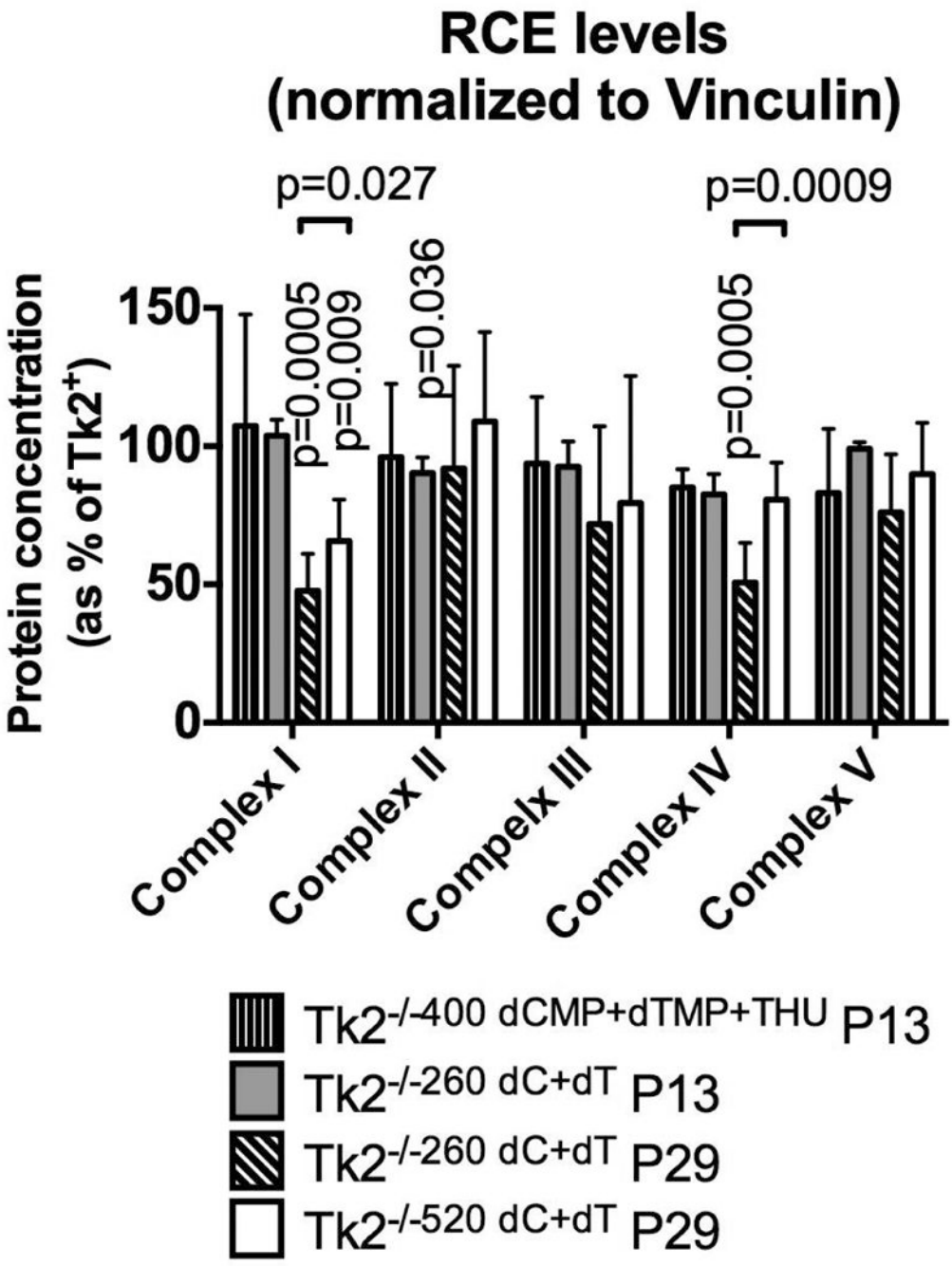

Figure 7. Effect of dC+dT and dCMP+dTMP+THU therapies on respiratory chain enzyme (RCE) protein levels in brain, normalized to Vinculin

RCE proteins (normalized to vinculin) are represented as percents of the RCE levels in $Tk2^{+}$ for each treatment. $TK2^{+}$ $^{400\ dCMP+dTMP+THU}$ P13 N= 5; $Tk2^{-/-\ 400\ dCMP+dTMP+THU}$ P13 N=4; $Tk2^{+260\ dC+dT}$ P13 N=5; $Tk2^{-/-260\ dC+dT}$ P13 N=3; $Tk2^{+260\ dC+dT}$ P29 N=5; $Tk2^{-/-260\ dC+dT}$ P29 N=8; $Tk2^{+520\ dC+dT}$ P29 N=5; $Tk2^{-/-520\ dC+dT}$ P29 N=10.

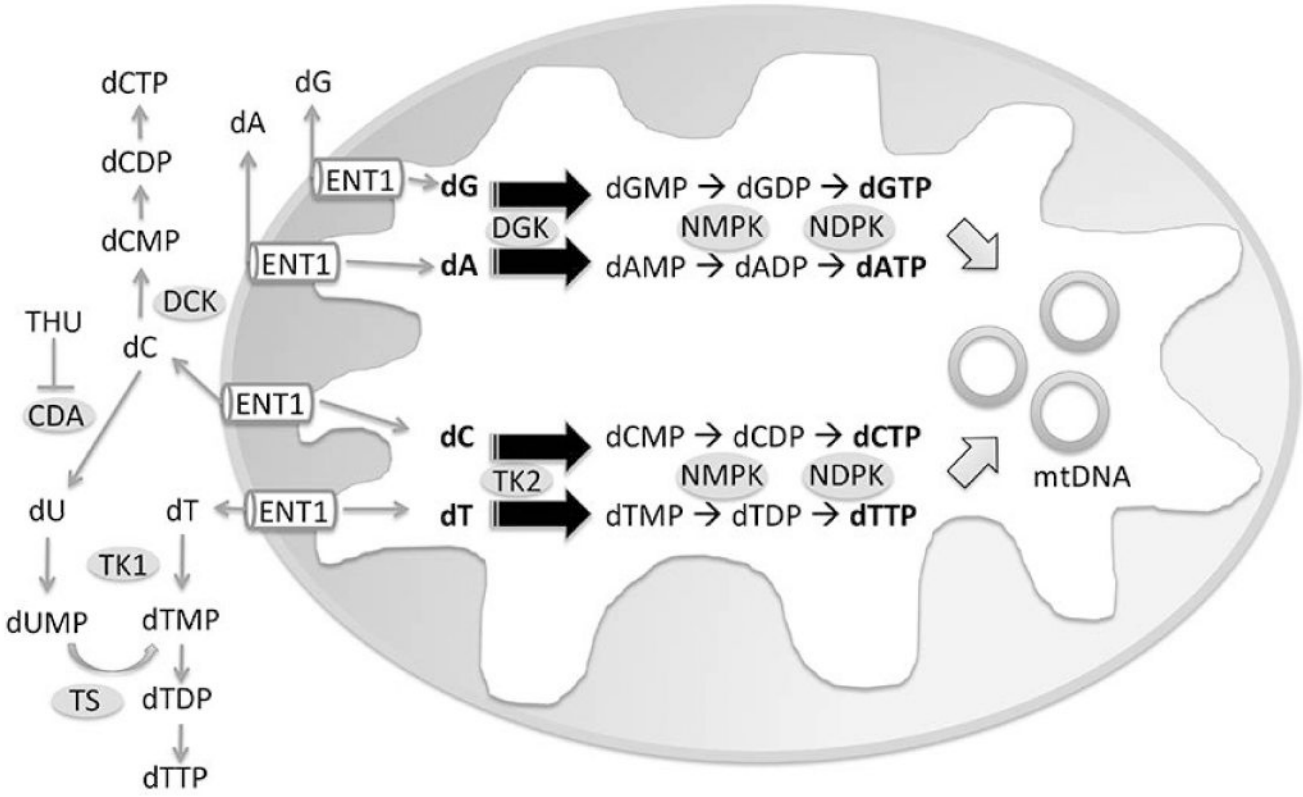

Figure 8. Mitochondrial and cytosolic deoxypyrimidine metabolic pathways

Enzymes are marked in ovals. CDA, cytidine deaminase; dA, deoxyadenosine; dC, deoxycytidine; DCK, deoxycytidine kinase; dG, deoxyguanosine; DGK, deoxyguanosine kinase; dT, thymidine; dU, deoxyuridine; ENT1, equilibrative nucleoside transporter 1; nDPK, nucleoside diphosphate kinase; nMPK, nucleoside monophosphate kinase; THU, tetrahydrouridine; TK1, thymidine kinase 1; TK2, thymidine kinase 2; TS, thymidylate synthase.