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(54) Title: DEOXYNUCLEOSIDE THERAPY FOR DISEASES CAUSED BY UNBALANCED NUCLEOTIDE POOLS INCLUDING MITOCHONDRIAL DNA DEPLETION SYNDROMES

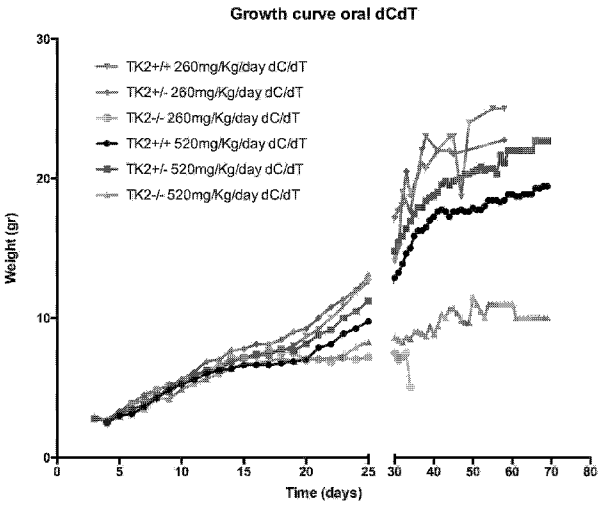

Figure 1

(57) Abstract: The invention relates generally to a pharmacological therapy for human genetic diseases, specifically those characterized by unbalance nucleotide pools, more specifically mitochondrial DNA depletion syndromes, and more specifically, thymidine kinase 2 (TK2) deficiency. The pharmacological therapy involves the administration of at least one deoxynucleoside, or mixtures thereof. For the treatment of TK2 deficiency, the pharmacological therapy involves the administration of either deoxythymidine (dT) or deoxycytidine (dC), or mixtures thereof. This administration of deoxynucleosides is applicable to other disorders of unbalanced nucleotide pools, especially those found in mitochondrial DNA depletion syndrome.

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NUCLEOTIDE POOLS INCLUDING MITOCHONDRIAL DNA DEPLETION SYNDROMES

GOVERNMENT SUPPORT

This invention was made with government support under HD080642 awarded by NIH. The government has certain rights in the invention.

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application claims priority to U.S. provisional patent application serial no. 62/180,194 filed June 17, 2015, which is hereby incorporated by reference.

FIELD OF THE INVENTION

The invention relates generally to a pharmacological therapy for a human genetic disease, specifically diseases characterized by unbalanced nucleotide pools, *e.g.*, mitochondrial DNA depletion syndromes, and more specifically, thymidine kinase 2 (TK2) deficiency. The pharmacological therapy involves the administration of at least one deoxynucleoside, or mixtures thereof. For the treatment of TK2 deficiency, the pharmacological therapy involves the administration of either deoxythymidine (dT) or deoxycytidine (dC), or mixtures thereof. This administration of one or more deoxynucleosides is applicable to other disorders of unbalanced nucleoside pools, especially those found in mitochondrial DNA depletion syndrome.

BACKGROUND OF THE INVENTION

Mitochondrial diseases are clinically heterogeneous diseases due to defects of the mitochondrial respiratory chain (RC) and oxidative phosphorylation, the biochemical pathways that convert energy in electrons into adenosine triphosphate (ATP). The respiratory chain is comprised of four multi-subunit enzymes (complexes I-IV) that transfer electrons to generate a proton gradient across the inner membrane of mitochondria and the flow of protons through complex V drives ATP synthesis (DiMauro and Schon 2003; DiMauro and Hirano 2005). Coenzyme Q_{10} (CoQ_{10}) is an essential molecule that shuttles electrons from complexes I and II to complex III. The respiratory chain is unique in eukaryotic, *e.g.*, mammalian, cells by virtue of being controlled by two genomes, mitochondrial DNA (mtDNA) and nuclear DNA (nDNA). As a consequence, mutations in either genome can

cause mitochondrial diseases. Most mitochondrial diseases affect multiple body organs and are typically fatal in childhood or early adult life. There are no proven effective treatments for mitochondrial diseases, only supportive therapies, such as the administration of CoQ_{10} and its analogs to enhance respiratory chain activity and to detoxify reactive oxygen species (ROS) that are toxic by-products of dysfunctional respiratory chain enzymes.

Mitochondrial DNA depletion syndrome (MDS), which is a subgroup of mitochondrial disease, is a frequent cause of severe childhood encephalomyopathy characterized molecularly by reduction of mitochondrial DNA (mtDNA) copy number in tissues and insufficient synthesis of mitochondrial RC complexes (Hirano, *et al.* 2001). Mutations in several nuclear genes have been identified as causes of infantile MDS, including: *TK2, DGUOK, POLG, POLG2, SCLA25A4, MPV17, RRM2B, SUCLA2, SUCLG1, TYMP, OPA1,* and *C10orf2 (PEO1).* (Bourdon, *et al.* 2007; Copeland 2008; Elpeleg, *et al.* 2005; Mandel, *et al.* 2001; Naviaux and Nguyen 2004; Ostergaard, *et al.* 2007; Saada, *et al.* 2003; Sarzi, *et al.* 2007; Spinazzola, *et al,* 2006). In addition, mutations in these nuclear genes can also cause multiple deletions of mtDNA with or without mtDNA depletion (Béhin, *et al.* 2012; Garone, *et al.* 2012; Longley, *et al.* 2006; Nishino, *et al.* 1999; Paradas, *et al.* 2012; Ronchi, *et al.* 2012; Spelbrink, *et al.* 2001; Tyynismaa, *et al.* 2009; Tyynismaa, *et al.* 2012; Van Goethem, *et al.* 2001).

One of these genes is *TK2,* which encodes thymidine kinase (TK2), a mitochondrial enzyme required for the phosphorylation of the pyrimidine nucleosides (thymidine and deoxycytidine) to generate deoxythymidine monophosphate (dTMP) and deoxycytidine monophosphate (dCMP) (Saada, *et al.* 2001). Mutations in *TK2* impair the mitochondrial nucleoside/nucleotide salvage pathways required for synthesis of deoxynucleotide triphosphate (dNTP), the building blocks for mDNA replication and repair.

TK2 deficiency was first described in 2001 by Saada and colleagues (Saada, *et a*l. 2001), in four affected children originating from four different families, who suffered from severe, devastating myopathy. After an uneventful early development, at ages 6-36 months the patients developed hyperCKemia, severe muscle hypotonia with subsequent loss of spontaneous activity. The disease was rapidly progressive and two patients were mechanically ventilated at 3 years, while two other patients were already dead by the time of the report.

After the first description, sixty additional patients have been reported in literature and at least twenty-six further patients have been diagnosed but not reported (Alston, *et al.* 2013; Bartesaghi, *et al.* 2010; Béhin, *et al.* 2012; Blakely, *et al.* 2008; Carrozzo, *et al.* 2003;

Chanprasert, *et al.* 2013; Collins, *et al.* 2009; Galbiati, *et al.* 2006; Gotz, *et al.* 2008; Leshinsky-Silver, *et al.* 2008; Lesko, *et al.* 2010; Mancuso, *et al.* 2002; Mancuso, *et al.* 2003; Marti, *et al.* 2010; Oskoui, *et al.* 2006; Paradas, *et al.* 2012; Roos, *et al.* 2014; Tulinius, *et al.* 2005; Tyynismaa, *et al.* 2012; Vilà, *et al.* 2003; Wang, *et al.* 2005), resulting in ninety patients, 53 males and 37 females.

The twenty-six patients recently diagnosed were identified through next-generation DNA sequencing. This large number of newly identified cases suggests that TK2 deficiency is an under diagnosed disorder.

TK2 deficiency manifests a wide clinical and molecular genetic spectrum with the majority of patients manifesting in early childhood with a devastating clinical course, while others have slowly progressive weakness over decades.

Treatment for TK2 deficiency, like most MDS and mitochondrial disorders, has been limited to supportive therapies. While the administration of deoxythymidine monophosphate (dTMP) and deoxycytidine monophosphate (dCMP) improved the conditions of both TK2 knock-in mutant mice and human patients with TK2 deficiency (US Application Serial No.15/082,207, which is incorporated herein in its entirety), there is still a need for therapeutic intervention for TK2 deficiency.

Additionally, there is a need for treatment for other forms of MDS and other diseases characterized by unbalanced nucleotide pools. For example, several mendelian disorders with mtDNA depletion or multiple deletions, or both are characterized by unbalanced deoxynucleotide triphosphate pools that lead to defects of mtDNA replication. One such disorder, *DGUOK* mutations impair the intramitochondrial enzyme deoxyguanosine kinase, which normally phosphorylates the deoxypurine nucleosides deoxguanosine and deoxycytidine to generate deoxguanosine monophosphate (dGMP) and deoxycytidine monophosphate (dCMP). Other nuclear genes that disrupt mitochondrial dNTP pools include *TYMP, RRM2B, SUCLA2, SUCLG1* and *MPV17*. Therapies that restore dNTP pool balance would be useful to treat these disorders as well.

SUMMARY OF THE INVENTION

In certain embodiments, the present invention relates to a method of treating a disease or disorder characterized by unbalanced nucleotide pools, comprising administering to a subject in need thereof a therapeutically effective amount of a composition comprising one or more deoxynucleosides.

Diseases or disorders characterized by unbalanced nucleotide pools that can be treated by the method of the current invention include, but are not limited to, those characterized by mutations in the following genes: *TK2; DGUOK; TYMP; RRM2B; SUCLA2; SUCLG1;* and *MPV17.*

In a preferred embodiment, the disorder is a mitochondrial DNA depletion syndrome (MDS). In a more preferred embodiment, the MDS includes disorders of a myopathic form characterized by mutations in *TK2*, an encephalomyopathic form characterized by mutations in *SUCLA2*, a neurogastrointestinal encephalopathic form characterized by mutations in *TYMP*, and a hepatopathic form characterized by mutations in *DGUOK*, *POLG*, and *MPV17.* In a most preferred embodiment, the disorder is a thymidine kinase 2 deficiency, characterized by mutation(s) in the *TK2* gene.

All mitochondrial DNA depletion syndromes can be treated with the method of the current invention which comprises administering deoxynucleosides. Examples of MDS that can be treated by the method of the current invention include but are not limited to, deficiencies in the: *DGUOK* gene, encoding deoxyguanosine kinase, dGK; *RRM2B* gene, encoding p53R2, the p53 inducible small subunit of ribonucleotide reductase, RNR; and *TYMP* gene, encoding thymidine phosphorylase, TP.

In a preferred embodiment, the deoxynucleoside is either deoxythymidine (dT) or deoxycytidine (dC) or mixtures thereof. Deoxyadenosine (dA) and deoxyguanosine (dG), alone or together, can also be used in the method of the invention. One deoxynucleoside (*i.e.*, dT, dC, dA, or dG) and mixtures of two or more of any of the four deoxynucleosides can be used in the method of the invention.

Preferred dosages of the deoxynucleoside(s) are between about 100 and about 1,000 mg/kg/day, more preferably between about 300 and about 800 mg/kg/day, and most preferably between about 250 and about 600 mg/kg/day. If the composition comprises a single deoxynucleoside, then the dosages are of the single deoxynucleoside. If the composition comprises more than one deoxynucleoside, the dosages can be of each deoxynucleoside or of the total deoxynucleosides in the composition.

Administration of the deoxynucleoside(s) can be once daily, twice daily, three times daily, four times daily, five times daily, up to six times daily, preferably at regular intervals.

Preferred methods of administration are oral, intrathecal, intravenous, and enteral.

Administration of the deoxynucleoside(s) should begin as soon as the disorder characterized by unbalanced nucleotide pools, *e.g.,* MDS, is suspected and continue

throughout the life of the patient. Test for the diagnosis of such disorders including TK2 deficiency are known in the art.

BRIEF DESCRIPTION OF THE DRAWINGS

For the purpose of illustrating the invention, there are depicted in drawings certain embodiments of the invention. However, the invention is not limited to the precise arrangements and instrumentalities of the embodiments depicted in the drawings.

Figure 1 depicts a growth curve of wild type ($Tk2^{+/+}$ and $Tk2^{+/-}$), and $Tk2^{-/-}$ mice treated with 260 mg/kg/day or 520 mg/kg/day of deoxycytidine (dC) and deoxythymidine (dT) from postnatal day 4. Each symbol represents the mean of weight at each time-point. N of each group is indicated in figure.

Figure 2 depicts the survival curve of wild type ($Tk2^{+/+}$), and $Tk2^{-/-}$ mice with the following treatments: $Tk2^{-/-milk}$ *vs* $Tk2^{-/-200\ mg/kg/day\ dCMP+dTMP}$, p=0.0013; $Tk2^{-/-milk}$ vs $Tk2^{-/-\ 260\ mg/kg/day\ dC+dT}$, p=0.0006; $Tk2^{-/-milk}$ vs $Tk2^{-/-\ 520\ mg/kg/day\ dC+dT}$, p<0.0001; $Tk2^{-/-\ 260\ mg/kg/day\ dC=dT}$ vs $Tk2^{-/-520mg/kg/day\ dCdT}$, p=0.0009, at postnatal day 4. N of each group indicated in figure. p-values determined by Mantel-Cox tests.

Figure 3 are graphs of the relative proportions of dNTPs in isolated mitochondria from brain and liver tissue of wild type ($Tk2^{+/+}$), and $Tk2^{-/-}$, untreated or treated with 200 mg/kg/day dCMP and dAMP, or 260 mg/kg/day or 520 mg/kg/day of deoxycytidine (dC) and deoxythymidine (dT) at ages postnatal day 13 (top panels) and postnatal day 29 (bottom panels).

Figure 4 are graphs showing the ratio of mtDNA/nDNA in brain, liver, intestine, and muscle in wild type Tk2 mice ($Tk2^{+/+}$) (left hand bar) as compared to $Tk2^{-/-}$ mice, untreated or treated with 260 mg/kg/day or 520 mg/kg/day of deoxycytidine (dC) and deoxythymidine (dT), at ages postnatal days 13 and 29. Data are represented as mean ± standard deviation (SD) of the percent of mtDNA copies relative to $Tk2^{+}$. p-values were assessed by Mann-Whitney tests. (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

Figure 5 are graphs depicting the results of HPLC measuring dT and uracil in plasma of untreated wild type ($Tk2^{+/+}$) mice, wild type ($Tk2^{+/+}$) mice treated with 260 mg/kg/day of deoxycytidine (dC) and deoxythymidine (dT), $Tk2^{-/-}$ mice treated with 260 mg/kg/day of deoxycytidine (dC) and deoxythymidine (dT), and $Tk2^{-/-}$ mice treated with 200 mg/kg/day of dCMP and dTMP, 30 minutes after treatment. Data are expressed as mean ± SD.

Figure 6 are graphs of levels of respiratory chain enzyme activities in $Tk2^{-/-}$ mice treated with 400 mg/kg/day of dCMP and dTMP and THU at 13 days postnatal, 260

mg/kg/day of deoxycytidine (dC) and deoxythymidine (dT) at 13 and 29 days postnatal, or 520 mg/kg/day of deoxycytidine (dC) and deoxythymidine (dT) 29 days postnatal. Data are represented as the percent of the RCE activities in $Tk2^{-/-}$ mouse tissues normalized to protein levels and relative to $Tk2^{+}$ for each treatment. p-values determined by Mann-Whitney tests. *$p<0.05$.

Figure 7A is an immunoblot of respiratory chain proteins in wild type mice treated with 260 mg/kg/day or 520 mg/kg/day of deoxycytidine (dC) and deoxythymidine (dT), and $Tk2^{-/-}$ mice treated with 260 mg/kg/day or 520 mg/kg/day of deoxycytidine (dC) and deoxythymidine (dT) at 29 days postnatal. Figure 7B are graphs showing the RCE levels normalized to complex II, represented as percent of the RCE levels in $TK2^{+/+}$ mice. p-values were assessed by Mann-Whitney tests.

Abbreviations:CS= citrate synthase; CI= NADH-dehydrogenase; CII= succinate dehydrogenase; CIII= cytochrome *c* reductase; CIV= cytochrome *c* oxidase (COX); CI+III= NADH-cytochrome *c* reductase; CII + III= succinate dehydrogenase-cytochrome *c* reductase.

DETAILED DESCRIPTION OF THE INVENTION

The current invention is based upon the surprising discovery that mitochondrial DNA depletion syndromes, including TK2 deficiency, can be treated with deoxynucleosides. As shown by the results herein, the administration of deoxynucleosides greatly improved the condition in both a mouse model of TK2 deficiency and human patients with TK2 deficiency.

Definitions

The terms used in this specification generally have their ordinary meanings in the art, within the context of this invention and the specific context where each term is used. Certain terms are discussed below, or elsewhere in the specification, to provide additional guidance to the practitioner in describing the methods of the invention and how to use them. Moreover, it will be appreciated that the same thing can be said in more than one way. Consequently, alternative language and synonyms may be used for any one or more of the terms discussed herein, nor is any special significance to be placed upon whether or not a term is elaborated or discussed herein. Synonyms for certain terms are provided. A recital of one or more synonyms does not exclude the use of the other synonyms. The use of examples anywhere in the specification, including examples of any terms discussed herein, is illustrative only, and in no way limits the scope and meaning of the invention or any exemplified term. Likewise, the invention is not limited to its preferred embodiments.

The term "subject" as used in this application means mammals. Mammals include canines, felines, rodents, bovine, equines, porcines, ovines, and primates. Thus, the invention can be used in veterinary medicine, *e.g.*, to treat companion animals, farm animals, laboratory animals in zoological parks, and animals in the wild. The invention is particularly desirable for human medical applications

The term "patient" as used in this application means a human subject. In some embodiments of the present invention, the "patient" is known or suspected of having a disease or disorder characterized by unbalanced nucleotide pools, mitochondrial disease, mitochondrial DNA depletion syndrome, or TK2 deficiency.

The phrase "therapeutically effective amount" is used herein to mean an amount sufficient to cause an improvement in a clinically significant condition in the subject, or delays or minimizes or mitigates one or more symptoms associated with the disease or disorder, or results in a desired beneficial change of physiology in the subject.

The terms "treat", "treatment", and the like refer to a means to slow down, relieve, ameliorate or alleviate at least one of the symptoms of the disease or disorder, or reverse the disease or disorder after its onset.

The terms "prevent", "prevention", and the like refer to acting prior to overt disease or disorder onset, to prevent the disease or disorder from developing or minimize the extent of the disease or disorder, or slow its course of development.

The term "in need thereof" would be a subject known or suspected of having or being at risk of having a disease or disorder characterized by unbalanced nucleotide pools, mitochondrial disease, mitochondrial DNA depletion syndrome, or TK2 deficiency.

The term "agent" as used herein means a substance that produces or is capable of producing an effect and would include, but is not limited to, chemicals, pharmaceuticals, biologics, small organic molecules, antibodies, nucleic acids, peptides, and proteins.

The term "deoxynucleoside" as used herein means deoxythymidine or dT, deoxycytidine or dC, deoxyadenosine or dA, and deoxyguanosine or dG. The full length name and common abbreviation for each will be used interchangeably. Such deoxynucleosides also include physiologically functional derivatives of the deoxynucleosides.

As used herein, the term "physiologically functional derivative" refers to a compound (*e.g,* a drug precursor) that is transformed *in vivo* to yield a deoxynucleoside. The transformation may occur by various mechanisms (*e.g.*, by metabolic or chemical processes), such as, for example, through hydrolysis in blood. Prodrugs are such derivatives, and a

discussion of the use of prodrugs is provided by T. Higuchi and W. Stella, "Pro-drugs as Novel Delivery Systems," Vol. 14 of the A.C.S. Symposium Series, and in Bioreversible Carriers in Drug Design, ed. Edward B. Roche, American Pharmaceutical Association and Pergamon Press, 1987.

As used herein "an adverse effect" is an unwanted reaction caused by the administration of a drug. In most cases, the administration of the deoxynucleosides caused no adverse effects. The most expected adverse effect would be a minor gastrointestinal intolerance.

The term "about" or "approximately" means within an acceptable error range for the particular value as determined by one of ordinary skill in the art, which will depend in part on how the value is measured or determined, *i.e.*, the limitations of the measurement system, *i.e.*, the degree of precision required for a particular purpose, such as a pharmaceutical formulation. For example, "about" can mean within 1 or more than 1 standard deviations, per the practice in the art. Alternatively, "about" can mean a range of up to 20%, preferably up to 10%, more preferably up to 5%, and more preferably still up to 1% of a given value. Alternatively, particularly with respect to biological systems or processes, the term can mean within an order of magnitude, preferably within 5-fold, and more preferably within 2-fold, of a value. Where particular values are described in the application and claims, unless otherwise stated, the term "about" meaning within an acceptable error range for the particular value should be assumed.

Administration of Deoxynucleosides for the Treatment of Mitochonodrial DNA Depletion Syndrome

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.

One form of MDS is thymidine kinase deficiency or TK2. TK2 encoded by the nuclear gene, *TK2*, is a mitochondrial matrix protein that phosphorylates thymidine and deoxycytidine nucleosides to generate deoxythymidine monophosphate (dTMP) and deoxycytidine monophosphate (dCMP), which in turn, are converted to deoxynucleotide triphosphates (dNTPs) required for mitochondrial DNA synthesis. As discussed in the background section, autosomal recessive *TK2* mutations cause devastating neuromuscular

weakness with severe depletion of mitochondrial DNA (mtDNA) in infants and children, as well as progressive external ophthalmoplegia with mtDNA multiple deletions in adults. Many patients cannot walk and require some type of mechanical ventilation and feeding tube. The central nervous system is variably involved in these disorders, with symptoms that include seizures, encephalopathy, cognitive impairment, and hearing loss. Less than 7% of patients live more than 42 years.

Based on clinical and molecular genetics findings of patients thus diagnosed, three disease presentations were identified: i) infantile-onset (≤1 year-old) myopathy with onset of weakness in the first year of life with severe mtDNA depletion and early mortality; ii) childhood-onset (>1-11 years-old) myopathy with severe mtDNA depletion; and iii) late-onset myopathy (≥12 years-old) with mild weakness at onset and slow progression to loss of ambulation, respiratory insufficiency, or both, often with chronic progressive external ophthalmoparesis in adolescence or adulthood in association with mtDNA multiple deletions, reduced mtDNA copy number, or both. See generally Garone, *et al.*, (2016) in preparation.

Attempts to study the pathogenesis and test therapies for TK2 deficiency using cultured fibroblasts from patients have been unsuccessful, because the replicating cells failed to manifest mtDNA depletion. In contrast, a homozygous Tk2 H126N knock-in mutant ($Tk2^{-/-}$) mouse model, manifests a phenotype that is strikingly similar to the human infantile encephalomyopathy caused by *TK2* mutations, characterized by onset at age 10 days with decreased ambulation, unstable gait, coarse tremor, growth retardation, and depletion of mitochondrial DNA (mtDNA) progressing rapidly to early death at age 14 to 16 days, which is a time period analogous to the human infantile-onset disease (Akman, *et al.* 2008; Dorado, *et al.* 2011).

The studies set forth herein with Tk2 knock-in mice have shown the administration of oral dC/dT prolonged delayed the onset of clinical symptoms of TK2 deficiency and prolonged the lives of the mice by two- to three-fold (Example 2).

Additional experiments showed tissue-specific effects. Measurement of the dNTP pool levels in mitochondria extracts showed that dCTP was rescued in brain and dTTP was rescued in liver (Example 3). Measurement of mtDNA depletion showed both dCMP+dTMP and dC+dT therapies rescued the mtDNA copy number in liver, muscle and tissue (Example 4). It was previously speculated that formation of the blood brain barrier might be compromising the treatment bioavailability in brain. Nevertheless, HPLC measurements showed that catalytic products of these compounds were found in higher concentrations after both nucleotides monophosphates and deoxynucleosides treatment, suggesting that they are

capable of crossing the blood brain barrier. mtDNA depletion measurements also showed a completely rescue of mtDNA copy number in intestine.

Thus, the experiments set forth herein using the mouse model of Tk2 deficiency show the administration of deoxynucleosides to be effective and safe for the treatment of the disease. Additionally, as shown in Example 5, the administration of dT and dC greatly improved the symptoms of TK2 deficiency in patients.

Thus, the present invention includes the administration of at least one deoxynucleoside to a patient in need thereof. In one embodiment, the present invention includes the administration of at least one deoxpyrimidine. In a further embodiment, the deoxypyrimidine is chosen from dC, dT and mixtures thereof. In yet another embodiment, the present invention includes the administration of at least one deoxypurine. In a further embodiment, the deoxypurine is chosen from dA, dG, and mixtures thereof.

Patients who would benefit from the administration of deoxynucleosides would be those diagnosed with TK2 deficiency. In these patients, at least one deoxypyrimidine, dC or dT, or mixtures thereof would be administered.

A parallel defect of deoxyguanosine kinase (dGK), due to autosomal recessive mutations in *DGUOK* with deficiencies in dGMP and dAMP, causes mtDNA depletion typically manifesting as early childhood-onset hepatocerebral disease (Mandel, *et al.* 2001). These patients would benefit from the administration of at least one deoxypurine, dG or dA, or mixtures thereof.

Other forms of MDS as well as other disorders related to unbalanced nucleotide pools can be treated by the administration of specific deoxynucleosides, *i.e.*, dA, dG, dC, or dT, or mixtures thereof. These disorders would include but are not limited to deficiencies related to *RRM2B* (encoding p53R2, the p53 inducible small subunit of ribonucleotide reductase, RNR) and mutations in *TYMP* (encoding thymidine phosphorylase, TP) which cause mitochondrial neurogastrointestinal encephalomyopathy (MNGIE). Additional nuclear genes that disrupt mitochondrial dNTP pools include but are not limited to *SUCLA2, SUCLG1* and *MPV17.* Disorders related to these genes can also be treated by the administration of one or more deoxynucleosides.

Additionally, as the mechanisms of other forms of MDS and other disorders become elucidated, the proper deoxynucleoside(s) for treatment can be determined by the skilled practitioner.

Patients that exhibit the phenotype discussed above for TK2 deficiency including the most typical presentation of progressive muscle disease characterized by generalized

hypotonia, proximal muscle weakness, loss of previously acquired motor skills, poor feeding, and respiratory difficulties, can be tested to definitively diagnose the disease.

If the clinical presentation is highly suspicious for mtDNA depletion syndrome, molecular genetic testing using a panel of genes known to cause mtDNA depletion syndrome should be performed (Chanprasert, *et al.* 2012). The *TK2* gene is the only gene in which mutations are known to cause TK2-related mitochondrial DNA depletion syndrome. This testing can include a sequence analysis of the entire coding and exon/intron junction regions of *TK2* for sequence variants and deletion/duplication. If compound heterozygous or homozygous deleterious mutations are identified in the sequence analysis, the diagnosis of TK2 deficiency is confirmed, and thus, the subject would benefit from the deoxynucleoside therapy. If sequence analysis does not identify two compound heterozygous or homozygous deleterious mutations, deletion/duplication analysis should be considered to determine and/or confirm a TK2 deficiency diagnosis.

Further tests to determine and/or confirm a TK2 deficiency diagnosis may include testing serum creatine kinase (CK) concentration, electromyography, histopathology on skeletal muscle, mitochondrial DNA (mtDNA) content (copy number), and electron transport chain (ETC) activity in skeletal muscle. If one or more of the following is found in these tests, the TK2 deficiency is determined and/or confirmed. Elevated CK concentration as compared to healthy controls can indicate TK2 deficiency. A skeletal muscle biopsy can be performed, and then a mtDNA content analysis in skeletal muscle performed. If the skeletal muscle biopsy shows prominent variance in fiber size, variable sarcoplasmic vacuoles, variable increased connective tissue, and ragged red fibers as well as increased succinate dehydrogenase (SDH) activity and low to absent cytochrome c oxidase (COX) activity, and mtDNA copy number is severely reduced (typically less than 20% of age- and tissue-matched healthy controls), a diagnosis of TK2 deficiency can be determined and/or confirmed (Chanprasert, *et al.* 2012).

Additionally, TK2 deficiency is inherited in an autosomal recessive manner. Thus, a sibling of an affected patient can be tested as early as possible after birth to diagnose the disease.

In all of these examples, deoxynucleoside therapy should be started as soon as possible after a diagnosis of TK2 deficiency.

Pharmaceutical Compositions, Methods of Administration, and Dosing

The present invention encompasses the administration of deoxynucleosides, more specifically one or more deoxynucleosides.

Most preferred methods of administration are oral, intrathecal and parental including intravenous. The deoxynucleosides must be in the appropriate form for administration of choice.

Deoxynucleosides are easily dissolved in liquid are easily dissolved in liquid (such as water, formula or milk) whereas the free acid form does not readily dissolve in liquid.

Such pharmaceutical compositions comprising one of more deoxynucleosides for administration may comprise a therapeutically effective amount of the deoxynucleosides and a pharmaceutically acceptable carrier. The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce an allergic or similar untoward reaction, such as gastric upset, dizziness and the like, when administered to a human, and approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. "Carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as saline solutions in water and oils, including those of petroleum, animal, vegetable, or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil, and the like. A saline solution is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol, and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents.

Oral administration is a preferred method of administration. The deoxynucleosides can be added to any form of liquid a patient would consume including but not limited to, milk, both cow's and human breast, infant formula, and water.

Additionally, pharmaceutical compositions adapted for oral administration may be capsules, tablets, powders, granules, solutions, syrups, suspensions (in non-aqueous or aqueous liquids), or emulsions. Tablets or hard gelatin capsules may comprise lactose, starch or derivatives thereof, magnesium stearate, sodium saccharine, cellulose, magnesium

carbonate, stearic acid or salts thereof. Soft gelatin capsules may comprise vegetable oils, waxes, fats, semi-solid, or liquid polyols. Solutions and syrups may comprise water, polyols, and sugars. An active agent intended for oral administration may be coated with or admixed with a material that delays disintegration and/or absorption of the active agent in the gastrointestinal tract. Thus, the sustained release may be achieved over many hours and if necessary, the active agent can be protected from degradation within the stomach. Pharmaceutical compositions for oral administration may be formulated to facilitate release of an active agent at a particular gastrointestinal location due to specific pH or enzymatic conditions.

In order to overcome any issue of the deoxynucleosides crossing the blood/brain barrier, intrathecal administration is a further preferred form of administration (Galbiati, *et al.* 2006; Gotz, *et al.* 2008). Intrathecal administration involves injection of the drug into the spinal canal, more specifically the subarachnoid space such that it reaches the cerebrospinal fluid. This method is commonly used for spinal anesthesia, chemotherapy, and pain medication. Intrathecal administration can be performed by lumbar puncture (bolus injection) or by a port-catheter system (bolus or infusion). The catheter is most commonly inserted between the laminae of the lumbar vertebrae and the tip is threaded up the thecal space to the desired level (generally L3-L4). Intrathecal formulations most commonly use water, and saline as excipients but EDTA and lipids have been used as well.

A further preferred form of administration is parenteral including intravenous administration. Pharmaceutical compositions adapted for parenteral administration, including intravenous administration, include aqueous and non-aqueous sterile injectable solutions or suspensions, which may contain anti-oxidants, buffers, bacteriostats, and solutes that render the compositions substantially isotonic with the blood of the subject. Other components which may be present in such compositions include water, alcohols, polyols, glycerine, and vegetable oils. Compositions adapted for parental administration may be presented in unit-dose or multi-dose containers, such as sealed ampules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of a sterile carrier, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules, and tablets. Suitable vehicles that can be used to provide parenteral dosage forms of the invention are well known to those skilled in the art. Examples include: Water for Injection USP; aqueous vehicles such as Sodium Chloride Injection, Ringer's Injection, Dextrose Injection, Dextrose and Sodium Chloride Injection, and Lactated Ringer's Injection; water-miscible vehicles such as ethyl alcohol, polyethylene

glycol, and polypropylene glycol; and non-aqueous vehicles such as corn oil, cottonseed oil, peanut oil, sesame oil, ethyl oleate, isopropyl myristate, and benzyl benzoate.

Additionally, since some patients may be receiving enteral nutrition by the time the deoxynucleoside treatment begins, the dNs can be administered through a gastronomy feeding tube or other enteral nutrition means.

Further methods of administration include mucosal, such as nasal, sublingual, vaginal, buccal, or rectal; or transdermal administration to a subject.

Pharmaceutical compositions adapted for nasal and pulmonary administration may comprise solid carriers such as powders, which can be administered by rapid inhalation through the nose. Compositions for nasal administration may comprise liquid carriers, such as sprays or drops. Alternatively, inhalation directly through into the lungs may be accomplished by inhalation deeply or installation through a mouthpiece. These compositions may comprise aqueous or oil solutions of the active ingredient. Compositions for inhalation may be supplied in specially adapted devices including, but not limited to, pressurized aerosols, nebulizers or insufflators, which can be constructed so as to provide predetermined dosages of the active ingredient.

Pharmaceutical compositions adapted for rectal administration may be provided as suppositories or enemas. Pharmaceutical compositions adapted for vaginal administration may be provided as pessaries, tampons, creams, gels, pastes, foams or spray formulations.

Pharmaceutical compositions adapted for transdermal administration may be provided as discrete patches intended to remain in intimate contact with the epidermis of the recipient over a prolonged period of time.

The deoxynucleoside therapy comprises the administration of one or more deoxynucleosides chosen from the group consisting of deoxythymidine (dT), deoxycytidine (dC), deoxyadenosine (dA) and deoxyguanosine (dG).

A skilled practitioner can determine which deoxynucleosides are beneficial based upon the deficiency. It is also within the skill of the art for the practitioner to determine if mixtures of the deoxynucleosides should be administered and in what ratio. If two deoxynucleosides are to be administered, they can be in a ratio of 50/50 of each deoxynucleoside, *e.g.*, dC and dT, or in ratios of about 5/95, 10/90, 15/85, 20/80, 25/75, 30/70, 35/65, 40/60, 45/55, 55/45, 60/40, 65/35, 70/30, 75/25, 80/20, 85/15, 90/10, and 95/5.

By way of example, dT and dC are administered in mixture of equal amounts for TK2 deficiency.

Selection of a therapeutically effective dose will be determined by the skilled artisan considering several factors, which will be known to one of ordinary skill in the art. Such factors include the particular form of the deoxynucleoside, and its pharmacokinetic parameters such as bioavailability, metabolism, and half-life, which will have been established during the usual development procedures typically employed in obtaining regulatory approval for a pharmaceutical compound. Further factors in considering the dose include the condition or disease to be treated or the benefit to be achieved in a normal individual, the body mass of the patient, the route of administration, whether the administration is acute or chronic, concomitant medications, and other factors well known to affect the efficacy of administered pharmaceutical agents. Thus, the precise dose should be decided according to the judgment of the person of skill in the art, and each patient's circumstances, and according to standard clinical techniques.

A preferred dose ranges from about 100 mg/kg/day to about 1,000 mg/kg/day. A further preferred dose ranges from about 200 mg/kg/day to about 800 mg/kg/day. A further preferred dose ranges from about 250 mg/kg/day to about 400 mg/kg/day. These dosage amounts are of individual deoxynucleosides or of a composition with a mixture of more than one deoxynucleosides, *e.g.*, dT and dC. For example, a dose can comprise 400 mg/kg/day of dT alone. In a further example, a dose can comprise a mixture of 200 mg/kg/day of dT and 200 mg/kg/day of dC. In a further example, a dose can comprise 400 mg/kg/day of a mixture of dT and dC.

Administration of the deoxynucleosides can be once a day, twice a day, three times a day, four times a day, five times a day, up to six times a day, preferably at regular intervals. For example, when the deoxynucleosides are administered four times daily, doses would be at 8:00 AM, 12:00 PM, 4:00 PM, and 8:00 PM.

Doses can also be lowered if being administered intravenously or intrathecally. Preferred dose ranges for such administration are from about 50 mg/kg/day to about 500 mg/kg/day.

As shown in Example 5, doses can be adjusted to optimize the effects in the subject. For example, the deoxynucleosides can be administered at 100 mg/kg/day to start, and then increased over time to 200 mg/kg/day, to 400 mg/kg/day, to 800 mg/kg/day, up to 1000 mg/kg/day, depending upon the subject's response and tolerability.

A subject can be monitored for improvement of their condition prior to increasing the dosage. A subject's response to the therapeutic administration of the deoxynucleosides can be monitored by observing a subject's muscle strength and control, and mobility as well as

changes in height and weight. If one or more of these parameters increase after the administration, the treatment can be continued. If one or more of these parameters stays the same or decreases, the dosage of the deoxynucleosides can be increased.

As shown in the Examples, the deoxynucleosides are well tolerated. Any observed adverse effects were minor and were mostly diarrhea, abdominal bloating and other gastrointestinal manifestations. A subject can also be monitored for any adverse effects, such as gastrointestinal intolerance, *e.g.,* diarrhea. If one or more adverse effects are observed after administration, then the dosage can be decreased. If no such adverse effects are observed, then the dosage can be increased. Additionally, once a dosage is decreased due to the observation of an adverse effect, and the adverse effect is no longer observed, the dosage can be increased.

The deoxynucleosides can also be co-administered with other agents. Such agents would include therapeutic agents for treating the symptoms of the particular form of MDS. In particular, for TK2 deficiency, the dT and dC can be co-administered with an inhibitor of ubiquitous nucleoside catabolic enzymes, including but not limited to enzyme inhibitors such as tetrahydrouridine (inhibitor of cytidine deaminase) and immucillin H (inhibitor of purine nucleoside phosphorylase) and tipiracil (inhibitor of thymidine phosphorylase). Such inhibitors are known and used in the treatment of some cancers.

EXAMPLES

The present invention may be better understood by reference to the following non-limiting examples, which are presented in order to more fully illustrate the preferred embodiments of the invention. They should in no way be construed to limit the broad scope of the invention.

Example 1- Materials and Methods

Mouse Model of TK2 Deficiency

A homozygous *Tk2* H126N knock-in mutant (*Tk2*$^{-/-}$) mouse that manifests a phenotype strikingly similar to the human infantile encephalomyopathy has been previously reported (Akman, *et al.* 2008). Between postnatal day 10 and 13, Tk2$^{-/-}$ mice rapidly develop fatal encephalomyopathy characterized by decreased ambulation, unstable gait, coarse tremor, growth retardation, and rapid progression to early death at age 14 to 16 days. Molecular and biochemical analyses of the mouse model demonstrated that the pathogenesis of the disease is due to loss of enzyme activity and ensuing dNTP pool imbalances with

decreased dTTP levels in brain and both dTTP and dCTP levels in liver, which, in turn, produces mtDNA depletion and defects of respiratory chain enzymes containing mtDNA-encoded subunits, most prominently in the brain and spinal cord.

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-hour light, 12-hour 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 eosin (H&E) for morphological study or processed for immunostaining studies with GFAP, COX I, or complex I subunit as detailed described in the supplemental procedures. Both heterozygous and homozygous wild type mice were considered as control group (*Tk2*$^{+}$) since no clinical and biochemical difference were previously described (Akman, *et al.* 2008; Dorado, *et al.* 2011).

Treatment administration and experimental plan

Deoxycytidine (dC) and deoxythymidine (dT) 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 control wild-type (*T*k2^{+}) using 2 doses, 260 mg/kg/day and 520 mg/kg/day, from post-natal day 4 to 29 days. At age 21 days, mice were separated from the mother and the treatment was continued by administration of dC and dT in drinking water using equimolar doses respectively of 1.6mM and 3.2mM. A negative control group of untreated *Tk2* mutant and control wild-type mice were weighed 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 (Akman, *et al.* 2008).

To define the degree of safety and efficacy of dT/dC, 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 were

compared. General 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 minutes at 4°C, followed by three centrifugations at 13,000g for 2 minutes at 4°C. Supernatant was precipitated with 60% methanol, kept 2 hours at -80°C, boiled 3 minutes, stored at -80°C (from 1 hour to overnight) and centrifuged at 20,800g for 10 minutes 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 (Ferraro, *et al.* 2010; Marti, *et al.* 2012a). 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 3H-dTTP or 3H-dATP (Moravek Biochemicals), 0.25 µM specific oligonucleotide, 40 mM Tris–HCl, pH 7.5, 10 mM MgCl2, 5mM DTT]. After 60 minutes at 48°C, 18 ml of reaction were spotted on Whatman DE81 filters, air dried and washed three times for 10 minutes with 5% Na2HPO4, once in distilled water and once in absolute ethanol. The retained radioactivity was determined by scintillation counting.

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 four buffers: eluent A (20 mM potassium phosphate, pH 5.6), eluent B (water) and eluent C (methanol). Samples were eluted over 60 minutes 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 eluates 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 deoxythymidine, deoxyuridine, uracil, and thymine peaks for each sample, a second aliquot was 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, nDNA) (Applied Biosystems, Invitrogen, Foster City, CA, USA) as described as described using ddCt method in a Step One Plus Real Time PCR 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 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). 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 (DiMauro, *et al.* 1987).

Statistical methods

Data are expressed as the mean ± SD of at least 3 experiments per group. Gehan-Breslow-Wilcoxon test was used to compare the survival proportion of each group of mice. A p-value of <0.05 was considered to be statistically significant.

Example 2- The Administration of dC/dT to $Tk2^{-/-}$ Mice Delayed the Clinical Onset of TK2 Deficiency and Increased Survival

A dose of 260 and 520 mg/kg/day each of deoxynucleosides (dC/dT) were administered to the $Tk2^{-/-}$ mice. These doses of deoxynucleosides were the molar equivalent of 400 and 800 mg/kg/day of dCMP+dTMP respectively.

Mice treated with oral dC+dT (260 or 520mg/kg/day from age 4 days) appeared normal until postnatal day 21 (Figure 1). After age 21 days, mutant mice treated with 260 mg/kg/day dose ($Tk2^{-/-\ 260\ mg/kg/day\ dC/dT}$) stopped gaining weight and developed mild head tremor and weakness that led to death at postnatal day 31±4.3 (Figure 2).

Mutant mice treated with the 520 mg/kg/day dC+dT ($Tk2^{-/-\ 520\ mg/Kg/day\ dC/dT}$) continued to gain weight for one additional week, but subsequently manifested deterioration similar to $Tk2^{-/-260\ mg/Kg/day\ dC/dT}$, and died at postnatal day 43±10. These results are comparable to those showed by $Tk2^{-/-}$ mice treated with 200 or 400mg/kg/day of oral dCMP/dTMP treatment. $Tk2^{+\ 260\ mg/kg/day\ dC/dT}$ and$Tk2^{+\ 520\ mg/kg/day\ dC/dT}$ were followed until postnatal day 60. No side effects were observed.

As shown, the lifespan of the treated $Tk2^{-/-}$ was significantly increased. Untreated $Tk2^{-/-}$ mice showed a mean lifespan of 13 days, while treated mice survived a mean of 31 and 40 days with the 260 and 520 mg/kg/day dose, respectively (Figure 2). Interestingly, one of the mice survived to postnatal day 56, which has been the longest lifespan for the Tk2 knock-in mouse model to date.

Example 3- Oral dC/dT Ameliorates Molecular Abnormalities in Brain and Liver

Measurement of dNTPs in mitochondrial extract showed that both $Tk2^{-/-\ 260\ mg/Kg/day\ dC/dT}$ and $Tk2^{-/-\ 520\ mg/Kg/day\ dC/dT}$ did not fully correct mitochondrial dNTP pool imbalances at postnatal day 13 and manifested variable effects in tissues with a completed rescue of dCTP deficits in brain, while dTTP was corrected in the liver. In contrast, deficiencies of dTTP in brain and dCTP in liver remained severe despite deoxynucleoside supplementation (Figure 3).

In $Tk2^{-/-\ 260\ mg/Kg/day\ dC/dT}$ and $Tk2^{-/-\ 520\ mg/Kg/day\ dC/dT}$ mice at postnatal day 13, the treatment prevented mtDNA depletion in heart, liver, kidney, intestine and muscle (Figure 4). In contrast, mtDNA copy number was only partially ameliorated in brain at postnatal day 13 in a dose-dependent manner with mtDNA/nDNA ratios relative to control brain reaching 39% with 260 mg/kg/day of dC+dT and 52% with 520 mg/kg/day. Measurements of the bases dT and uracil in brain by HPLC showed higher levels in animals treated with dC+dT or with dCMP+dTMP (Figure 5), further indicating that both deoxynucleosides and deoxynucleoside monophosphates cross the blood brain barrier. At postnatal day 29, mtDNA depletion was partially rescued by 260 and 520 mg/kg/day of dC+dT therapy in heart (40 and 35%), liver (46 and 45%), kidney (38 and 42%) and muscle (24 and 35%), but strikingly was fully rescued in intestine (82 and 84%) (Figure 4).

Example 4- Oral dC/dT Ameliorates Biochemical Abnormalities in Brain

Respiratory chain enzyme (RCE) activities and protein levels were completely rescued in brain of $TK2^{-/-\ 260\ mg/Kg/day\ dC/dT}$ at postnatal day 13 (Figure 6). RCE activities were also restored at postnatal day 29, and only a slight decrease of complex I activity could be observed in $TK2^{-/-52\ 0mg/Kg/day\ dC/dT}$ (Figure 6). RCE protein levels in brain were partially restored at postnatal day 29 with higher levels in $TK2^{-/-520\ mg/Kg/day\ dC/dT}$ than in $TK2^{-/-260\ mg/Kg/day\ dC/dT}$ (Figure 7). These differences in protein levels were consistent with the differences in mtDNA depletion in brains of treated mutant mice at postnatal day 29, and likely accounted for the prolonged survival observed with the higher dose.

Example 5- Administration of dC/dT in Patients with TK2 Deficiency Was Efficacious

Symptoms, dosages, and outcomes of patients with TK2 deficiency who have received deoxynucleoside therapy under the supervision and control of the inventors are summarized below.

Patient 1

This patient was born in the United States in February 2011. His symptoms manifested at 12 months with hypotonic and a floppy head. He has never walked. He also has respiratory muscle weakness and was put on mechanical ventilation at 19 months, of which he is still on 24 hours/day. He has also been on a feeding tube since 19 months.

He was previously on 100 mg/kg/day and then 200 mg/kg/day of dCMP and dTMP. On this therapy, he was able to grip small objects and his weight increased from 10.4 kg to 19.5 kg.

In October of 2015, he began on 260 mg/kg/day of dC and dT which was increased to 340 mg/kg/day of dC and dT. After two months, he was moving his hands and head better, able to stand 5 minutes with support of a person, starting to cough, and his heart rate was slower (down from 140-170 bpm during day, to 100-120 bpm during day).

On March 23, 2016, the dose was increased to 400 mg/kg/day of dC and dT. After 6 weeks on this therapy, he showed further improvements: he was able to sit in a chair about 5 hours/day; stood in a "Stander" for 1.5 hours; about to grab and hold small stuffed animals; pressed computer buttons; untied his diapers and aimed his penis to wet the person changing the diaper; and held his knees flexed for a few seconds.

The only adverse effect seen during the treatment was diarrhea.

Patient 2

This patient was born in Spain in 1987. He began showing symptoms at 3 years of age including proximal muscle weakness. He lost the ability to walk at age 13 and was ventilated 24 hours a day. He was previously taking dAMP and dCMP at 200 mg/kg/day and showed a weight increase and a decrease of 24 to 22 hours a day on ventilation.

He has been on deoxynucleoside therapy since June of 2015 at 400 mg/kg/day dC and dT, and has shown improvement in muscle strength, his weight and ventilation have stabilized, and he is enjoying a better quality of life.

The only adverse effects seen during the treatment was diarrhea and hair loss.

Patient 3

This patient was born in Spain in 1985. His symptoms began at 6 years old with facial, proximal, and axial muscle weakness. He started 200 mg/kg/day of dT and dC in June of 2015 and to date, his condition has improved with improvements in 6 minute walk test, time to get up and go, and climb up and down 4 steps.

The only adverse effect seen during the treatment was diarrhea.

Patient 4

This patient was born in Spain in February 2009. His symptoms manifested at six months with failure to thrive. He started on 230 mg/kg/ day of dC and dT in July of 2015. By January of 2016, he showed improvement in his condition and was eating better.

There were no observed adverse effects.

Patient 5

This patient was born in Spain in 1957 and began to have symptoms at 50 years old of orthopnea, and diaphragmatic weakness. He is on BiPAP at night. He started on 200 mg/kg/day of dC and dT in November of 2015.

There were no observed adverse effects.

Patient 6

This patient was born in Spain in October 2011, and starting showing symptoms at 15 months, including hypotonia and weakness. He lost ambulation at 22 months, and has respiratory muscle weakness. He started mechanical ventilation at 16 months and is currently on BiPAP twelve hours a day. He was previously on dCMP and dAMP at 100 mg/kg/day that was increased to 400 mg/kg/day. His strength as shown by Egen Klassification scale improved (28/30 to 13/30) and his weight increased from 9.8 kg to 12.3 kg.

He began deoxynucleoside therapy in April 2015 at 400 mg/kg/day of dC and dT. In October of 2015, his change in Egen Klassification scale went from 13/30 to 11/30 and his weight increased to 16.5 kg from 12.3 kg.

There were no observed adverse effects.

Patient 7

This patient was born in Spain in November of 2012. He started showing symptoms at 17 months including weakness and hypotonia. He lost ambulation at 22 months and started mechanical ventilation at 29 months. He was previously on dCMP and dAMP at 100 mg/kg/day that was increased to 400 mg/kg/day. His strength as shown by Egen Klassification scale improved (30/30 to 24/30) and his weight increased from 11 kg to 15.7 kg.

He started deoxynucleoside therapy in April of 2015 with a dose of 400 mg/kg/day dT and dC. In November of 2015, his change in Egen Klassification scale went from 24/30 to 19/30 and his weight increased to 17 kg from 15.7 kg.

There were no observed adverse effects.

Patient 8

This patient was born in Chile in September of 1989 and started showing symptoms at 11 months with frequent falls and progressive gait impairment. She lost the ability to walk alone at about 4 years of age. She had been on nucleotide therapy previously and showed improvement in her mobility, including walking unassisted, standing longer, climbing stairs, attending gym class, and attending to personal needs.

She switched to deoxynucleoside therapy in February of 2016 at a dose of 260 mg/kg/day of dC and dT, and then increased to a dose of 400 mg/kg/day of dC and dT in May of 2016 and continued to show improvement.

There were no observed adverse effects.

Patient 9

This patient was born in Guatemala in September of 1989. He began 130 mg/kg/day of dC and dT in August of 2015 and increased to 260 mg/kg/day in February of 2016. He has shown improved energy.

There were no observed adverse effects.

REFERENCES

Akman, *et al.* (2008) Thymidine kinase 2 (H126N) knock in mice show the essential role of balanced deoxynucleotide pools for mitochondrial DNA maintenance. *Hum. Mol. Genet.* 17:2433-2440

Alston, *et al.* (2013) Late-onset respiratory failure due to TK2 mutations causing multiple mtDNA deletions. *Neurology* 81:2051-3

Bartesaghi, *et al.* (2010) Loss of thymidine kinase 2 alters neuronal bioenergetics and leads to neurodegeneration. *Hum. Mol. Genet.* 19:1669-77

Béhin, *et al.* (2012) Adult cases of mitochondrial DNA depletion due to TK2 defect An expanding spectrum. *Neurology* 78:644-648

Blakely, *et al.* (2008) Novel mutations in the TK2 gene associated with fatal mitochondrial DNA depletion myopathy. *Neuromuscular Disorders* 18:557-560

Bourdon, *et al.* (2007) Mutation of RRM2B, encoding p53-controlled ribonucleotide reductase (p53R2), causes severe mitochondrial DNA depletion. *Nature Genetics* 39**:**776-780

Carrozzo, *et al.* (2003) Mutation analysis in 16 patients with mtDNA depletion. *Hum. Mutat.* 21:453-454

Chanprasert, *et al.* (2013) Molecular and clinical characterization of the myopathic form of mitochondrial DNA depletion syndrome caused by mutations in the thymidine kinase (TK2) gene. *Mol. Genet. Metab.* 110:153-61

Chanprasert, *et al.* (2012) TK2-Related Mitochondrial DNA Depletion Syndrome, Myopathic Form. *GeneReviews® Internet*, December 6, 2012

Collins, *et al.* (2009) Progressive myofiber loss with extensive fibro-fatty replacement in a child with mitochondrial DNA depletion syndrome and novel thymidine kinase 2 gene mutations. *Neuromuscular Disorders* 19:784-787

Copeland (2008) Inherited mitochondrial diseases of DNA replication. *Ann. Rev. Med.* 59:131-146

DiMauro, *et al.* (1987) Cytochrome c oxidase deficiency in Leigh syndrome. *Ann. Neurol.* 22:498-506

DiMauro, Schon. (2003) Mitochondrial respiratory-chain diseases. *New England Journal of Medicine* 348:2656-2668

DiMauro, Hirano. (2005) Mitochondrial encephalomyopathies: an update. *Neuromuscul. Disord.* 15:276-286

Dorado, *et al.* (2011) Onset and organ specificity of Tk2 deficiency depends on Tk1 down-regulation and transcriptional compensation. *Hum. Mol. Genet.* 20:155-64

Elpeleg, *et al.* (2005) Deficiency of the ADP-forming succinyl-CoA synthase activity is associated with encephalomyopathy and mitochondrial DNA depletion. *Am. J. Hum. Genet.* 76:1081-1086

Ferraro, *et al.* (2010) Quantitation of cellular deoxynucleoside triphosphates. *Nucleic Acids Research* 38:e85

Galbiati, *et al.* (2006) New mutations in TK2 gene associated with mitochondrial DNA depletion. *Pediatr. Neurol.* 34:177-185

Garone, *et al.* (2012). MPV17 Mutations Causing Adult-Onset Multisystemic Disorder With Multiple Mitochondrial DNA Deletions. *Arch Neurol* 69:1648-1651

Garone, et al. (2014). Deoxypyrimidine monophosphate bypass therapy for thymidine kinase 2 deficiency. *EMBO Mol Med* 6:1016-1027

Garone, *et al.* (2016) in preparation, "Phenotypic Spectrum and Retrospective Natural History of Thymidine Kinase 2 Deficiency"

Gotz, *et al.* (2008) Thymidine kinase 2 defects can cause multi-tissue mtDNA depletion syndrome. *Brain* 131:2841-2850

Hirano, *et al.* (2001) Defects of intergenomic communication: autosomal disorders that cause multiple deletions and depletion of mitochondrial DNA. *Semin. Cell. Develop. Biol.* 12:417-427

Hirano, *et al.* (2004) MtDNA maintenance and stability genes: MNGIE and mtDNA depletion syndromes. In: Köhler, Bauer, editors. Mitochondrial Function and Biogenetics. Berlin: Springer-Verlag. p. 177-200

Leshinsky-Silver, *et al.*.(2008) A defect in the thymidine kinase 2 gene causing isolated mitochondrial myopathy without mtDNA depletion. *Eur. J. Paediatr. Neurol.* 12:309-13

Lesko, *et al.* (2010) Two novel mutations in thymidine kinase-2 cause early onset fatal encephalomyopathy and severe mtDNA depletion. *Neuromuscul. Disord.* 20:198-203

Longley, *et al.* (2006). Mutant POLG2 disrupts DNA polymerase gamma subunits and causes progressive external ophthalmoplegia. *Am J Hum Genet.* 78:1026-1034

Lopez, *et al.* (2009) Unbalanced deoxynucleotide pools cause mitochondrial DNA instability in thymidine phosphorylase deficient mice. *Human Molecular Genetics* 18:714-22

Mancuso, *et al.* (2003) Mitochondrial myopathy of childhood associated with mitochondrial DNA depletion and a homozygous mutation (T77M) in the TK2 gene. *Arch. Neurol.* 60:1007-9

Mancuso, *et al.* (2002) Mitochondrial DNA depletion: mutations in thymidine kinase gene with myopathy and SMA. *Neurology.* 59:1197-202

Mandel, *et al.* (2001) The deoxyguanosine kinase gene is mutated in individuals with depleted hepatocerebral mitochondrial DNA. *Nature Genet.* 29:337-341

Martí, *et al.* (2010) Hearing loss in a patient with the myopathic form of mitochondrial DNA depletion syndrome and a novel mutation in the TK2 gene. *Pediatr. Res.* 68:151-4

Martí, *et al.* (2012a) Measurement of mitochondrial dNTP pools. *Methods Mol. Biol.* 837:135-148

Martí, *et al.* (2012b) Assessment of thymidine phosphorylase function: measurement of plasma thymidine (and deoxyuridine) and thymidine phosphorylase activity. *Methods Mol. Biol.* 837: 121-133

Naviaux, Nguyen. (2004) POLG mutations associated with Alpers' syndrome and mitochondrial DNA depletion. *Ann. Neurol.* 55:706-712

Nishino, *et al.* (1999). Thymidine phosphorylase gene mutations in MNGIE, a human mitochondrial disorder. *Science* 283:689-692.

Oskoui, *et al.* (2006) Clinical spectrum of mitochondrial DNA depletion due to mutations in the thymidine kinase 2 gene. *Arch. Neurol.* 63:1122-1126.

Ostergaard, *et al.* (2007) Deficiency of the alpha subunit of succinate-coenzyme A ligase causes fatal infantile lactic acidosis with mitochondrial DNA depletion. *Am. J. Hum. Genet.* 81: 383-387

Paradas, *et al.* (2012) TK2 mutation presenting as indolent myopathy. *Neurology* 29:504-506

Ronchi, *et al.* (2012). Next-generation sequencing reveals DGUOK mutations in adult patients with mitochondrial DNA multiple deletions. *Brain* 135:3404-3415.

Roos, *et al.* (2014) Mitochondrial DNA depletion in single fibers in a patient with novel TK2 mutations. *Neuromuscul. Disord.* 24:713-20

Saada, *et al.* (2001) Mutant mitochondrial thymidine kinase in mitochondrial DNA depletion myopathy. *Nature Genet.* 29:342-344

Saada, *et al.* (2003) Mitochondrial deoxyribonucleoside triphosphate pools in thymidine kinase 2 deficiency. *Biochem. Biophys. Res. Commun.* 310:963-966

Sarzi, *et al.* (2007) Twinkle helicase (PEO1) gene mutation causes mitochondrial DNA depletion. *Ann. Neurol.* 62**:** 579-587

Spelbrink, *et al.* (2001). Human mitochondrial DNA deletions associated with mutations in the gene encoding Twinkle, a phage T7 gene 4-like protein localized in mitochondria. *Nature Genet.* 28:223-231

Spinazzola, *et al.* (2006) MPV17 encodes an inner mitochondrial membrane protein and is mutated in infantile hepatic mitochondrial DNA depletion. *Nature Genet.* 38**:**570-575

Tulinius, *et al.* (2005) Novel mutations in the thymidine kinase 2 gene (TK2) associated with fatal mitochondrial myopathy and mitochondrial DNA depletion. *Neuromuscul. Disord.* 15:412-415

Tyynismaa, *et al.* (2012) Thymidine kinase 2 mutations in autosomal recessive progressive external ophthalmoplegia with multiple mitochondrial DNA deletions. *Hum. Mol. Genet.* 21:66-75

Tyynismaa, *et al.* (2009). A heterozygous truncating mutation in RRM2B causes autosomal-dominant progressive external ophthalmoplegia with multiple mtDNA deletions. *Am. J. Hum. Genet.* 85: 290-295

Van Goethem, *et al.* (2001) Mutation of POLG is associated with progressive external ophthalmoplegia characterized by mtDNA deletions. *Nature Genet.* 28:211-212.

Vilà, *et al.* (2003) Reversion of mtDNA depletion in a patient with TK2 deficiency. *Neurology* 60:1203-1205

Wang, *et al.* (2005) Molecular insight into mitochondrial DNA depletion syndrome in two patients with novel mutations in the deoxyguanosine kinase and thymidine kinase 2 genes. *Mol. Genet. Metab.* 84:75-82.

CLAIMS:

1. A method of treating a disease or disorder characterized by unbalanced nucleotide pools in a subject in need thereof comprising administering to the subject a therapeutically effective amount of a composition comprising at least one deoxynucleoside or a physiologically functional derivative thereof.

2. The method of claim 1, wherein the disease or disorder characterized by unbalanced nucleotide pools is a mitochondrial DNA depletion syndrome.

3. The method of claim 2, wherein the mitochondrial DNA depletion syndrome is thymidine kinase 2 (TK2) deficiency.

4. The method of claim 1, wherein the disease or disorder characterized by unbalanced nucleotide pools is characterized by at least one mutation in a gene chosen from the group consisting of: *TK2; DGUOK; TYMP; RRM2B; SUCLA2; SUCLG1;* and *MPV17.*

5. The method of claim 2, wherein the mitochondrial DNA depletion syndrome is chosen from the group consisting of deoxyguanosine kinase (dGK) deficiency, thymidine phosphorylase (TP) deficiency, and at least one mutation in a gene chosen from the group consisting of *DGUOK, TYMP, RRM2B, POLG,* and *MPV17* gene.

6. The method of claim 1, wherein the subject is a mammal.

7. The method of claim 1, wherein the subject is a human.

8. The method of claim 1, wherein the composition comprises two or more deoxynucleosides.

9. The method of claim 1, wherein the deoxynucleoside is a deoxypyrimidine.

10. The method of claim 9, wherein the deoxypyrimidine is chosen from the group consisting of deoxycytidine (dC), deoxythymidine (dT), and mixtures thereof.

11. The method of claim 1, wherein the deoxynucleoside is a deoxypurine.

12. The method of claim 19, wherein the deoxypurine is chosen from the group consisting of deoxyadenosine (dA), deoxyguanosine (dG), and mixtures thereof.

13. The method of claim 1, wherein the therapeutically effective amount is between about 100 mg/kg/day and about 1000 mg/kg/day.

14. The method of claim 1, wherein the therapeutically effective amount is between about 200 mg/kg/day and about 800 mg/kg/day.

15. The method of claim 1, wherein the therapeutically effective amount is between about 250 mg/kg/day and about 400 mg/kg/day.

16. The method of claim 13, wherein the composition comprises more than one deoxynucleoside and the therapeutically effective amount is between about 100 mg/kg/day and about 1000 mg/kg/day of each deoxynucleoside in the composition.

17. The method of claim 13, wherein the composition comprises more than one deoxynucleoside and the therapeutically effective amount is between about 100 mg/kg/day and about 1000 mg/kg/day of the total deoxynucleoside in the composition.

18. The method of claim 14, wherein the composition comprises more than one deoxynucleoside and the therapeutically effective amount is between about 200 mg/kg/day and about 800 mg/kg/day of each deoxynucleoside in the composition.

21. The method of claim 15, wherein the composition comprises more than one deoxynucleoside and the therapeutically effective amount is between about 250 mg/kg/day and about 400 mg/kg/day of the total deoxynucleosides in the composition.

22. The method of claim 1, wherein the composition is administered once daily, twice daily, three times daily, four times daily, five times daily or six times daily.

23. The method of claim 1, wherein the composition administered orally, intrathecally, enterally, or intravenously.

24. The method of claim 23, wherein the composition is administered orally and comprises deoxynucleoside mixed with cow's milk, human breast milk, infant formula or water.

25. The method of claim 1, further comprising administering to the subject an inhibitor of thymidine phosphorylase.

26. The method of claim 25, wherein the inhibitor of thymidine phosphorylase is tipiracil.

27. The method of claim 1, further comprising administering to the subject an inhibitor of cytidine deaminase.

28. The method of claim 27, wherein the inhibitor of cytidine deaminase is tetrahydrouridine [THU].

29. The method of claim 1, wherein the therapeutically effective amount of the composition administered to the subject is increased over time.

30. The method of claim 29, wherein a first therapeutically effective amount of the composition administered to the subject is about 100 mg/kg/day of composition, and wherein the therapeutically effective amount of the composition is increased over time to 200 mg/kg/day, to 400 mg/kg/day, to 800 mg/kg/day, up to 1000 mg/kg/day.

31. The method of claim 1, wherein the composition comprises a pharmaceutically acceptable carrier.

32. A method for the treatment of TK deficiency in a subject comprising:

a. obtaining a sample from the subject, said sample comprising nucleic acid;

b. performing sequence analysis of the *TK2* gene in the nucleic acid of the subject;

c. determining the subject has TK2 deficiency when a homozygous mutation or compound heterozygous mutations in the *TK2* gene is detected; and

d. administering a therapeutically effective amount of a composition comprising deoxycytidine (dC), deoxythymidine (dT), and mixtures thereof to the subject.

33. The method of claim 32, further comprising:

a. detecting the level of creatine kinase concentration in a sample from the subject;

b. performing a biopsy on skeletal muscle of the subject;

c. measuring mitochondrial DNA count in skeletal muscle of the subject; and

d. further determining and/or confirming the subject has TK2 deficiency if one or more of the following is detected: the levels of creatine kinase concentration are increased or elevated compared to healthy controls; the skeletal muscle of the subject comprises prominent variance in fiber size, variable sarcoplasmic vacuoles, variable increased connective tissue, ragged red fibers, and cytochrome *c* oxidase (COX) deficient fibers: and mitochondrial DNA levels are decreased compared to healthy controls.

34. The method of claim 3, further comprising monitoring the subject after the administration of the composition, comprising:

a. observing muscle strength and control;

b. observing differences in height and weight;

c. observing mobility; and

d. determining an improvement in condition of the subject if any of observations (a) – (c) are increased after administration of the composition, and determining no improvement if any of observations (a) – (c) are the same or decreased after administration of the composition.

35. The method of claim 34, wherein if the determination of no improvement is made in step (d), the therapeutically effective amount of the composition is increased.

36. The method of claim 1, further comprising monitoring the subject for an adverse effect after the administration of the composition, wherein if an adverse effect is observed, the therapeutically effective amount of the composition is decreased.

37. The method of claim 36, further comprising monitoring the subject for the observed adverse effect after the therapeutically effective amount of the composition is decreased, wherein if the adverse effect is no longer observed, the therapeutically effective amount of the composition is increased.

38. The method of claim 36, wherein an adverse effect is a gastrointestinal intolerance.

39. The method of claim 36, wherein the adverse effect is chosen from the group consisting of diarrhea and abdominal bloating.

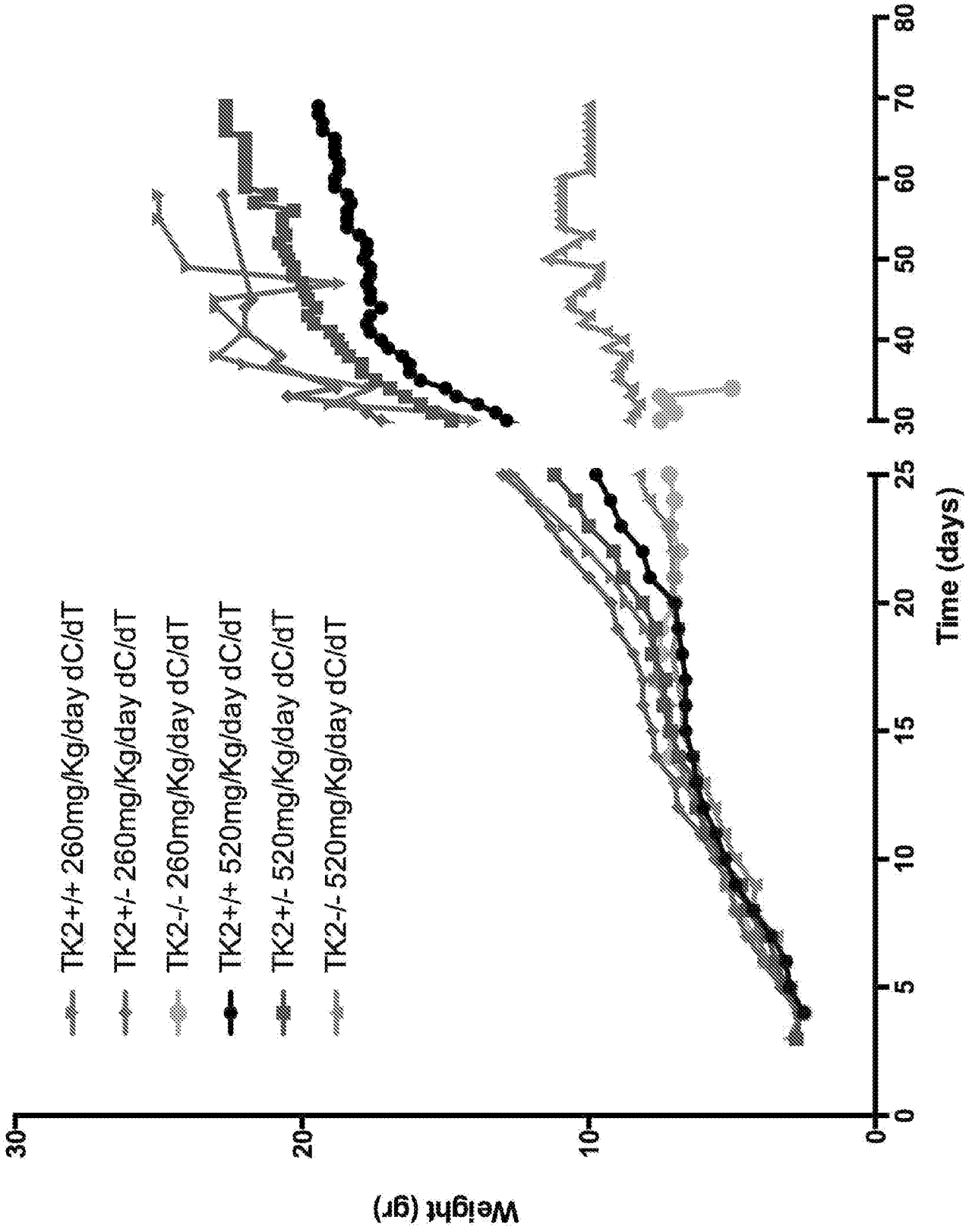

Growth curve oral dCdT
TK2+/+ 260mg/Kg/day dC/dT
TK2+/- 260mg/Kg/day dC/dT
TK2-/- 260mg/Kg/day dC/dT
TK2+/+ 520mg/Kg/day dC/dT
TK2+/- 520mg/Kg/day dC/dT
TK2-/- 520mg/Kg/day dC/dT
Weight (gr)
30
20
10
0
0
5
10
15
20
25
30
40
50
60
70
80
Time (days)

Figure 1

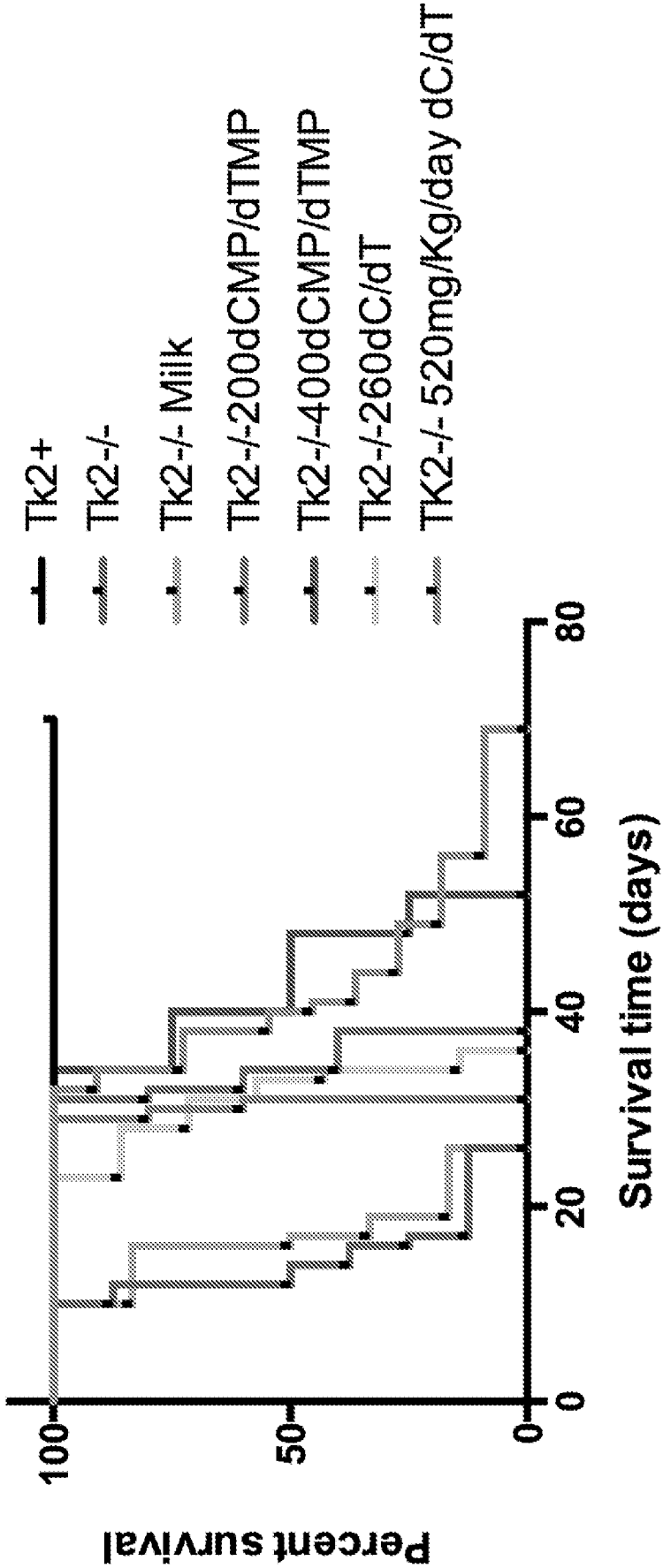

Tk2+
Tk2-/-
Tk2-/- Milk
Tk2-/-200dCMP/dTMP
Tk2-/-400dCMP/dTMP
Tk2-/-260dC/dT
TK2-/- 520mg/Kg/day dC/dT
Percent survival
100
50
0
0
20
40
60
80
Survival time (days)

Figure 2

Figure 3

LIVER
BRAIN
P13
P29
dATP
dTTP
dGTP
dCTP
100%
80%
60%
40%
20%
0%
untreated TK2+/+ P13
TK2-/- P13
TK2+ 2ddC/dT P13
TK2-/- 2ddC/dT P13
TK2+ 2ddC/dT/dTMP P13
TK2-/- 2ddC/dT/dTMP P13
TK2+ 5ddC dC/dT P13
TK2-/- 5ddC dC/dT P13
untreated TK2+/+ P29
TK2-/- P29
TK2+ 2ddC/dT P29
TK2-/- 2ddC/dT P29
TK2+ 2ddC/dT/dTMP P29
TK2-/- 2ddC/dT/dTMP P29
TK2+ 5ddC dC/dT P29
TK2-/- 5ddC dC/dT P29

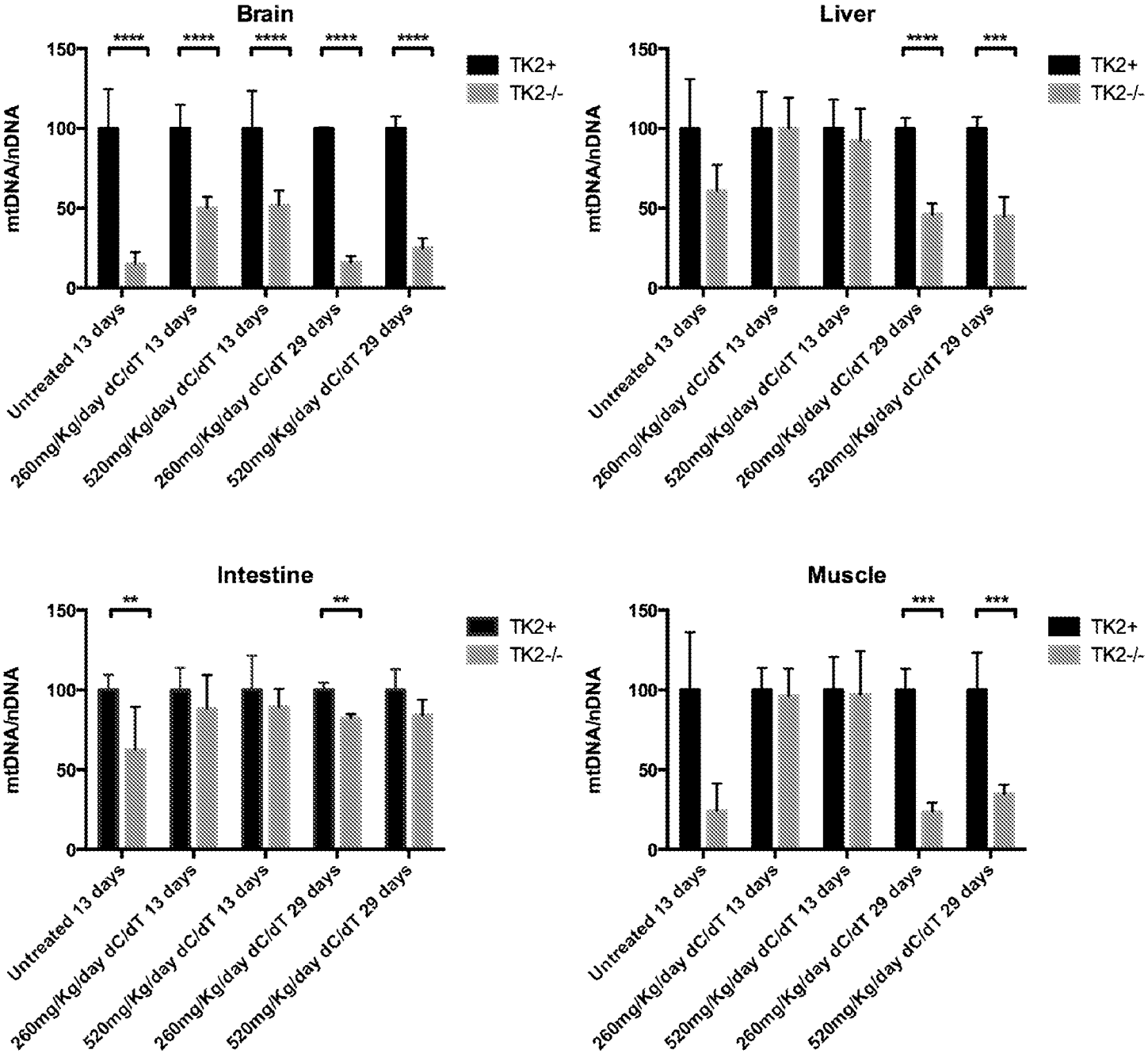

Brain
Liver
Intestine
Muscle
mtDNA/nDNA
TK2+
TK2-/-
Untreated 13 days
260mg/Kg/day dC/dT 13 days
520mg/Kg/day dC/dT 13 days
260mg/Kg/day dC/dT 29 days
520mg/Kg/day dC/dT 29 days

Figure 4

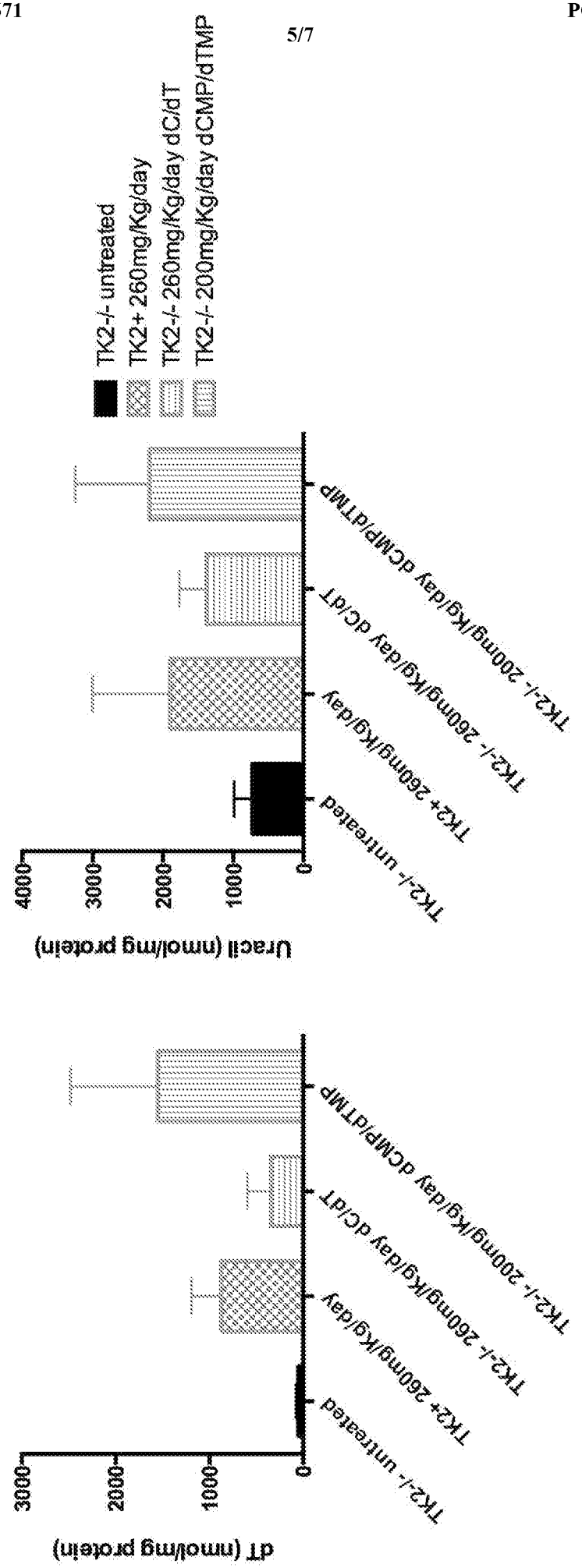

TK2-/- untreated
TK2+ 260mg/Kg/day
TK2-/- 260mg/Kg/day dC/dT
TK2-/- 200mg/Kg/day dCMP/dTMP
Uracil (nmol/mg protein)
4000
3000
2000
1000
0
TK2-/- untreated
TK2+ 260mg/Kg/day
TK2-/- 260mg/Kg/day dC/dT
TK2-/- 200mg/Kg/day dCMP/dTMP
dT (nmol/mg protein)
3000
2000
1000
0
TK2-/- untreated
TK2+ 260mg/Kg/day
TK2-/- 260mg/Kg/day dC/dT
TK2-/- 200mg/Kg/day dCMP/dTMP

Figure 5

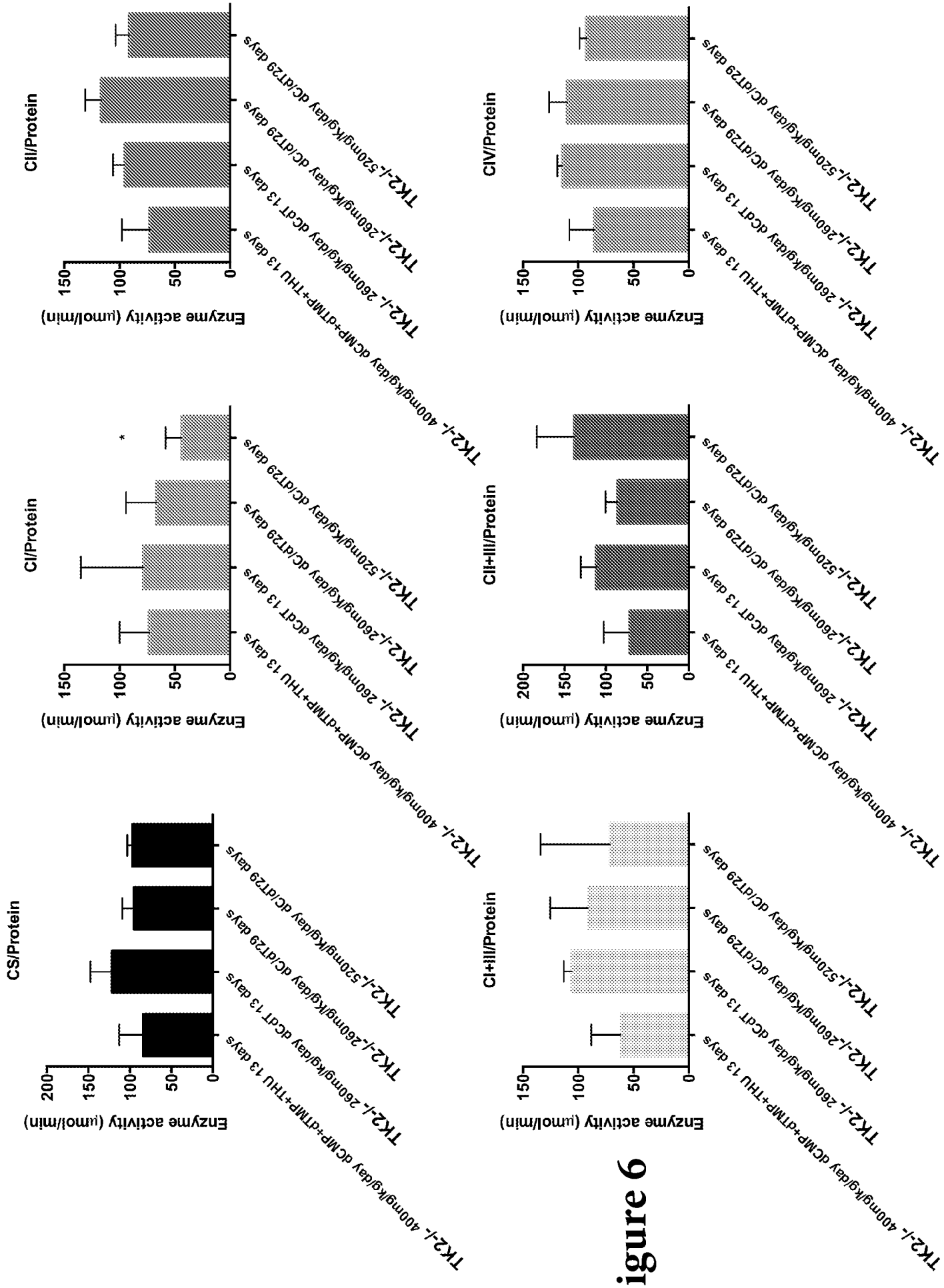
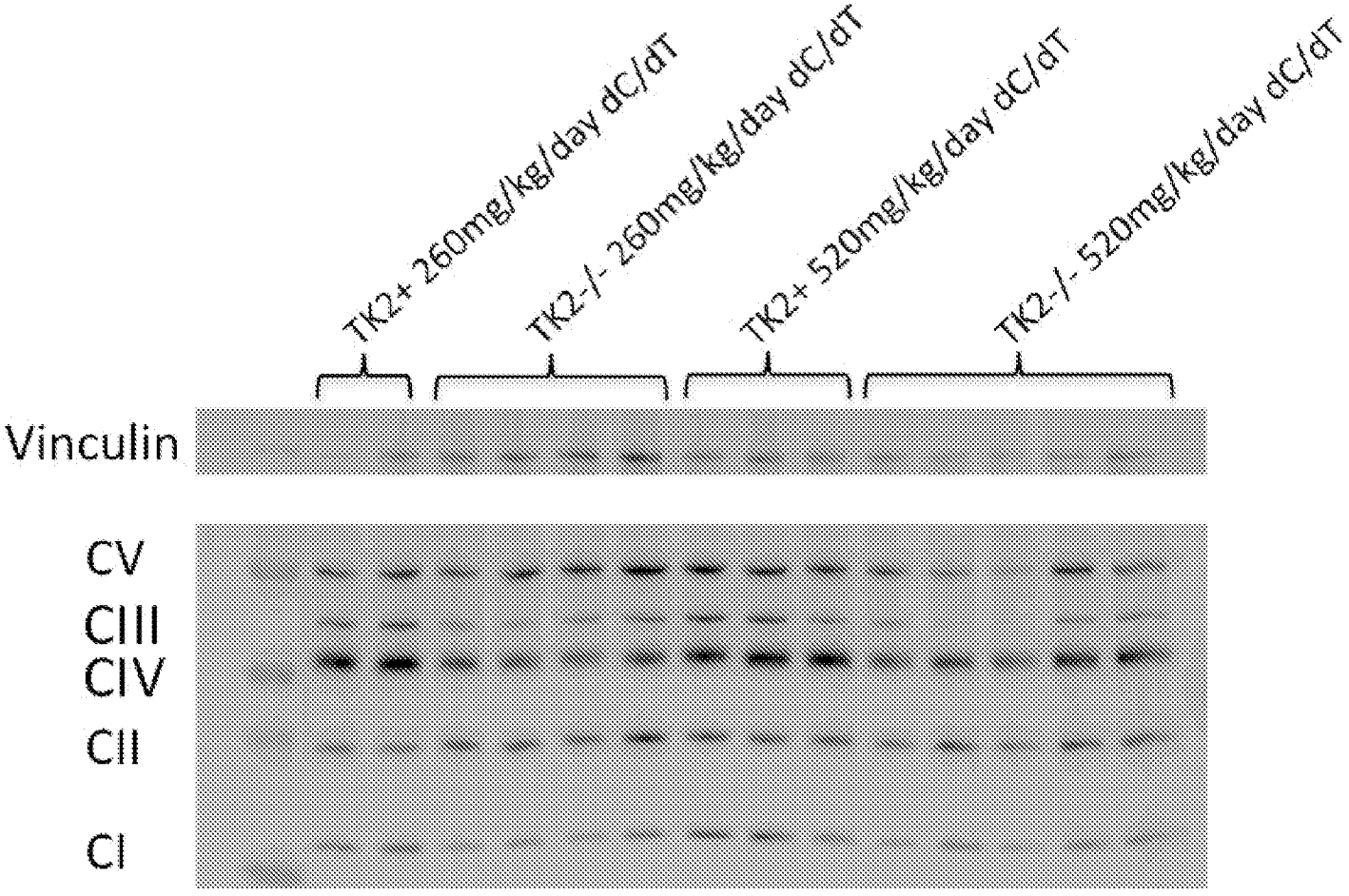

CS/Protein
Enzyme activity (μmol/min)
200
150
100
50
0
TK2-/- 400mg/kg/day dCMP+dTMP+THU 13 days
TK2-/- 260mg/kg/day dCdT 13 days
TK2-/- 520mg/kg/day dC/dT29 days
CI/Protein
Enzyme activity (μmol/min)
150
100
50
0
*
TK2-/- 400mg/kg/day dCMP+dTMP+THU 13 days
TK2-/- 260mg/kg/day dCdT 13 days
TK2-/- 520mg/kg/day dC/dT29 days
CII/Protein
Enzyme activity (μmol/min)
150
100
50
0
TK2-/- 400mg/kg/day dCMP+dTMP+THU 13 days
TK2-/- 260mg/kg/day dCdT 13 days
TK2-/- 520mg/kg/day dC/dT29 days
CIV/Protein
Enzyme activity (μmol/min)
150
100
50
0
TK2-/- 400mg/kg/day dCMP+dTMP+THU 13 days
TK2-/- 260mg/kg/day dCdT 13 days
TK2-/- 520mg/kg/day dC/dT29 days
CI+III/Protein
Enzyme activity (μmol/min)
200
150
100
50
0
TK2-/- 400mg/kg/day dCMP+dTMP+THU 13 days
TK2-/- 260mg/kg/day dCdT 13 days
TK2-/- 520mg/kg/day dC/dT29 days
CII+III/Protein
Enzyme activity (μmol/min)
150
100
50
0
TK2-/- 400mg/kg/day dCMP+dTMP+THU 13 days
TK2-/- 260mg/kg/day dCdT 13 days
TK2-/- 520mg/kg/day dC/dT29 days

Figure 6

Figure 7A



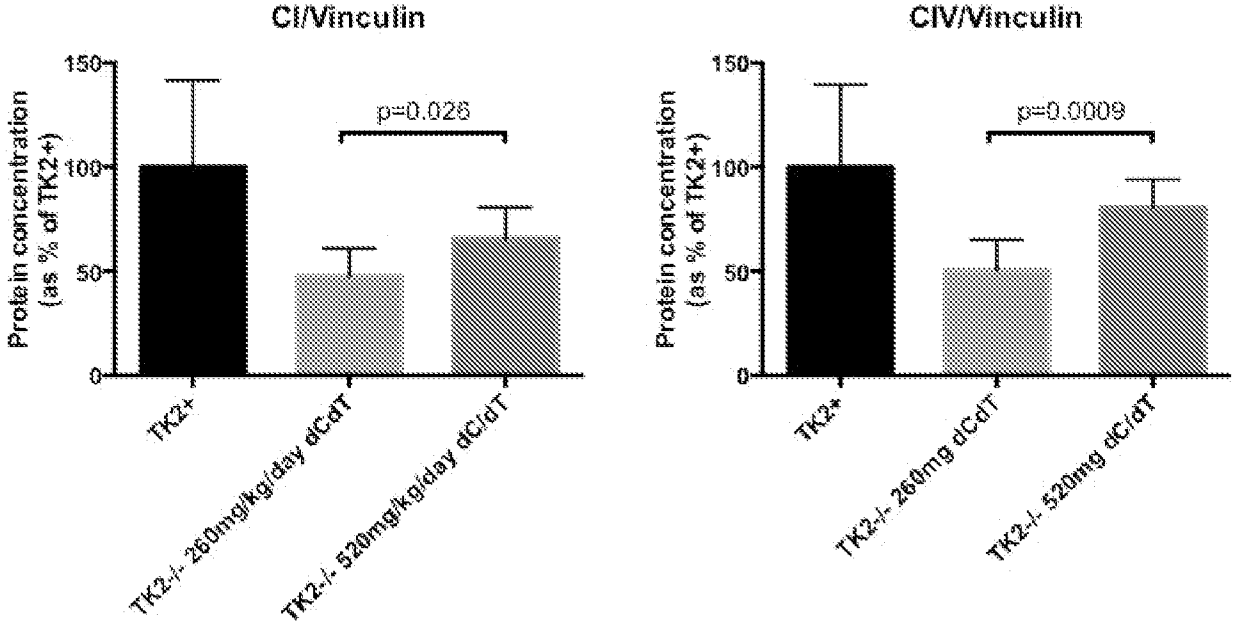


Figure 7B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 16/38110

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 31/7068, 31/7064, 31/7072; C07D 403/06 (2016.01)
CPC - A61K 31/7068, 31/7064, 31/7072; C12Q 1/6869; C07D 403/06
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8): A61K 31/7068, 31/7064, 31/7072; C07D 403/06 (2016.01)
CPC: A61K 31/7068, 31/7064, 31/7072; C12Q 1/6869; C07D 403/06

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
CPC: A61K 31/7068, 31/7064, 31/7072; C12Q 1/6869; C07D 403/06 (text search)
USPC: 514/49(text search)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Electronic data bases: PatBase; Google Patents; Google Scholar
Search terms: Mitochondrial DNA depletion syndrome (MDS), thymidine kinase 2 (TK2) deficiency, mutation TK2 gene, sequence TK2 gene, administer deoxypyrimidine (deoxythymidine or deoxycytidine),

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X — Y	GARONE et al. Deoxypyrimidine monophosphate bypass therapy for thymidine kinase 2 deficiency. EMBO Mol Med 26 June 2014 Vol 6 No 8 Pages 1016-1027. Especially abstract, pg 1016 col 2, pg 1017 col 1 para 2, para 2, pg 1024 col 1 para 1, pg 1024 col 1 para 5	1-4, 6, 7, 9, 10, 13-15, 22-25, 29-31 ---------- 26-28, 32-39
Y	MedChem Express. Tipiracil hydrochloride (online) 2014 [retrieved 26 October 2016] Available on the internet: <ULR: https://www.medchemexpress.com/Tipiracil-hydrochloride.html?gclid=Cj0KEQjwqMHABRDVl6_hqKGDyNIBEiQAN-O9hEkNyE4wS-bWnZLDeUwtN_gQ5bhgZHaeJwqW6uzbxVkaAsPf8P8HAQ>. Especially pg 1	26
Y	CAMARA et al. Feeding the deoxyribonucleoside salvage pathway to rescue mitochondrial DNA. Drug Discov Today October 2013 Vol 18 No 19-20 Pages 950-957. Especially Pg 955 col 2 para 3-4.	27, 28
Y	WO 2012/125848 A2 (Baylor College of Medicine) 30 September 2012 (30.09.2012). Especially para [0044], [0090]	32-39
Y	GARONE et al. Clinical and genetic spectrum of mitochondrial neurogastrointestinal encephalomyopathy. Brain November 2011 Vol 134 Pt 11 pages 3326-3332. Especially pg 3328 col 1 para 3	38, 39

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 October 2016	16 NOV 2016
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 16/38110

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
----Go to Extra Sheet for continuation------

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-4, 6, 7, 9, 10, 13-15, 22-39 limited to TK2 defects and therapeutical deoxycytidine

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

----continuation of Box III (Lack of Unity of Invention)----

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I+: Claims 1-39, drawn to a method of treating a disease or disorder characterized by unbalanced nucleotide pools in a subject comprising administering at least one deoxynucleoside. The method will be searched to the extent that the DNA depletion syndrome encompasses the defect in the first named gene, thymidine kinase 2 (TK2) (claims 3-4), and the first named therapeutical deoxynucleotide, deoxycytidine (dC) (claim 10). It is believed that claims 1-4, 6, 7, 9, 10, 13-15, 22-39 read on this first named invention and thus these claims will be searched without fee to the extent that they encompass TK2 defects and therapeutical deoxycytidine (dC). Additional depletion syndromes, mutant genes, and therapeutic deoxynucleosides will be searched upon payment of additional fees. Applicant must specify the claims that encompass any additional elected depletion syndromes, mutant genes, and therapeutic deoxynucleosides. Applicants must further indicate, if applicable, the claims which read on the first named invention if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined. An exemplary election would be deoxyguanosine kinase (dGK), DGUOK and deoxyguanosine (dG): (Claims 1, 2, 5-8, 11-24, 29-31).

The inventions listed as Group I+ do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features:

Among the inventions listed as Groups I+ are the specific nucleotide deficiencies and mutant genes recited therein. The inventions do not share a special technical feature, because no significant structural similarities can readily be ascertained among the nucleotide deficiencies or genes containing a mutation.

Common Technical Features:

Group I+ shares the common technical feature of independent claims 1 and 32.

However, said common technical features do not represent a contribution over the prior art and is obvious over the technical publication titled "Deoxypyrimidine monophosphate bypass therapy for thymidine kinase 2 deficiency" by Garone et al. (hereinafter "Garone") [published 26 June 2014 EMBO Mol Med Vol 6 No 8 Pages 1016-1027.], in view of WO 2012/125848 A2 to Baylor College of Medicine (hereinafter "Baylor).

As to claim 1, Garone teaches a method of treating a disease or disorder characterized by unbalanced nucleotide pools in a subject in need thereof comprising administering to the subject a therapeutically effective amount of a composition comprising at least one deoxynucleoside or a physiologically functional derivative thereof (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 phenotypically normal, but biochemically affected. Assessment of 13-day-old Tk2-/- mice treated with dCMP+dTMP 200 mg/kg/day each (Tk2-/-200dCMP/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)").

As to claim 32, Baylor teaches a method for the treatment of TK deficiency in a subject comprising:
a. obtaining a sample from the subject, said sample comprising nucleic acid (para [0090]);
b. performing sequence analysis of the TK2 gene in the nucleic acid of the subject (para [0090]; The Depletion Panel is a panel that may be performed using the deep sequencing technique described above. It contains 14 nuclear genes (C10ORF2, DGUOK, MPV17, OPA1, OP A3, POLG, POLG2, RRM2B, SLC25A4, SUCLA2, SUCLG1, SUCLG2, TK2 and TYMP) that are involved in the maintenance of mtDNA integrity and deoxynucleotide salvage pathway. These genes are analyzed by the "deep sequencing technique" by the application of Massive Parallel Sequencing (MPS) utility to the clinical diagnosis")
c. determining the subject has TK2 deficiency when a homozygous mutation or compound heterozygous mutations in the TK2 gene is detected (Para [0044]; For the identification of mutations in nuclear genes, coverage of greater than 30X sequence reads would usually be considered adequate for making homozygous or heterozygous base calls and the detection of small indel variations for research purposes"). Baylor does not teach d. administering a therapeutically effective amount of a composition comprising deoxycytidine (dC), deoxythymidine (dT), and mixtures thereof to the subject. However, Garone teaches administering a therapeutically effective amount of a composition comprising deoxycytidine (dC), deoxythymidine (dT), and mixtures thereof to the subject. It would have been obvious to combine claim limitations (a), (b), and (c), as taught by Baylor, with claim limitation (d), as taught by Garone because it would have enabled a combination of diagnosis and treatment in a subject suffering from TK deficiency.

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As the common technical features were known in the art at the time of the invention, they cannot be considered common special technical features that would otherwise unify the groups. The inventions lack unity with one another.

Therefore, Groups I+ lacks unity of invention under PCT Rule 13 because they do not share a same or corresponding special technical feature.

Note concerning claim 34. Claim 34 is written to depend from claim 3, is objected, because claim 3 does not have required antecedent "monitoring the subject". For the purposes of the International Search & Opinion, claim 34 is interpreted to depend from claim 32.

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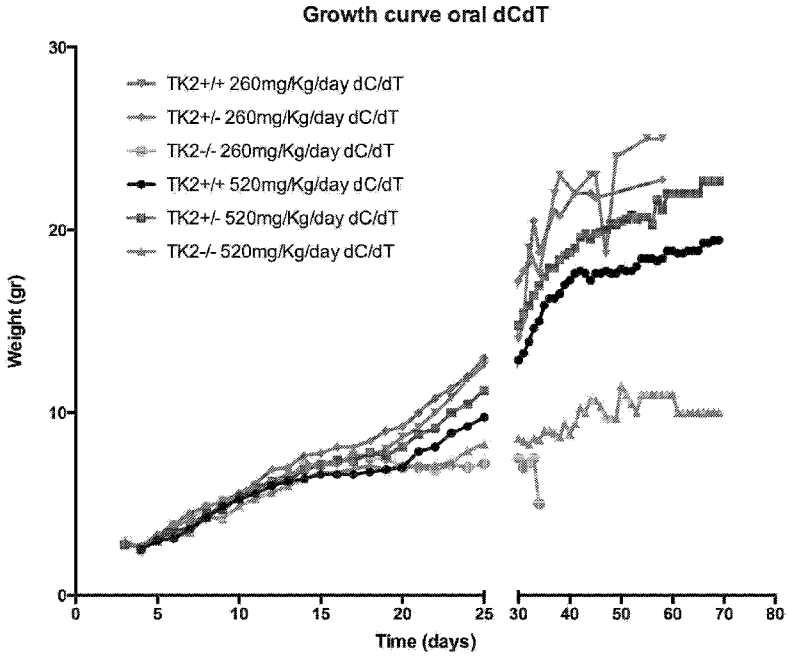

Figure 1

(57) Abstract: The invention relates generally to a pharmacological therapy for human genetic diseases, specifically those characterized by unbalance nucleotide pools, more specifically mitochondrial DNA depletion syndromes, and more specifically, thymidine kinase 2 (TK2) deficiency. The pharmacological therapy involves the administration of at least one deoxynucleoside, or mixtures thereof. For the treatment of TK2 deficiency, the pharmacological therapy involves the administration of either deoxythymidine (dT) or deoxycytidine (dC), or mixtures thereof. This administration of deoxynucleosides is applicable to other disorders of unbalanced nucleotide pools, especially those found in mitochondrial DNA depletion syndrome.

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NUCLEOTIDE POOLS INCLUDING MITOCHONDRIAL DNA DEPLETION SYNDROMES

GOVERNMENT SUPPORT

This invention was made with government support under HD080642 awarded by NIH. The government has certain rights in the invention.

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application claims priority to U.S. provisional patent application serial no. 62/180,194 filed June 17, 2015, which is hereby incorporated by reference.

FIELD OF THE INVENTION

The invention relates generally to a pharmacological therapy for a human genetic disease, specifically diseases characterized by unbalanced nucleotide pools, *e.g.*, mitochondrial DNA depletion syndromes, and more specifically, thymidine kinase 2 (TK2) deficiency. The pharmacological therapy involves the administration of at least one deoxynucleoside, or mixtures thereof. For the treatment of TK2 deficiency, the pharmacological therapy involves the administration of either deoxythymidine (dT) or deoxycytidine (dC), or mixtures thereof. This administration of one or more deoxynucleosides is applicable to other disorders of unbalanced nucleoside pools, especially those found in mitochondrial DNA depletion syndrome.

BACKGROUND OF THE INVENTION

Mitochondrial diseases are clinically heterogeneous diseases due to defects of the mitochondrial respiratory chain (RC) and oxidative phosphorylation, the biochemical pathways that convert energy in electrons into adenosine triphosphate (ATP). The respiratory chain is comprised of four multi-subunit enzymes (complexes I-IV) that transfer electrons to generate a proton gradient across the inner membrane of mitochondria and the flow of protons through complex V drives ATP synthesis (DiMauro and Schon 2003; DiMauro and Hirano 2005). Coenzyme Q_{10} (CoQ_{10}) is an essential molecule that shuttles electrons from complexes I and II to complex III. The respiratory chain is unique in eukaryotic, *e.g.*, mammalian, cells by virtue of being controlled by two genomes, mitochondrial DNA (mtDNA) and nuclear DNA (nDNA). As a consequence, mutations in either genome can

cause mitochondrial diseases. Most mitochondrial diseases affect multiple body organs and are typically fatal in childhood or early adult life. There are no proven effective treatments for mitochondrial diseases, only supportive therapies, such as the administration of CoQ_{10} and its analogs to enhance respiratory chain activity and to detoxify reactive oxygen species (ROS) that are toxic by-products of dysfunctional respiratory chain enzymes.

Mitochondrial DNA depletion syndrome (MDS), which is a subgroup of mitochondrial disease, is a frequent cause of severe childhood encephalomyopathy characterized molecularly by reduction of mitochondrial DNA (mtDNA) copy number in tissues and insufficient synthesis of mitochondrial RC complexes (Hirano, *et al.* 2001). Mutations in several nuclear genes have been identified as causes of infantile MDS, including: *TK2, DGUOK, POLG, POLG2, SCLA25A4, MPV17, RRM2B, SUCLA2, SUCLG1, TYMP, OPA1,* and *C10orf2 (PEO1).* (Bourdon, *et al.* 2007; Copeland 2008; Elpeleg, *et al.* 2005; Mandel, *et al.* 2001; Naviaux and Nguyen 2004; Ostergaard, *et al.* 2007; Saada, *et al.* 2003; Sarzi, *et al.* 2007; Spinazzola, *et al,* 2006). In addition, mutations in these nuclear genes can also cause multiple deletions of mtDNA with or without mtDNA depletion (Béhin, *et al.* 2012; Garone, *et al.* 2012; Longley, *et al.* 2006; Nishino, *et al.* 1999; Paradas, *et al.* 2012; Ronchi, *et al.* 2012; Spelbrink, *et al.* 2001; Tyynismaa, *et al.* 2009; Tyynismaa, *et al.* 2012; Van Goethem, *et al.* 2001).

One of these genes is *TK2,* which encodes thymidine kinase (TK2), a mitochondrial enzyme required for the phosphorylation of the pyrimidine nucleosides (thymidine and deoxycytidine) to generate deoxythymidine monophosphate (dTMP) and deoxycytidine monophosphate (dCMP) (Saada, *et al.* 2001). Mutations in *TK2* impair the mitochondrial nucleoside/nucleotide salvage pathways required for synthesis of deoxynucleotide triphosphate (dNTP), the building blocks for mDNA replication and repair.

TK2 deficiency was first described in 2001 by Saada and colleagues (Saada, *et a*l. 2001), in four affected children originating from four different families, who suffered from severe, devastating myopathy. After an uneventful early development, at ages 6-36 months the patients developed hyperCKemia, severe muscle hypotonia with subsequent loss of spontaneous activity. The disease was rapidly progressive and two patients were mechanically ventilated at 3 years, while two other patients were already dead by the time of the report.

After the first description, sixty additional patients have been reported in literature and at least twenty-six further patients have been diagnosed but not reported (Alston, *et al.* 2013; Bartesaghi, *et al.* 2010; Béhin, *et al.* 2012; Blakely, *et al.* 2008; Carrozzo, *et al.* 2003;

Chanprasert, *et al.* 2013; Collins, *et al.* 2009; Galbiati, *et al.* 2006; Gotz, *et al.* 2008; Leshinsky-Silver, *et al.* 2008; Lesko, *et al.* 2010; Mancuso, *et al.* 2002; Mancuso, *et al.* 2003; Marti, *et al.* 2010; Oskoui, *et al.* 2006; Paradas, *et al.* 2012; Roos, *et al.* 2014; Tulinius, *et al.* 2005; Tyynismaa, *et al.* 2012; Vilà, *et al.* 2003; Wang, *et al.* 2005), resulting in ninety patients, 53 males and 37 females.

The twenty-six patients recently diagnosed were identified through next-generation DNA sequencing. This large number of newly identified cases suggests that TK2 deficiency is an under diagnosed disorder.

TK2 deficiency manifests a wide clinical and molecular genetic spectrum with the majority of patients manifesting in early childhood with a devastating clinical course, while others have slowly progressive weakness over decades.

Treatment for TK2 deficiency, like most MDS and mitochondrial disorders, has been limited to supportive therapies. While the administration of deoxythymidine monophosphate (dTMP) and deoxycytidine monophosphate (dCMP) improved the conditions of both TK2 knock-in mutant mice and human patients with TK2 deficiency (US Application Serial No.15/082,207, which is incorporated herein in its entirety), there is still a need for therapeutic intervention for TK2 deficiency.

Additionally, there is a need for treatment for other forms of MDS and other diseases characterized by unbalanced nucleotide pools. For example, several mendelian disorders with mtDNA depletion or multiple deletions, or both are characterized by unbalanced deoxynucleotide triphosphate pools that lead to defects of mtDNA replication. One such disorder, *DGUOK* mutations impair the intramitochondrial enzyme deoxyguanosine kinase, which normally phosphorylates the deoxypurine nucleosides deoxguanosine and deoxycytidine to generate deoxguanosine monophosphate (dGMP) and deoxycytidine monophosphate (dCMP). Other nuclear genes that disrupt mitochondrial dNTP pools include *TYMP, RRM2B, SUCLA2, SUCLG1* and *MPV17*. Therapies that restore dNTP pool balance would be useful to treat these disorders as well.

SUMMARY OF THE INVENTION

In certain embodiments, the present invention relates to a method of treating a disease or disorder characterized by unbalanced nucleotide pools, comprising administering to a subject in need thereof a therapeutically effective amount of a composition comprising one or more deoxynucleosides.

Diseases or disorders characterized by unbalanced nucleotide pools that can be treated by the method of the current invention include, but are not limited to, those characterized by mutations in the following genes: *TK2; DGUOK; TYMP; RRM2B; SUCLA2; SUCLG1;* and *MPV17.*

In a preferred embodiment, the disorder is a mitochondrial DNA depletion syndrome (MDS). In a more preferred embodiment, the MDS includes disorders of a myopathic form characterized by mutations in *TK2*, an encephalomyopathic form characterized by mutations in *SUCLA2*, a neurogastrointestinal encephalopathic form characterized by mutations in *TYMP*, and a hepatopathic form characterized by mutations in *DGUOK*, *POLG*, and *MPV17.* In a most preferred embodiment, the disorder is a thymidine kinase 2 deficiency, characterized by mutation(s) in the *TK2* gene.

All mitochondrial DNA depletion syndromes can be treated with the method of the current invention which comprises administering deoxynucleosides. Examples of MDS that can be treated by the method of the current invention include but are not limited to, deficiencies in the: *DGUOK* gene, encoding deoxyguanosine kinase, dGK; *RRM2B* gene, encoding p53R2, the p53 inducible small subunit of ribonucleotide reductase, RNR; and *TYMP* gene, encoding thymidine phosphorylase, TP.

In a preferred embodiment, the deoxynucleoside is either deoxythymidine (dT) or deoxycytidine (dC) or mixtures thereof. Deoxyadenosine (dA) and deoxyguanosine (dG), alone or together, can also be used in the method of the invention. One deoxynucleoside (*i.e.*, dT, dC, dA, or dG) and mixtures of two or more of any of the four deoxynucleosides can be used in the method of the invention.

Preferred dosages of the deoxynucleoside(s) are between about 100 and about 1,000 mg/kg/day, more preferably between about 300 and about 800 mg/kg/day, and most preferably between about 250 and about 600 mg/kg/day. If the composition comprises a single deoxynucleoside, then the dosages are of the single deoxynucleoside. If the composition comprises more than one deoxynucleoside, the dosages can be of each deoxynucleoside or of the total deoxynucleosides in the composition.

Administration of the deoxynucleoside(s) can be once daily, twice daily, three times daily, four times daily, five times daily, up to six times daily, preferably at regular intervals.

Preferred methods of administration are oral, intrathecal, intravenous, and enteral.

Administration of the deoxynucleoside(s) should begin as soon as the disorder characterized by unbalanced nucleotide pools, *e.g.,* MDS, is suspected and continue

throughout the life of the patient. Test for the diagnosis of such disorders including TK2 deficiency are known in the art.

BRIEF DESCRIPTION OF THE DRAWINGS

For the purpose of illustrating the invention, there are depicted in drawings certain embodiments of the invention. However, the invention is not limited to the precise arrangements and instrumentalities of the embodiments depicted in the drawings.

Figure 1 depicts a growth curve of wild type ($Tk2^{+/+}$ and $Tk2^{+/-}$), and $Tk2^{-/-}$ mice treated with 260 mg/kg/day or 520 mg/kg/day of deoxycytidine (dC) and deoxythymidine (dT) from postnatal day 4. Each symbol represents the mean of weight at each time-point. N of each group is indicated in figure.

Figure 2 depicts the survival curve of wild type ($Tk2^{+/+}$), and $Tk2^{-/-}$ mice with the following treatments: $Tk2^{-/-milk}$ *vs* $Tk2^{-/-200\ mg/kg/day\ dCMP+dTMP}$, p=0.0013; $Tk2^{-/-milk}$ vs $Tk2^{-/-\ 260\ mg/kg/day\ dC+dT}$, p=0.0006; $Tk2^{-/-milk}$ vs $Tk2^{-/-\ 520\ mg/kg/day\ dC+dT}$, p<0.0001; $Tk2^{-/-\ 260\ mg/kg/day\ dC=dT}$ vs $Tk2^{-/-520mg/kg/day\ dCdT}$, p=0.0009, at postnatal day 4. N of each group indicated in figure. p-values determined by Mantel-Cox tests.

Figure 3 are graphs of the relative proportions of dNTPs in isolated mitochondria from brain and liver tissue of wild type ($Tk2^{+/+}$), and $Tk2^{-/-}$, untreated or treated with 200 mg/kg/day dCMP and dAMP, or 260 mg/kg/day or 520 mg/kg/day of deoxycytidine (dC) and deoxythymidine (dT) at ages postnatal day 13 (top panels) and postnatal day 29 (bottom panels).

Figure 4 are graphs showing the ratio of mtDNA/nDNA in brain, liver, intestine, and muscle in wild type Tk2 mice ($Tk2^{+/+}$) (left hand bar) as compared to $Tk2^{-/-}$ mice, untreated or treated with 260 mg/kg/day or 520 mg/kg/day of deoxycytidine (dC) and deoxythymidine (dT), at ages postnatal days 13 and 29. Data are represented as mean ± standard deviation (SD) of the percent of mtDNA copies relative to $Tk2^{+}$. p-values were assessed by Mann-Whitney tests. (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

Figure 5 are graphs depicting the results of HPLC measuring dT and uracil in plasma of untreated wild type ($Tk2^{+/+}$) mice, wild type ($Tk2^{+/+}$) mice treated with 260 mg/kg/day of deoxycytidine (dC) and deoxythymidine (dT), $Tk2^{-/-}$ mice treated with 260 mg/kg/day of deoxycytidine (dC) and deoxythymidine (dT), and $Tk2^{-/-}$ mice treated with 200 mg/kg/day of dCMP and dTMP, 30 minutes after treatment. Data are expressed as mean ± SD.

Figure 6 are graphs of levels of respiratory chain enzyme activities in $Tk2^{-/-}$ mice treated with 400 mg/kg/day of dCMP and dTMP and THU at 13 days postnatal, 260

mg/kg/day of deoxycytidine (dC) and deoxythymidine (dT) at 13 and 29 days postnatal, or 520 mg/kg/day of deoxycytidine (dC) and deoxythymidine (dT) 29 days postnatal. Data are represented as the percent of the RCE activities in $Tk2^{-/-}$ mouse tissues normalized to protein levels and relative to $Tk2^{+}$ for each treatment. p-values determined by Mann-Whitney tests. *$p<0.05$.

Figure 7A is an immunoblot of respiratory chain proteins in wild type mice treated with 260 mg/kg/day or 520 mg/kg/day of deoxycytidine (dC) and deoxythymidine (dT), and $Tk2^{-/-}$ mice treated with 260 mg/kg/day or 520 mg/kg/day of deoxycytidine (dC) and deoxythymidine (dT) at 29 days postnatal. Figure 7B are graphs showing the RCE levels normalized to complex II, represented as percent of the RCE levels in $TK2^{+/+}$ mice. p-values were assessed by Mann-Whitney tests.

Abbreviations:CS= citrate synthase; CI= NADH-dehydrogenase; CII= succinate dehydrogenase; CIII= cytochrome *c* reductase; CIV= cytochrome *c* oxidase (COX); CI+III= NADH-cytochrome *c* reductase; CII + III= succinate dehydrogenase-cytochrome *c* reductase.

DETAILED DESCRIPTION OF THE INVENTION

The current invention is based upon the surprising discovery that mitochondrial DNA depletion syndromes, including TK2 deficiency, can be treated with deoxynucleosides. As shown by the results herein, the administration of deoxynucleosides greatly improved the condition in both a mouse model of TK2 deficiency and human patients with TK2 deficiency.

Definitions

The terms used in this specification generally have their ordinary meanings in the art, within the context of this invention and the specific context where each term is used. Certain terms are discussed below, or elsewhere in the specification, to provide additional guidance to the practitioner in describing the methods of the invention and how to use them. Moreover, it will be appreciated that the same thing can be said in more than one way. Consequently, alternative language and synonyms may be used for any one or more of the terms discussed herein, nor is any special significance to be placed upon whether or not a term is elaborated or discussed herein. Synonyms for certain terms are provided. A recital of one or more synonyms does not exclude the use of the other synonyms. The use of examples anywhere in the specification, including examples of any terms discussed herein, is illustrative only, and in no way limits the scope and meaning of the invention or any exemplified term. Likewise, the invention is not limited to its preferred embodiments.

The term "subject" as used in this application means mammals. Mammals include canines, felines, rodents, bovine, equines, porcines, ovines, and primates. Thus, the invention can be used in veterinary medicine, *e.g.*, to treat companion animals, farm animals, laboratory animals in zoological parks, and animals in the wild. The invention is particularly desirable for human medical applications

The term "patient" as used in this application means a human subject. In some embodiments of the present invention, the "patient" is known or suspected of having a disease or disorder characterized by unbalanced nucleotide pools, mitochondrial disease, mitochondrial DNA depletion syndrome, or TK2 deficiency.

The phrase "therapeutically effective amount" is used herein to mean an amount sufficient to cause an improvement in a clinically significant condition in the subject, or delays or minimizes or mitigates one or more symptoms associated with the disease or disorder, or results in a desired beneficial change of physiology in the subject.

The terms "treat", "treatment", and the like refer to a means to slow down, relieve, ameliorate or alleviate at least one of the symptoms of the disease or disorder, or reverse the disease or disorder after its onset.

The terms "prevent", "prevention", and the like refer to acting prior to overt disease or disorder onset, to prevent the disease or disorder from developing or minimize the extent of the disease or disorder, or slow its course of development.

The term "in need thereof" would be a subject known or suspected of having or being at risk of having a disease or disorder characterized by unbalanced nucleotide pools, mitochondrial disease, mitochondrial DNA depletion syndrome, or TK2 deficiency.

The term "agent" as used herein means a substance that produces or is capable of producing an effect and would include, but is not limited to, chemicals, pharmaceuticals, biologics, small organic molecules, antibodies, nucleic acids, peptides, and proteins.

The term "deoxynucleoside" as used herein means deoxythymidine or dT, deoxycytidine or dC, deoxyadenosine or dA, and deoxyguanosine or dG. The full length name and common abbreviation for each will be used interchangeably. Such deoxynucleosides also include physiologically functional derivatives of the deoxynucleosides.

As used herein, the term "physiologically functional derivative" refers to a compound (*e.g,* a drug precursor) that is transformed *in vivo* to yield a deoxynucleoside. The transformation may occur by various mechanisms (*e.g.*, by metabolic or chemical processes), such as, for example, through hydrolysis in blood. Prodrugs are such derivatives, and a

discussion of the use of prodrugs is provided by T. Higuchi and W. Stella, "Pro-drugs as Novel Delivery Systems," Vol. 14 of the A.C.S. Symposium Series, and in Bioreversible Carriers in Drug Design, ed. Edward B. Roche, American Pharmaceutical Association and Pergamon Press, 1987.

As used herein "an adverse effect" is an unwanted reaction caused by the administration of a drug. In most cases, the administration of the deoxynucleosides caused no adverse effects. The most expected adverse effect would be a minor gastrointestinal intolerance.

The term "about" or "approximately" means within an acceptable error range for the particular value as determined by one of ordinary skill in the art, which will depend in part on how the value is measured or determined, *i.e.*, the limitations of the measurement system, *i.e.*, the degree of precision required for a particular purpose, such as a pharmaceutical formulation. For example, "about" can mean within 1 or more than 1 standard deviations, per the practice in the art. Alternatively, "about" can mean a range of up to 20%, preferably up to 10%, more preferably up to 5%, and more preferably still up to 1% of a given value. Alternatively, particularly with respect to biological systems or processes, the term can mean within an order of magnitude, preferably within 5-fold, and more preferably within 2-fold, of a value. Where particular values are described in the application and claims, unless otherwise stated, the term "about" meaning within an acceptable error range for the particular value should be assumed.

Administration of Deoxynucleosides for the Treatment of Mitochonodrial DNA Depletion Syndrome

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.

One form of MDS is thymidine kinase deficiency or TK2. TK2 encoded by the nuclear gene, *TK2*, is a mitochondrial matrix protein that phosphorylates thymidine and deoxycytidine nucleosides to generate deoxythymidine monophosphate (dTMP) and deoxycytidine monophosphate (dCMP), which in turn, are converted to deoxynucleotide triphosphates (dNTPs) required for mitochondrial DNA synthesis. As discussed in the background section, autosomal recessive *TK2* mutations cause devastating neuromuscular

weakness with severe depletion of mitochondrial DNA (mtDNA) in infants and children, as well as progressive external ophthalmoplegia with mtDNA multiple deletions in adults. Many patients cannot walk and require some type of mechanical ventilation and feeding tube. The central nervous system is variably involved in these disorders, with symptoms that include seizures, encephalopathy, cognitive impairment, and hearing loss. Less than 7% of patients live more than 42 years.

Based on clinical and molecular genetics findings of patients thus diagnosed, three disease presentations were identified: i) infantile-onset (≤1 year-old) myopathy with onset of weakness in the first year of life with severe mtDNA depletion and early mortality; ii) childhood-onset (>1-11 years-old) myopathy with severe mtDNA depletion; and iii) late-onset myopathy (≥12 years-old) with mild weakness at onset and slow progression to loss of ambulation, respiratory insufficiency, or both, often with chronic progressive external ophthalmoparesis in adolescence or adulthood in association with mtDNA multiple deletions, reduced mtDNA copy number, or both. See generally Garone, *et al.*, (2016) in preparation.

Attempts to study the pathogenesis and test therapies for TK2 deficiency using cultured fibroblasts from patients have been unsuccessful, because the replicating cells failed to manifest mtDNA depletion. In contrast, a homozygous Tk2 H126N knock-in mutant ($Tk2^{-/-}$) mouse model, manifests a phenotype that is strikingly similar to the human infantile encephalomyopathy caused by *TK2* mutations, characterized by onset at age 10 days with decreased ambulation, unstable gait, coarse tremor, growth retardation, and depletion of mitochondrial DNA (mtDNA) progressing rapidly to early death at age 14 to 16 days, which is a time period analogous to the human infantile-onset disease (Akman, *et al.* 2008; Dorado, *et al.* 2011).

The studies set forth herein with Tk2 knock-in mice have shown the administration of oral dC/dT prolonged delayed the onset of clinical symptoms of TK2 deficiency and prolonged the lives of the mice by two- to three-fold (Example 2).

Additional experiments showed tissue-specific effects. Measurement of the dNTP pool levels in mitochondria extracts showed that dCTP was rescued in brain and dTTP was rescued in liver (Example 3). Measurement of mtDNA depletion showed both dCMP+dTMP and dC+dT therapies rescued the mtDNA copy number in liver, muscle and tissue (Example 4). It was previously speculated that formation of the blood brain barrier might be compromising the treatment bioavailability in brain. Nevertheless, HPLC measurements showed that catalytic products of these compounds were found in higher concentrations after both nucleotides monophosphates and deoxynucleosides treatment, suggesting that they are

capable of crossing the blood brain barrier. mtDNA depletion measurements also showed a completely rescue of mtDNA copy number in intestine.

Thus, the experiments set forth herein using the mouse model of Tk2 deficiency show the administration of deoxynucleosides to be effective and safe for the treatment of the disease. Additionally, as shown in Example 5, the administration of dT and dC greatly improved the symptoms of TK2 deficiency in patients.

Thus, the present invention includes the administration of at least one deoxynucleoside to a patient in need thereof. In one embodiment, the present invention includes the administration of at least one deoxpyrimidine. In a further embodiment, the deoxypyrimidine is chosen from dC, dT and mixtures thereof. In yet another embodiment, the present invention includes the administration of at least one deoxypurine. In a further embodiment, the deoxypurine is chosen from dA, dG, and mixtures thereof.

Patients who would benefit from the administration of deoxynucleosides would be those diagnosed with TK2 deficiency. In these patients, at least one deoxypyrimidine, dC or dT, or mixtures thereof would be administered.

A parallel defect of deoxyguanosine kinase (dGK), due to autosomal recessive mutations in *DGUOK* with deficiencies in dGMP and dAMP, causes mtDNA depletion typically manifesting as early childhood-onset hepatocerebral disease (Mandel, *et al.* 2001). These patients would benefit from the administration of at least one deoxypurine, dG or dA, or mixtures thereof.

Other forms of MDS as well as other disorders related to unbalanced nucleotide pools can be treated by the administration of specific deoxynucleosides, *i.e.*, dA, dG, dC, or dT, or mixtures thereof. These disorders would include but are not limited to deficiencies related to *RRM2B* (encoding p53R2, the p53 inducible small subunit of ribonucleotide reductase, RNR) and mutations in *TYMP* (encoding thymidine phosphorylase, TP) which cause mitochondrial neurogastrointestinal encephalomyopathy (MNGIE). Additional nuclear genes that disrupt mitochondrial dNTP pools include but are not limited to *SUCLA2, SUCLG1* and *MPV17.* Disorders related to these genes can also be treated by the administration of one or more deoxynucleosides.

Additionally, as the mechanisms of other forms of MDS and other disorders become elucidated, the proper deoxynucleoside(s) for treatment can be determined by the skilled practitioner.

Patients that exhibit the phenotype discussed above for TK2 deficiency including the most typical presentation of progressive muscle disease characterized by generalized

hypotonia, proximal muscle weakness, loss of previously acquired motor skills, poor feeding, and respiratory difficulties, can be tested to definitively diagnose the disease.

If the clinical presentation is highly suspicious for mtDNA depletion syndrome, molecular genetic testing using a panel of genes known to cause mtDNA depletion syndrome should be performed (Chanprasert, *et al.* 2012). The *TK2* gene is the only gene in which mutations are known to cause TK2-related mitochondrial DNA depletion syndrome. This testing can include a sequence analysis of the entire coding and exon/intron junction regions of *TK2* for sequence variants and deletion/duplication. If compound heterozygous or homozygous deleterious mutations are identified in the sequence analysis, the diagnosis of TK2 deficiency is confirmed, and thus, the subject would benefit from the deoxynucleoside therapy. If sequence analysis does not identify two compound heterozygous or homozygous deleterious mutations, deletion/duplication analysis should be considered to determine and/or confirm a TK2 deficiency diagnosis.

Further tests to determine and/or confirm a TK2 deficiency diagnosis may include testing serum creatine kinase (CK) concentration, electromyography, histopathology on skeletal muscle, mitochondrial DNA (mtDNA) content (copy number), and electron transport chain (ETC) activity in skeletal muscle. If one or more of the following is found in these tests, the TK2 deficiency is determined and/or confirmed. Elevated CK concentration as compared to healthy controls can indicate TK2 deficiency. A skeletal muscle biopsy can be performed, and then a mtDNA content analysis in skeletal muscle performed. If the skeletal muscle biopsy shows prominent variance in fiber size, variable sarcoplasmic vacuoles, variable increased connective tissue, and ragged red fibers as well as increased succinate dehydrogenase (SDH) activity and low to absent cytochrome c oxidase (COX) activity, and mtDNA copy number is severely reduced (typically less than 20% of age- and tissue-matched healthy controls), a diagnosis of TK2 deficiency can be determined and/or confirmed (Chanprasert, *et al.* 2012).

Additionally, TK2 deficiency is inherited in an autosomal recessive manner. Thus, a sibling of an affected patient can be tested as early as possible after birth to diagnose the disease.

In all of these examples, deoxynucleoside therapy should be started as soon as possible after a diagnosis of TK2 deficiency.

Pharmaceutical Compositions, Methods of Administration, and Dosing

The present invention encompasses the administration of deoxynucleosides, more specifically one or more deoxynucleosides.

Most preferred methods of administration are oral, intrathecal and parental including intravenous. The deoxynucleosides must be in the appropriate form for administration of choice.

Deoxynucleosides are easily dissolved in liquid are easily dissolved in liquid (such as water, formula or milk) whereas the free acid form does not readily dissolve in liquid.

Such pharmaceutical compositions comprising one of more deoxynucleosides for administration may comprise a therapeutically effective amount of the deoxynucleosides and a pharmaceutically acceptable carrier. The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce an allergic or similar untoward reaction, such as gastric upset, dizziness and the like, when administered to a human, and approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. “Carrier” refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as saline solutions in water and oils, including those of petroleum, animal, vegetable, or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil, and the like. A saline solution is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol, and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents.

Oral administration is a preferred method of administration. The deoxynucleosides can be added to any form of liquid a patient would consume including but not limited to, milk, both cow’s and human breast, infant formula, and water.

Additionally, pharmaceutical compositions adapted for oral administration may be capsules, tablets, powders, granules, solutions, syrups, suspensions (in non-aqueous or aqueous liquids), or emulsions. Tablets or hard gelatin capsules may comprise lactose, starch or derivatives thereof, magnesium stearate, sodium saccharine, cellulose, magnesium

carbonate, stearic acid or salts thereof. Soft gelatin capsules may comprise vegetable oils, waxes, fats, semi-solid, or liquid polyols. Solutions and syrups may comprise water, polyols, and sugars. An active agent intended for oral administration may be coated with or admixed with a material that delays disintegration and/or absorption of the active agent in the gastrointestinal tract. Thus, the sustained release may be achieved over many hours and if necessary, the active agent can be protected from degradation within the stomach. Pharmaceutical compositions for oral administration may be formulated to facilitate release of an active agent at a particular gastrointestinal location due to specific pH or enzymatic conditions.

In order to overcome any issue of the deoxynucleosides crossing the blood/brain barrier, intrathecal administration is a further preferred form of administration (Galbiati, *et al.* 2006; Gotz, *et al.* 2008). Intrathecal administration involves injection of the drug into the spinal canal, more specifically the subarachnoid space such that it reaches the cerebrospinal fluid. This method is commonly used for spinal anesthesia, chemotherapy, and pain medication. Intrathecal administration can be performed by lumbar puncture (bolus injection) or by a port-catheter system (bolus or infusion). The catheter is most commonly inserted between the laminae of the lumbar vertebrae and the tip is threaded up the thecal space to the desired level (generally L3-L4). Intrathecal formulations most commonly use water, and saline as excipients but EDTA and lipids have been used as well.

A further preferred form of administration is parenteral including intravenous administration. Pharmaceutical compositions adapted for parenteral administration, including intravenous administration, include aqueous and non-aqueous sterile injectable solutions or suspensions, which may contain anti-oxidants, buffers, bacteriostats, and solutes that render the compositions substantially isotonic with the blood of the subject. Other components which may be present in such compositions include water, alcohols, polyols, glycerine, and vegetable oils. Compositions adapted for parental administration may be presented in unit-dose or multi-dose containers, such as sealed ampules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of a sterile carrier, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules, and tablets. Suitable vehicles that can be used to provide parenteral dosage forms of the invention are well known to those skilled in the art. Examples include: Water for Injection USP; aqueous vehicles such as Sodium Chloride Injection, Ringer's Injection, Dextrose Injection, Dextrose and Sodium Chloride Injection, and Lactated Ringer's Injection; water-miscible vehicles such as ethyl alcohol, polyethylene

glycol, and polypropylene glycol; and non-aqueous vehicles such as corn oil, cottonseed oil, peanut oil, sesame oil, ethyl oleate, isopropyl myristate, and benzyl benzoate.

Additionally, since some patients may be receiving enteral nutrition by the time the deoxynucleoside treatment begins, the dNs can be administered through a gastronomy feeding tube or other enteral nutrition means.

Further methods of administration include mucosal, such as nasal, sublingual, vaginal, buccal, or rectal; or transdermal administration to a subject.

Pharmaceutical compositions adapted for nasal and pulmonary administration may comprise solid carriers such as powders, which can be administered by rapid inhalation through the nose. Compositions for nasal administration may comprise liquid carriers, such as sprays or drops. Alternatively, inhalation directly through into the lungs may be accomplished by inhalation deeply or installation through a mouthpiece. These compositions may comprise aqueous or oil solutions of the active ingredient. Compositions for inhalation may be supplied in specially adapted devices including, but not limited to, pressurized aerosols, nebulizers or insufflators, which can be constructed so as to provide predetermined dosages of the active ingredient.

Pharmaceutical compositions adapted for rectal administration may be provided as suppositories or enemas. Pharmaceutical compositions adapted for vaginal administration may be provided as pessaries, tampons, creams, gels, pastes, foams or spray formulations.

Pharmaceutical compositions adapted for transdermal administration may be provided as discrete patches intended to remain in intimate contact with the epidermis of the recipient over a prolonged period of time.

The deoxynucleoside therapy comprises the administration of one or more deoxynucleosides chosen from the group consisting of deoxythymidine (dT), deoxycytidine (dC), deoxyadenosine (dA) and deoxyguanosine (dG).

A skilled practitioner can determine which deoxynucleosides are beneficial based upon the deficiency. It is also within the skill of the art for the practitioner to determine if mixtures of the deoxynucleosides should be administered and in what ratio. If two deoxynucleosides are to be administered, they can be in a ratio of 50/50 of each deoxynucleoside, *e.g.*, dC and dT, or in ratios of about 5/95, 10/90, 15/85, 20/80, 25/75, 30/70, 35/65, 40/60, 45/55, 55/45, 60/40, 65/35, 70/30, 75/25, 80/20, 85/15, 90/10, and 95/5.

By way of example, dT and dC are administered in mixture of equal amounts for TK2 deficiency.

Selection of a therapeutically effective dose will be determined by the skilled artisan considering several factors, which will be known to one of ordinary skill in the art. Such factors include the particular form of the deoxynucleoside, and its pharmacokinetic parameters such as bioavailability, metabolism, and half-life, which will have been established during the usual development procedures typically employed in obtaining regulatory approval for a pharmaceutical compound. Further factors in considering the dose include the condition or disease to be treated or the benefit to be achieved in a normal individual, the body mass of the patient, the route of administration, whether the administration is acute or chronic, concomitant medications, and other factors well known to affect the efficacy of administered pharmaceutical agents. Thus, the precise dose should be decided according to the judgment of the person of skill in the art, and each patient's circumstances, and according to standard clinical techniques.

A preferred dose ranges from about 100 mg/kg/day to about 1,000 mg/kg/day. A further preferred dose ranges from about 200 mg/kg/day to about 800 mg/kg/day. A further preferred dose ranges from about 250 mg/kg/day to about 400 mg/kg/day. These dosage amounts are of individual deoxynucleosides or of a composition with a mixture of more than one deoxynucleosides, *e.g.*, dT and dC. For example, a dose can comprise 400 mg/kg/day of dT alone. In a further example, a dose can comprise a mixture of 200 mg/kg/day of dT and 200 mg/kg/day of dC. In a further example, a dose can comprise 400 mg/kg/day of a mixture of dT and dC.

Administration of the deoxynucleosides can be once a day, twice a day, three times a day, four times a day, five times a day, up to six times a day, preferably at regular intervals. For example, when the deoxynucleosides are administered four times daily, doses would be at 8:00 AM, 12:00 PM, 4:00 PM, and 8:00 PM.

Doses can also be lowered if being administered intravenously or intrathecally. Preferred dose ranges for such administration are from about 50 mg/kg/day to about 500 mg/kg/day.

As shown in Example 5, doses can be adjusted to optimize the effects in the subject. For example, the deoxynucleosides can be administered at 100 mg/kg/day to start, and then increased over time to 200 mg/kg/day, to 400 mg/kg/day, to 800 mg/kg/day, up to 1000 mg/kg/day, depending upon the subject's response and tolerability.

A subject can be monitored for improvement of their condition prior to increasing the dosage. A subject's response to the therapeutic administration of the deoxynucleosides can be monitored by observing a subject's muscle strength and control, and mobility as well as

changes in height and weight. If one or more of these parameters increase after the administration, the treatment can be continued. If one or more of these parameters stays the same or decreases, the dosage of the deoxynucleosides can be increased.

As shown in the Examples, the deoxynucleosides are well tolerated. Any observed adverse effects were minor and were mostly diarrhea, abdominal bloating and other gastrointestinal manifestations. A subject can also be monitored for any adverse effects, such as gastrointestinal intolerance, *e.g.,* diarrhea. If one or more adverse effects are observed after administration, then the dosage can be decreased. If no such adverse effects are observed, then the dosage can be increased. Additionally, once a dosage is decreased due to the observation of an adverse effect, and the adverse effect is no longer observed, the dosage can be increased.

The deoxynucleosides can also be co-administered with other agents. Such agents would include therapeutic agents for treating the symptoms of the particular form of MDS. In particular, for TK2 deficiency, the dT and dC can be co-administered with an inhibitor of ubiquitous nucleoside catabolic enzymes, including but not limited to enzyme inhibitors such as tetrahydrouridine (inhibitor of cytidine deaminase) and immucillin H (inhibitor of purine nucleoside phosphorylase) and tipiracil (inhibitor of thymidine phosphorylase). Such inhibitors are known and used in the treatment of some cancers.

EXAMPLES

The present invention may be better understood by reference to the following non-limiting examples, which are presented in order to more fully illustrate the preferred embodiments of the invention. They should in no way be construed to limit the broad scope of the invention.

Example 1- Materials and Methods

Mouse Model of TK2 Deficiency

A homozygous *Tk2* H126N knock-in mutant ($Tk2^{-/-}$) mouse that manifests a phenotype strikingly similar to the human infantile encephalomyopathy has been previously reported (Akman, *et al.* 2008). Between postnatal day 10 and 13, $Tk2^{-/-}$ mice rapidly develop fatal encephalomyopathy characterized by decreased ambulation, unstable gait, coarse tremor, growth retardation, and rapid progression to early death at age 14 to 16 days. Molecular and biochemical analyses of the mouse model demonstrated that the pathogenesis of the disease is due to loss of enzyme activity and ensuing dNTP pool imbalances with

decreased dTTP levels in brain and both dTTP and dCTP levels in liver, which, in turn, produces mtDNA depletion and defects of respiratory chain enzymes containing mtDNA-encoded subunits, most prominently in the brain and spinal cord.

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-hour light, 12-hour 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 eosin (H&E) for morphological study or processed for immunostaining studies with GFAP, COX I, or complex I subunit as detailed described in the supplemental procedures. Both heterozygous and homozygous wild type mice were considered as control group (*Tk2*$^{+}$) since no clinical and biochemical difference were previously described (Akman, *et al.* 2008; Dorado, *et al.* 2011).

Treatment administration and experimental plan

Deoxycytidine (dC) and deoxythymidine (dT) 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 control wild-type (*T*k2^{+}) using 2 doses, 260 mg/kg/day and 520 mg/kg/day, from post-natal day 4 to 29 days. At age 21 days, mice were separated from the mother and the treatment was continued by administration of dC and dT in drinking water using equimolar doses respectively of 1.6mM and 3.2mM. A negative control group of untreated *Tk2* mutant and control wild-type mice were weighed 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 (Akman, *et al.* 2008).

To define the degree of safety and efficacy of dT/dC, 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 were

compared. General 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 minutes at 4°C, followed by three centrifugations at 13,000g for 2 minutes at 4°C. Supernatant was precipitated with 60% methanol, kept 2 hours at -80°C, boiled 3 minutes, stored at -80°C (from 1 hour to overnight) and centrifuged at 20,800g for 10 minutes 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 (Ferraro, *et al.* 2010; Marti, *et al.* 2012a). 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 3H-dTTP or 3H-dATP (Moravek Biochemicals), 0.25 µM specific oligonucleotide, 40 mM Tris–HCl, pH 7.5, 10 mM MgCl2, 5mM DTT]. After 60 minutes at 48°C, 18 ml of reaction were spotted on Whatman DE81 filters, air dried and washed three times for 10 minutes with 5% Na2HPO4, once in distilled water and once in absolute ethanol. The retained radioactivity was determined by scintillation counting.

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 four buffers: eluent A (20 mM potassium phosphate, pH 5.6), eluent B (water) and eluent C (methanol). Samples were eluted over 60 minutes 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 eluates 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 deoxythymidine, deoxyuridine, uracil, and thymine peaks for each sample, a second aliquot was 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, nDNA) (Applied Biosystems, Invitrogen, Foster City, CA, USA) as described as described using ddCt method in a Step One Plus Real Time PCR 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 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). 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 (DiMauro, *et al.* 1987).

Statistical methods

Data are expressed as the mean ± SD of at least 3 experiments per group. Gehan-Breslow-Wilcoxon test was used to compare the survival proportion of each group of mice. A p-value of <0.05 was considered to be statistically significant.

Example 2- The Administration of dC/dT to $Tk2^{-/-}$ Mice Delayed the Clinical Onset of TK2 Deficiency and Increased Survival

A dose of 260 and 520 mg/kg/day each of deoxynucleosides (dC/dT) were administered to the $Tk2^{-/-}$ mice. These doses of deoxynucleosides were the molar equivalent of 400 and 800 mg/kg/day of dCMP+dTMP respectively.

Mice treated with oral dC+dT (260 or 520mg/kg/day from age 4 days) appeared normal until postnatal day 21 (Figure 1). After age 21 days, mutant mice treated with 260 mg/kg/day dose ($Tk2^{-/-\ 260\ mg/kg/day\ dC/dT}$) stopped gaining weight and developed mild head tremor and weakness that led to death at postnatal day 31±4.3 (Figure 2).

Mutant mice treated with the 520 mg/kg/day dC+dT ($Tk2^{-/-\ 520\ mg/Kg/day\ dC/dT}$) continued to gain weight for one additional week, but subsequently manifested deterioration similar to $Tk2^{-/-260\ mg/Kg/day\ dC/dT}$, and died at postnatal day 43±10. These results are comparable to those showed by $Tk2^{-/-}$ mice treated with 200 or 400mg/kg/day of oral dCMP/dTMP treatment. $Tk2^{+\ 260\ mg/kg/day\ dC/dT}$ and$Tk2^{+\ 520\ mg/kg/day\ dC/dT}$ were followed until postnatal day 60. No side effects were observed.

As shown, the lifespan of the treated $Tk2^{-/-}$ was significantly increased. Untreated $Tk2^{-/-}$ mice showed a mean lifespan of 13 days, while treated mice survived a mean of 31 and 40 days with the 260 and 520 mg/kg/day dose, respectively (Figure 2). Interestingly, one of the mice survived to postnatal day 56, which has been the longest lifespan for the Tk2 knock-in mouse model to date.

Example 3- Oral dC/dT Ameliorates Molecular Abnormalities in Brain and Liver

Measurement of dNTPs in mitochondrial extract showed that both $Tk2^{-/-\ 260\ mg/Kg/day\ dC/dT}$ and $Tk2^{-/-\ 520\ mg/Kg/day\ dC/dT}$ did not fully correct mitochondrial dNTP pool imbalances at postnatal day 13 and manifested variable effects in tissues with a completed rescue of dCTP deficits in brain, while dTTP was corrected in the liver. In contrast, deficiencies of dTTP in brain and dCTP in liver remained severe despite deoxynucleoside supplementation (Figure 3).

In $Tk2^{-/-\ 260\ mg/Kg/day\ dC/dT}$ and $Tk2^{-/-\ 520\ mg/Kg/day\ dC/dT}$ mice at postnatal day 13, the treatment prevented mtDNA depletion in heart, liver, kidney, intestine and muscle (Figure 4). In contrast, mtDNA copy number was only partially ameliorated in brain at postnatal day 13 in a dose-dependent manner with mtDNA/nDNA ratios relative to control brain reaching 39% with 260 mg/kg/day of dC+dT and 52% with 520 mg/kg/day. Measurements of the bases dT and uracil in brain by HPLC showed higher levels in animals treated with dC+dT or with dCMP+dTMP (Figure 5), further indicating that both deoxynucleosides and deoxynucleoside monophosphates cross the blood brain barrier. At postnatal day 29, mtDNA depletion was partially rescued by 260 and 520 mg/kg/day of dC+dT therapy in heart (40 and 35%), liver (46 and 45%), kidney (38 and 42%) and muscle (24 and 35%), but strikingly was fully rescued in intestine (82 and 84%) (Figure 4).

Example 4- Oral dC/dT Ameliorates Biochemical Abnormalities in Brain

Respiratory chain enzyme (RCE) activities and protein levels were completely rescued in brain of $TK2^{-/-\ 260\ mg/Kg/day\ dC/dT}$ at postnatal day 13 (Figure 6). RCE activities were also restored at postnatal day 29, and only a slight decrease of complex I activity could be observed in $TK2^{-/-52\ 0mg/Kg/day\ dC/dT}$ (Figure 6). RCE protein levels in brain were partially restored at postnatal day 29 with higher levels in $TK2^{-/-520\ mg/Kg/day\ dC/dT}$ than in $TK2^{-/-260\ mg/Kg/day\ dC/dT}$ (Figure 7). These differences in protein levels were consistent with the differences in mtDNA depletion in brains of treated mutant mice at postnatal day 29, and likely accounted for the prolonged survival observed with the higher dose.

Example 5- Administration of dC/dT in Patients with TK2 Deficiency Was Efficacious

Symptoms, dosages, and outcomes of patients with TK2 deficiency who have received deoxynucleoside therapy under the supervision and control of the inventors are summarized below.

Patient 1

This patient was born in the United States in February 2011. His symptoms manifested at 12 months with hypotonic and a floppy head. He has never walked. He also has respiratory muscle weakness and was put on mechanical ventilation at 19 months, of which he is still on 24 hours/day. He has also been on a feeding tube since 19 months.

He was previously on 100 mg/kg/day and then 200 mg/kg/day of dCMP and dTMP. On this therapy, he was able to grip small objects and his weight increased from 10.4 kg to 19.5 kg.

In October of 2015, he began on 260 mg/kg/day of dC and dT which was increased to 340 mg/kg/day of dC and dT. After two months, he was moving his hands and head better, able to stand 5 minutes with support of a person, starting to cough, and his heart rate was slower (down from 140-170 bpm during day, to 100-120 bpm during day).

On March 23, 2016, the dose was increased to 400 mg/kg/day of dC and dT. After 6 weeks on this therapy, he showed further improvements: he was able to sit in a chair about 5 hours/day; stood in a “Stander” for 1.5 hours; about to grab and hold small stuffed animals; pressed computer buttons; untied his diapers and aimed his penis to wet the person changing the diaper; and held his knees flexed for a few seconds.

The only adverse effect seen during the treatment was diarrhea.

Patient 2

This patient was born in Spain in 1987. He began showing symptoms at 3 years of age including proximal muscle weakness. He lost the ability to walk at age 13 and was ventilated 24 hours a day. He was previously taking dAMP and dCMP at 200 mg/kg/day and showed a weight increase and a decrease of 24 to 22 hours a day on ventilation.

He has been on deoxynucleoside therapy since June of 2015 at 400 mg/kg/day dC and dT, and has shown improvement in muscle strength, his weight and ventilation have stabilized, and he is enjoying a better quality of life.

The only adverse effects seen during the treatment was diarrhea and hair loss.

Patient 3

This patient was born in Spain in 1985. His symptoms began at 6 years old with facial, proximal, and axial muscle weakness. He started 200 mg/kg/day of dT and dC in June of 2015 and to date, his condition has improved with improvements in 6 minute walk test, time to get up and go, and climb up and down 4 steps.

The only adverse effect seen during the treatment was diarrhea.

Patient 4

This patient was born in Spain in February 2009. His symptoms manifested at six months with failure to thrive. He started on 230 mg/kg/ day of dC and dT in July of 2015. By January of 2016, he showed improvement in his condition and was eating better.

There were no observed adverse effects.

Patient 5

This patient was born in Spain in 1957 and began to have symptoms at 50 years old of orthopnea, and diaphragmatic weakness. He is on BiPAP at night. He started on 200 mg/kg/day of dC and dT in November of 2015.

There were no observed adverse effects.

Patient 6

This patient was born in Spain in October 2011, and starting showing symptoms at 15 months, including hypotonia and weakness. He lost ambulation at 22 months, and has respiratory muscle weakness. He started mechanical ventilation at 16 months and is currently on BiPAP twelve hours a day. He was previously on dCMP and dAMP at 100 mg/kg/day that was increased to 400 mg/kg/day. His strength as shown by Egen Klassification scale improved (28/30 to 13/30) and his weight increased from 9.8 kg to 12.3 kg.

He began deoxynucleoside therapy in April 2015 at 400 mg/kg/day of dC and dT. In October of 2015, his change in Egen Klassification scale went from 13/30 to 11/30 and his weight increased to 16.5 kg from 12.3 kg.

There were no observed adverse effects.

Patient 7

This patient was born in Spain in November of 2012. He started showing symptoms at 17 months including weakness and hypotonia. He lost ambulation at 22 months and started mechanical ventilation at 29 months. He was previously on dCMP and dAMP at 100 mg/kg/day that was increased to 400 mg/kg/day. His strength as shown by Egen Klassification scale improved (30/30 to 24/30) and his weight increased from 11 kg to 15.7 kg.

He started deoxynucleoside therapy in April of 2015 with a dose of 400 mg/kg/day dT and dC. In November of 2015, his change in Egen Klassification scale went from 24/30 to 19/30 and his weight increased to 17 kg from 15.7 kg.

There were no observed adverse effects.

Patient 8

This patient was born in Chile in September of 1989 and started showing symptoms at 11 months with frequent falls and progressive gait impairment. She lost the ability to walk alone at about 4 years of age. She had been on nucleotide therapy previously and showed improvement in her mobility, including walking unassisted, standing longer, climbing stairs, attending gym class, and attending to personal needs.

She switched to deoxynucleoside therapy in February of 2016 at a dose of 260 mg/kg/day of dC and dT, and then increased to a dose of 400 mg/kg/day of dC and dT in May of 2016 and continued to show improvement.

There were no observed adverse effects.

Patient 9

This patient was born in Guatemala in September of 1989. He began 130 mg/kg/day of dC and dT in August of 2015 and increased to 260 mg/kg/day in February of 2016. He has shown improved energy.

There were no observed adverse effects.

REFERENCES

Akman, *et al.* (2008) Thymidine kinase 2 (H126N) knock in mice show the essential role of balanced deoxynucleotide pools for mitochondrial DNA maintenance. *Hum. Mol. Genet.* 17:2433-2440

Alston, *et al.* (2013) Late-onset respiratory failure due to TK2 mutations causing multiple mtDNA deletions. *Neurology* 81:2051-3

Bartesaghi, *et al.* (2010) Loss of thymidine kinase 2 alters neuronal bioenergetics and leads to neurodegeneration. *Hum. Mol. Genet.* 19:1669-77

Béhin, *et al.* (2012) Adult cases of mitochondrial DNA depletion due to TK2 defect An expanding spectrum. *Neurology* 78:644-648

Blakely, *et al.* (2008) Novel mutations in the TK2 gene associated with fatal mitochondrial DNA depletion myopathy. *Neuromuscular Disorders* 18:557-560

Bourdon, *et al.* (2007) Mutation of RRM2B, encoding p53-controlled ribonucleotide reductase (p53R2), causes severe mitochondrial DNA depletion. *Nature Genetics* 39**:**776-780

Carrozzo, *et al.* (2003) Mutation analysis in 16 patients with mtDNA depletion. *Hum. Mutat.* 21:453-454

Chanprasert, *et al.* (2013) Molecular and clinical characterization of the myopathic form of mitochondrial DNA depletion syndrome caused by mutations in the thymidine kinase (TK2) gene. *Mol. Genet. Metab.* 110:153-61

Chanprasert, *et al.* (2012) TK2-Related Mitochondrial DNA Depletion Syndrome, Myopathic Form. *GeneReviews® Internet*, December 6, 2012

Collins, *et al.* (2009) Progressive myofiber loss with extensive fibro-fatty replacement in a child with mitochondrial DNA depletion syndrome and novel thymidine kinase 2 gene mutations. *Neuromuscular Disorders* 19:784-787

Copeland (2008) Inherited mitochondrial diseases of DNA replication. *Ann. Rev. Med.* 59:131-146

DiMauro, *et al.* (1987) Cytochrome c oxidase deficiency in Leigh syndrome. *Ann. Neurol.* 22:498-506

DiMauro, Schon. (2003) Mitochondrial respiratory-chain diseases. *New England Journal of Medicine* 348:2656-2668

DiMauro, Hirano. (2005) Mitochondrial encephalomyopathies: an update. *Neuromuscul. Disord.* 15:276-286

Dorado, *et al.* (2011) Onset and organ specificity of Tk2 deficiency depends on Tk1 down-regulation and transcriptional compensation. *Hum. Mol. Genet.* 20:155-64

Elpeleg, *et al.* (2005) Deficiency of the ADP-forming succinyl-CoA synthase activity is associated with encephalomyopathy and mitochondrial DNA depletion. *Am. J. Hum. Genet.* 76:1081-1086

Ferraro, *et al.* (2010) Quantitation of cellular deoxynucleoside triphosphates. *Nucleic Acids Research* 38:e85

Galbiati, *et al.* (2006) New mutations in TK2 gene associated with mitochondrial DNA depletion. *Pediatr. Neurol.* 34:177-185

Garone, *et al.* (2012). MPV17 Mutations Causing Adult-Onset Multisystemic Disorder With Multiple Mitochondrial DNA Deletions. *Arch Neurol* 69:1648-1651

Garone, et al. (2014). Deoxypyrimidine monophosphate bypass therapy for thymidine kinase 2 deficiency. *EMBO Mol Med* 6:1016-1027

Garone, *et al.* (2016) in preparation, "Phenotypic Spectrum and Retrospective Natural History of Thymidine Kinase 2 Deficiency"

Gotz, *et al.* (2008) Thymidine kinase 2 defects can cause multi-tissue mtDNA depletion syndrome. *Brain* 131:2841-2850

Hirano, *et al.* (2001) Defects of intergenomic communication: autosomal disorders that cause multiple deletions and depletion of mitochondrial DNA. *Semin. Cell. Develop. Biol.* 12:417-427

Hirano, *et al.* (2004) MtDNA maintenance and stability genes: MNGIE and mtDNA depletion syndromes. In: Köhler, Bauer, editors. Mitochondrial Function and Biogenetics. Berlin: Springer-Verlag. p. 177-200

Leshinsky-Silver, *et al.*.(2008) A defect in the thymidine kinase 2 gene causing isolated mitochondrial myopathy without mtDNA depletion. *Eur. J. Paediatr. Neurol.* 12:309-13

Lesko, *et al.* (2010) Two novel mutations in thymidine kinase-2 cause early onset fatal encephalomyopathy and severe mtDNA depletion. *Neuromuscul. Disord.* 20:198-203

Longley, *et al.* (2006). Mutant POLG2 disrupts DNA polymerase gamma subunits and causes progressive external ophthalmoplegia. *Am J Hum Genet.* 78:1026-1034

Lopez, *et al.* (2009) Unbalanced deoxynucleotide pools cause mitochondrial DNA instability in thymidine phosphorylase deficient mice. *Human Molecular Genetics* 18:714-22

Mancuso, *et al.* (2003) Mitochondrial myopathy of childhood associated with mitochondrial DNA depletion and a homozygous mutation (T77M) in the TK2 gene. *Arch. Neurol.* 60:1007-9

Mancuso, *et al.* (2002) Mitochondrial DNA depletion: mutations in thymidine kinase gene with myopathy and SMA. *Neurology.* 59:1197-202

Mandel, *et al.* (2001) The deoxyguanosine kinase gene is mutated in individuals with depleted hepatocerebral mitochondrial DNA. *Nature Genet.* 29**:**337-341

Martí, *et al.* (2010) Hearing loss in a patient with the myopathic form of mitochondrial DNA depletion syndrome and a novel mutation in the TK2 gene. *Pediatr. Res.* 68:151-4

Martí, *et al.* (2012a) Measurement of mitochondrial dNTP pools. *Methods Mol. Biol.* 837:135-148

Martí, *et al.* (2012b) Assessment of thymidine phosphorylase function: measurement of plasma thymidine (and deoxyuridine) and thymidine phosphorylase activity. *Methods Mol. Biol.* 837: 121-133

Naviaux, Nguyen. (2004) POLG mutations associated with Alpers' syndrome and mitochondrial DNA depletion. *Ann. Neurol.* 55:706-712

Nishino, *et al.* (1999). Thymidine phosphorylase gene mutations in MNGIE, a human mitochondrial disorder. *Science* 283:689-692.

Oskoui, *et al.* (2006) Clinical spectrum of mitochondrial DNA depletion due to mutations in the thymidine kinase 2 gene. *Arch. Neurol.* 63:1122-1126.

Ostergaard, *et al.* (2007) Deficiency of the alpha subunit of succinate-coenzyme A ligase causes fatal infantile lactic acidosis with mitochondrial DNA depletion. *Am. J. Hum. Genet.* 81: 383-387

Paradas, *et al.* (2012) TK2 mutation presenting as indolent myopathy. *Neurology* 29:504-506

Ronchi, *et al.* (2012). Next-generation sequencing reveals DGUOK mutations in adult patients with mitochondrial DNA multiple deletions. *Brain* 135:3404-3415.

Roos, *et al.* (2014) Mitochondrial DNA depletion in single fibers in a patient with novel TK2 mutations. *Neuromuscul. Disord.* 24:713-20

Saada, *et al.* (2001) Mutant mitochondrial thymidine kinase in mitochondrial DNA depletion myopathy. *Nature Genet.* 29:342-344

Saada, *et al.* (2003) Mitochondrial deoxyribonucleoside triphosphate pools in thymidine kinase 2 deficiency. *Biochem. Biophys. Res. Commun.* 310:963-966

Sarzi, *et al.* (2007) Twinkle helicase (PEO1) gene mutation causes mitochondrial DNA depletion. *Ann. Neurol.* 62**:** 579-587

Spelbrink, *et al.* (2001). Human mitochondrial DNA deletions associated with mutations in the gene encoding Twinkle, a phage T7 gene 4-like protein localized in mitochondria. *Nature Genet.* 28:223-231

Spinazzola, *et al.* (2006) MPV17 encodes an inner mitochondrial membrane protein and is mutated in infantile hepatic mitochondrial DNA depletion. *Nature Genet.* 38**:**570-575

Tulinius, *et al.* (2005) Novel mutations in the thymidine kinase 2 gene (TK2) associated with fatal mitochondrial myopathy and mitochondrial DNA depletion. *Neuromuscul. Disord.* 15:412-415

Tyynismaa, *et al.* (2012) Thymidine kinase 2 mutations in autosomal recessive progressive external ophthalmoplegia with multiple mitochondrial DNA deletions. *Hum. Mol. Genet.* 21:66-75

Tyynismaa, *et al.* (2009). A heterozygous truncating mutation in RRM2B causes autosomal-dominant progressive external ophthalmoplegia with multiple mtDNA deletions. *Am. J. Hum. Genet.* 85: 290-295

Van Goethem, *et al.* (2001) Mutation of POLG is associated with progressive external ophthalmoplegia characterized by mtDNA deletions. *Nature Genet.* 28:211-212.

Vilà, *et al.* (2003) Reversion of mtDNA depletion in a patient with TK2 deficiency. *Neurology* 60:1203-1205

Wang, *et al.* (2005) Molecular insight into mitochondrial DNA depletion syndrome in two patients with novel mutations in the deoxyguanosine kinase and thymidine kinase 2 genes. *Mol. Genet. Metab.* 84:75-82.

CLAIMS:

1. A method of treating a disease or disorder characterized by unbalanced nucleotide pools in a subject in need thereof comprising administering to the subject a therapeutically effective amount of a composition comprising at least one deoxynucleoside or a physiologically functional derivative thereof.

2. The method of claim 1, wherein the disease or disorder characterized by unbalanced nucleotide pools is a mitochondrial DNA depletion syndrome.

3. The method of claim 2, wherein the mitochondrial DNA depletion syndrome is thymidine kinase 2 (TK2) deficiency.

4. The method of claim 1, wherein the disease or disorder characterized by unbalanced nucleotide pools is characterized by at least one mutation in a gene chosen from the group consisting of: *TK2; DGUOK; TYMP; RRM2B; SUCLA2; SUCLG1;* and *MPV17.*

5. The method of claim 2, wherein the mitochondrial DNA depletion syndrome is chosen from the group consisting of deoxyguanosine kinase (dGK) deficiency, thymidine phosphorylase (TP) deficiency, and at least one mutation in a gene chosen from the group consisting of *DGUOK, TYMP, RRM2B, POLG,* and *MPV17* gene.

6. The method of claim 1, wherein the subject is a mammal.

7. The method of claim 1, wherein the subject is a human.

8. The method of claim 1, wherein the composition comprises two or more deoxynucleosides.

9. The method of claim 1, wherein the deoxynucleoside is a deoxypyrimidine.

10. The method of claim 9, wherein the deoxypyrimidine is chosen from the group consisting of deoxycytidine (dC), deoxythymidine (dT), and mixtures thereof.

11. The method of claim 1, wherein the deoxynucleoside is a deoxypurine.

12. The method of claim 19, wherein the deoxypurine is chosen from the group consisting of deoxyadenosine (dA), deoxyguanosine (dG), and mixtures thereof.

13. The method of claim 1, wherein the therapeutically effective amount is between about 100 mg/kg/day and about 1000 mg/kg/day.

14. The method of claim 1, wherein the therapeutically effective amount is between about 200 mg/kg/day and about 800 mg/kg/day.

15. The method of claim 1, wherein the therapeutically effective amount is between about 250 mg/kg/day and about 400 mg/kg/day.

16. The method of claim 13, wherein the composition comprises more than one deoxynucleoside and the therapeutically effective amount is between about 100 mg/kg/day and about 1000 mg/kg/day of each deoxynucleoside in the composition.

17. The method of claim 13, wherein the composition comprises more than one deoxynucleoside and the therapeutically effective amount is between about 100 mg/kg/day and about 1000 mg/kg/day of the total deoxynucleoside in the composition.

18. The method of claim 14, wherein the composition comprises more than one deoxynucleoside and the therapeutically effective amount is between about 200 mg/kg/day and about 800 mg/kg/day of each deoxynucleoside in the composition.

19. The method of claim 14, wherein the composition comprises more than one deoxynucleoside and the therapeutically effective amount is between about 200 mg/kg/day and about 800 mg/kg/day of the total deoxynucleosides in the composition.

20. The method of claim 15, wherein the composition comprises more than one deoxynucleoside and the therapeutically effective amount is between about 250 mg/kg/day and about 400 mg/kg/day of each deoxynucleoside in the composition.

21. The method of claim 15, wherein the composition comprises more than one deoxynucleoside and the therapeutically effective amount is between about 250 mg/kg/day and about 400 mg/kg/day of the total deoxynucleosides in the composition.

22. The method of claim 1, wherein the composition is administered once daily, twice daily, three times daily, four times daily, five times daily or six times daily.

23. The method of claim 1, wherein the composition administered orally, intrathecally, enterally, or intravenously.

24. The method of claim 23, wherein the composition is administered orally and comprises deoxynucleoside mixed with cow's milk, human breast milk, infant formula or water.

25. The method of claim 1, further comprising administering to the subject an inhibitor of thymidine phosphorylase.

26. The method of claim 25, wherein the inhibitor of thymidine phosphorylase is tipiracil.

27. The method of claim 1, further comprising administering to the subject an inhibitor of cytidine deaminase.

28. The method of claim 27, wherein the inhibitor of cytidine deaminase is tetrahydrouridine [THU].

29. The method of claim 1, wherein the therapeutically effective amount of the composition administered to the subject is increased over time.

30. The method of claim 29, wherein a first therapeutically effective amount of the composition administered to the subject is about 100 mg/kg/day of composition, and wherein the therapeutically effective amount of the composition is increased over time to 200 mg/kg/day, to 400 mg/kg/day, to 800 mg/kg/day, up to 1000 mg/kg/day.

31. The method of claim 1, wherein the composition comprises a pharmaceutically acceptable carrier.

32. A method for the treatment of TK deficiency in a subject comprising:

a. obtaining a sample from the subject, said sample comprising nucleic acid;

b. performing sequence analysis of the *TK2* gene in the nucleic acid of the subject;

c. determining the subject has TK2 deficiency when a homozygous mutation or compound heterozygous mutations in the *TK2* gene is detected; and

d. administering a therapeutically effective amount of a composition comprising deoxycytidine (dC), deoxythymidine (dT), and mixtures thereof to the subject.

33. The method of claim 32, further comprising:

a. detecting the level of creatine kinase concentration in a sample from the subject;

b. performing a biopsy on skeletal muscle of the subject;

c. measuring mitochondrial DNA count in skeletal muscle of the subject; and

d. further determining and/or confirming the subject has TK2 deficiency if one or more of the following is detected: the levels of creatine kinase concentration are increased or elevated compared to healthy controls; the skeletal muscle of the subject comprises prominent variance in fiber size, variable sarcoplasmic vacuoles, variable increased connective tissue, ragged red fibers, and cytochrome *c* oxidase (COX) deficient fibers: and mitochondrial DNA levels are decreased compared to healthy controls.

34. The method of claim 3, further comprising monitoring the subject after the administration of the composition, comprising:

a. observing muscle strength and control;

b. observing differences in height and weight;

c. observing mobility; and

d. determining an improvement in condition of the subject if any of observations (a) – (c) are increased after administration of the composition, and determining no improvement if any of observations (a) – (c) are the same or decreased after administration of the composition.

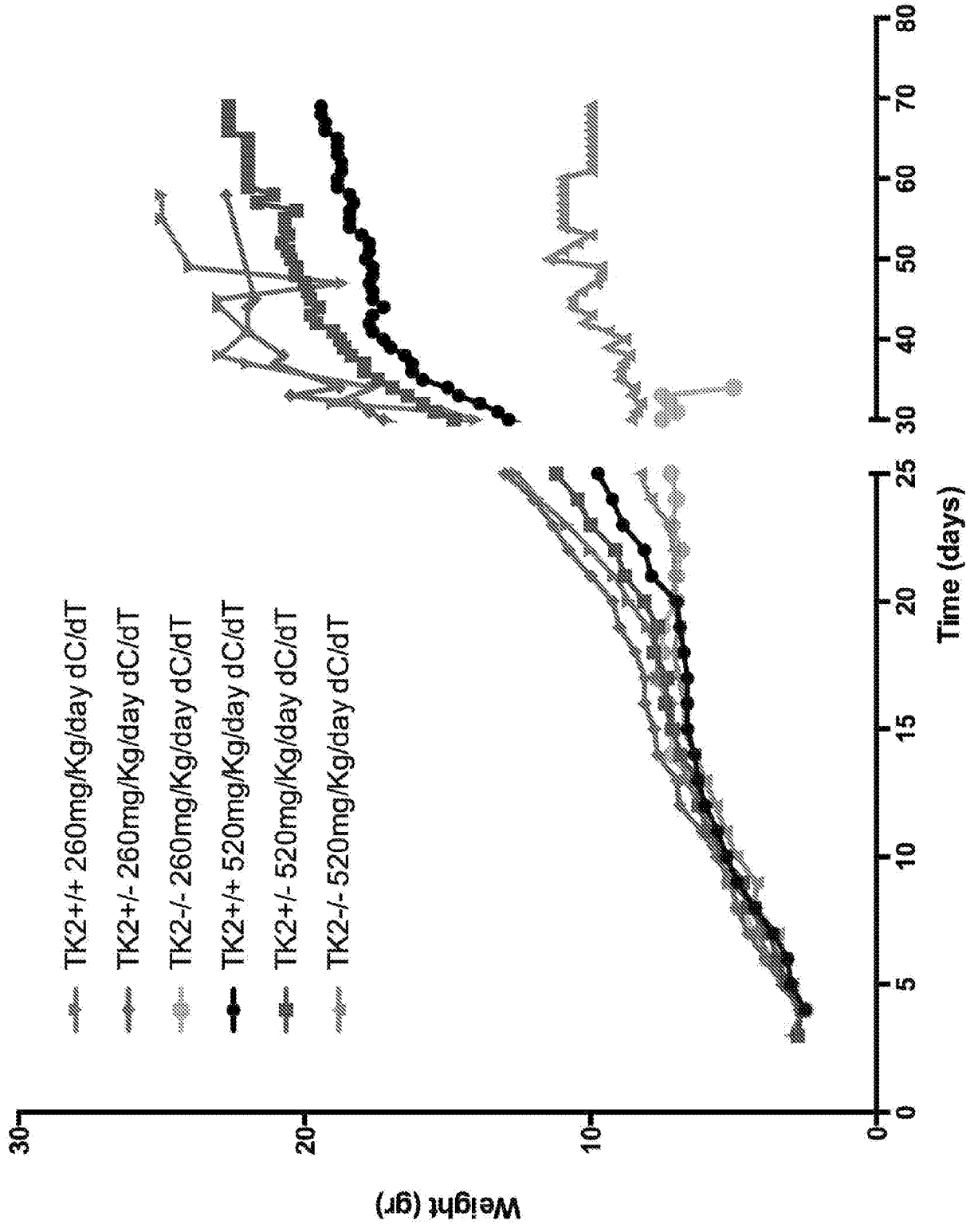
35. The method of claim 34, wherein if the determination of no improvement is made in step (d), the therapeutically effective amount of the composition is increased.

36. The method of claim 1, further comprising monitoring the subject for an adverse effect after the administration of the composition, wherein if an adverse effect is observed, the therapeutically effective amount of the composition is decreased.

37. The method of claim 36, further comprising monitoring the subject for the observed adverse effect after the therapeutically effective amount of the composition is decreased, wherein if the adverse effect is no longer observed, the therapeutically effective amount of the composition is increased.

38. The method of claim 36, wherein an adverse effect is a gastrointestinal intolerance.

39. The method of claim 36, wherein the adverse effect is chosen from the group consisting of diarrhea and abdominal bloating.


Growth curve oral dCdT
TK2+/+ 260mg/Kg/day dC/dT
TK2+/- 260mg/Kg/day dC/dT
TK2-/- 260mg/Kg/day dC/dT
TK2+/+ 520mg/Kg/day dC/dT
TK2+/- 520mg/Kg/day dC/dT
TK2-/- 520mg/Kg/day dC/dT
Weight (gr)
Time (days)
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20
30
0
5
10
15
20
25
30
40
50
60
70
80

Figure 1

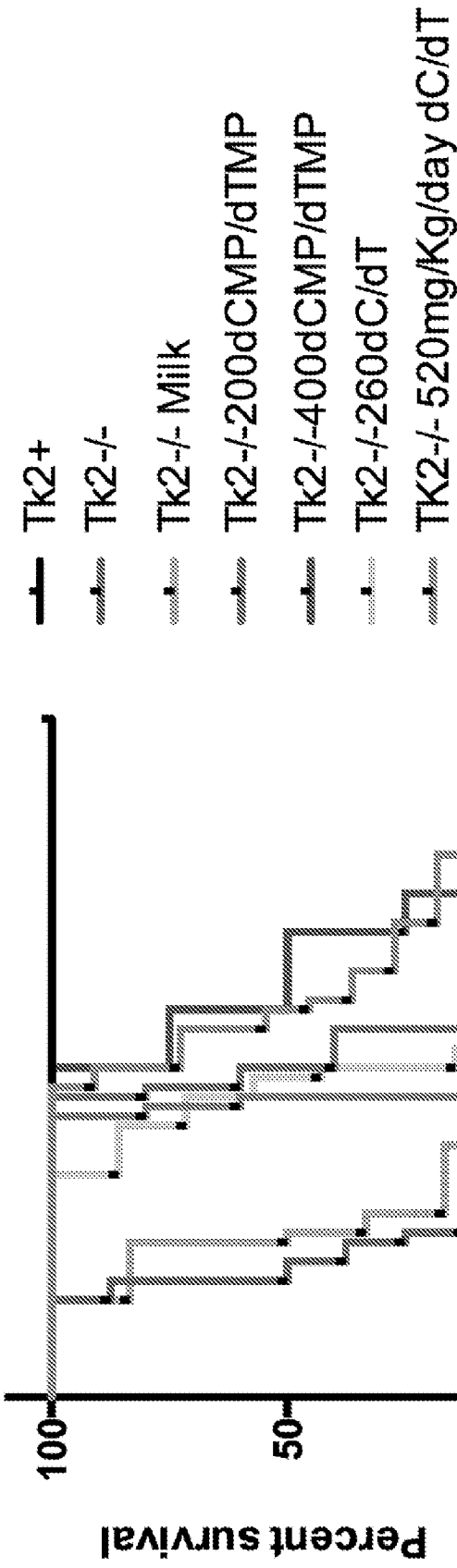
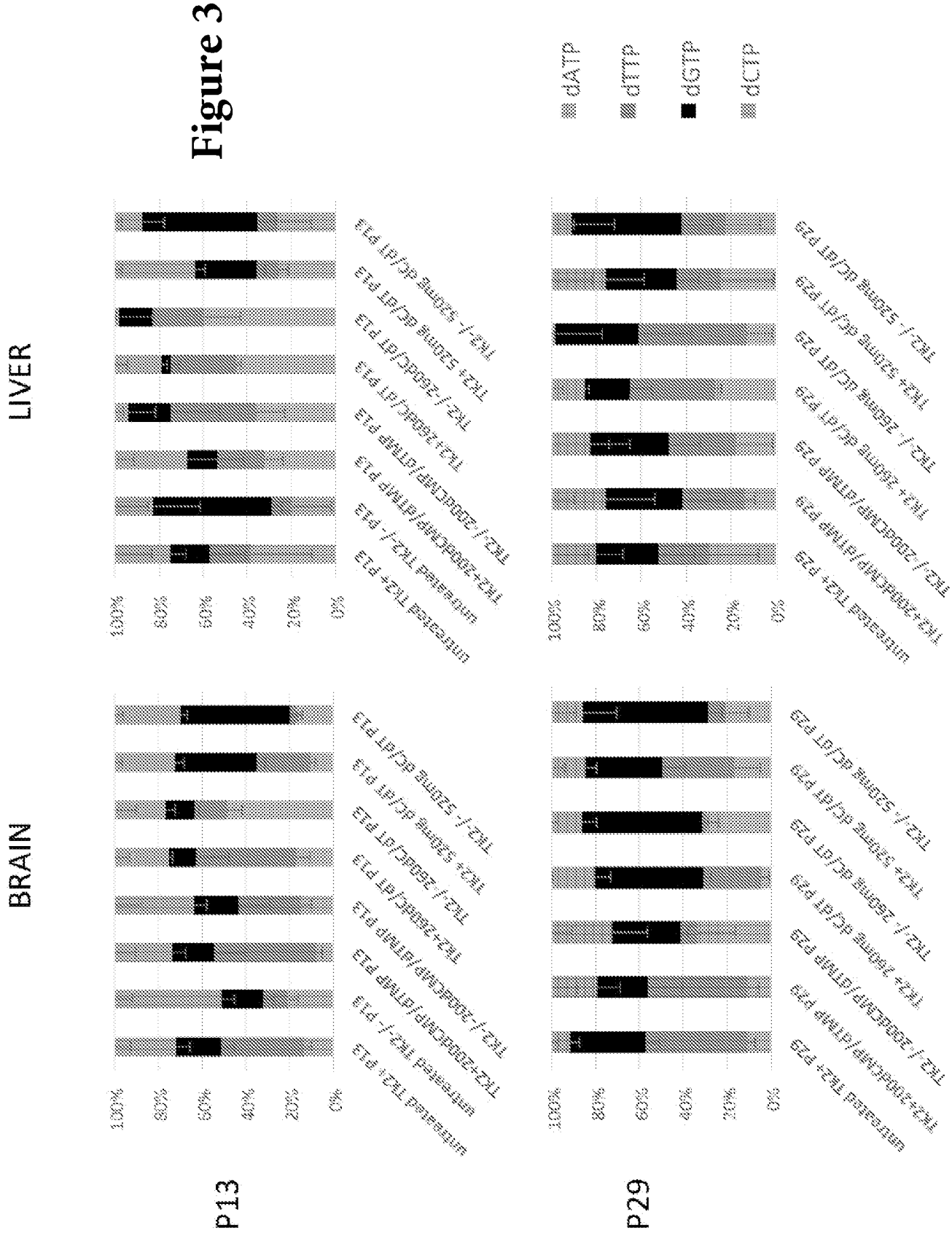

Tk2+
Tk2-/-
Tk2-/- Milk
Tk2-/-200dCMP/dTMP
Tk2-/-400dCMP/dTMP
Tk2-/-260dC/dT
TK2-/- 520mg/Kg/day dC/dT
Percent survival
100
50
0
0
20
40
60
80
Survival time (days)

Figure 2

Figure 3


LIVER
BRAIN
P13
P29
dATP
dTTP
dGTP
dCTP
100%
80%
60%
40%
20%
0%
untreated TK2+ P13
TK2+ 2500dC/dT/dGTP/dATP P13
TK2-/- 2500dC/dT/dGTP/dATP P13
TK2+ 2500dC/dT/dGTP/dATP P13
TK2-/- 2500dC/dT P13
TK2+ 2500dC/dT P13
TK2-/- 52DMF dC/dT P13
TK2+ 52DMF dC/dT P13
untreated TK2+ P29
TK2+ 2500dC/dT/dGTP/dATP P29
TK2-/- 2500dC/dT/dGTP/dATP P29
TK2+ 2500dC/dT/dGTP/dATP P29
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TK2+ 52DMF dC/dT P29

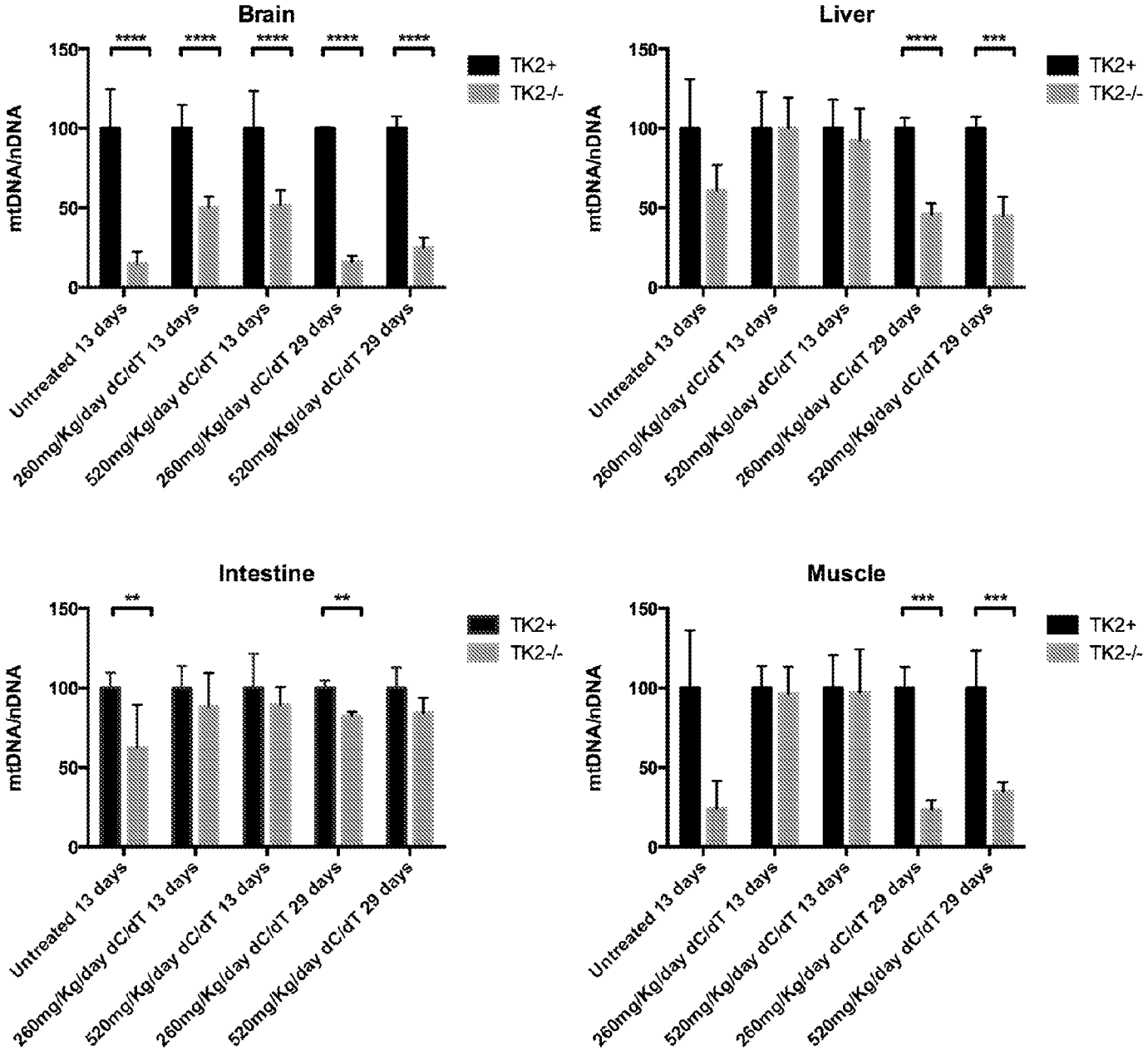

Brain
Liver
Intestine
Muscle
mtDNA/nDNA
TK2+
TK2-/-
Untreated 13 days
260mg/Kg/day dC/dT 13 days
520mg/Kg/day dC/dT 13 days
260mg/Kg/day dC/dT 29 days
520mg/Kg/day dC/dT 29 days

Figure 4

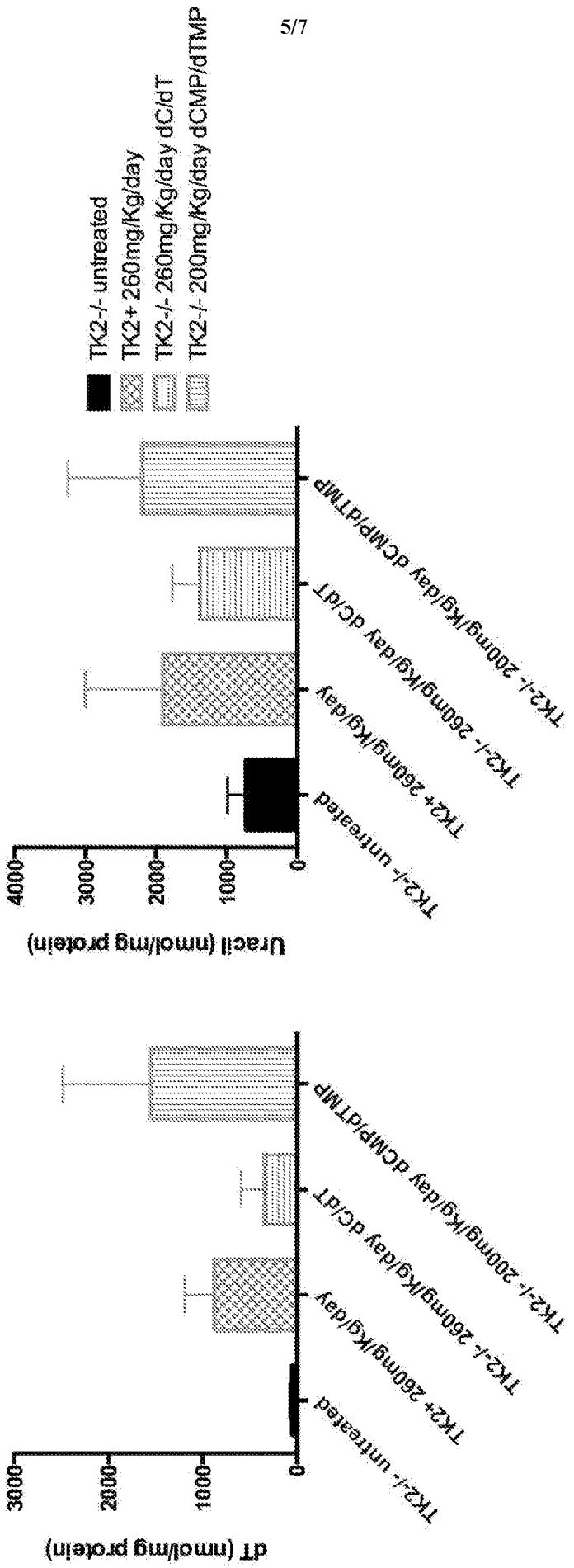

TK2-/- untreated
TK2+ 260mg/Kg/day
TK2-/- 260mg/Kg/day dC/dT
TK2-/- 200mg/Kg/day dCMP/dTMP
Uracil (nmol/mg protein)
4000
3000
2000
1000
0
TK2-/- untreated
TK2+ 260mg/Kg/day
TK2-/- 260mg/Kg/day dC/dT
TK2-/- 200mg/Kg/day dCMP/dTMP
dT (nmol/mg protein)
3000
2000
1000
0
TK2-/- untreated
TK2+ 260mg/Kg/day
TK2-/- 260mg/Kg/day dC/dT
TK2-/- 200mg/Kg/day dCMP/dTMP

Figure 5

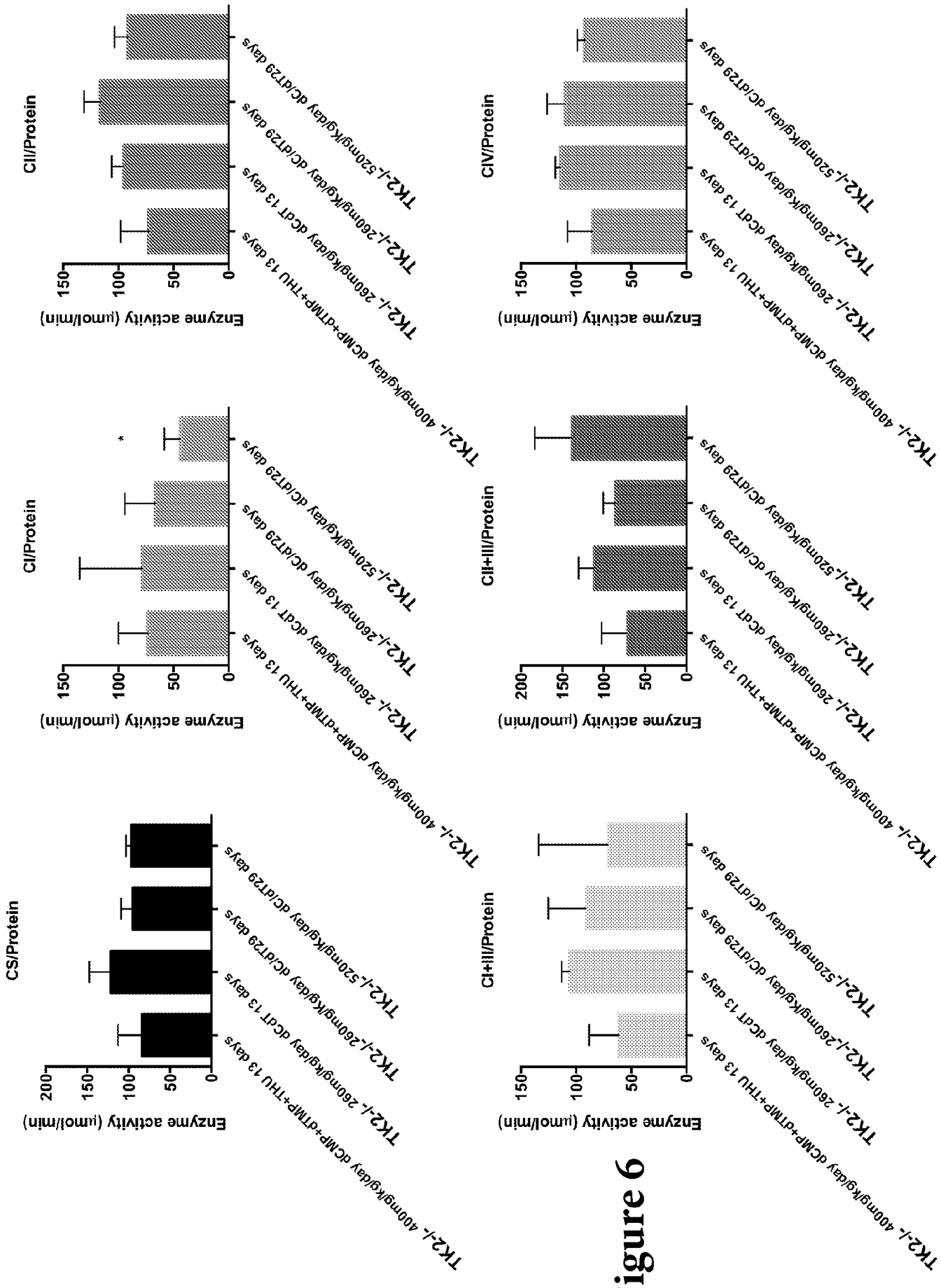
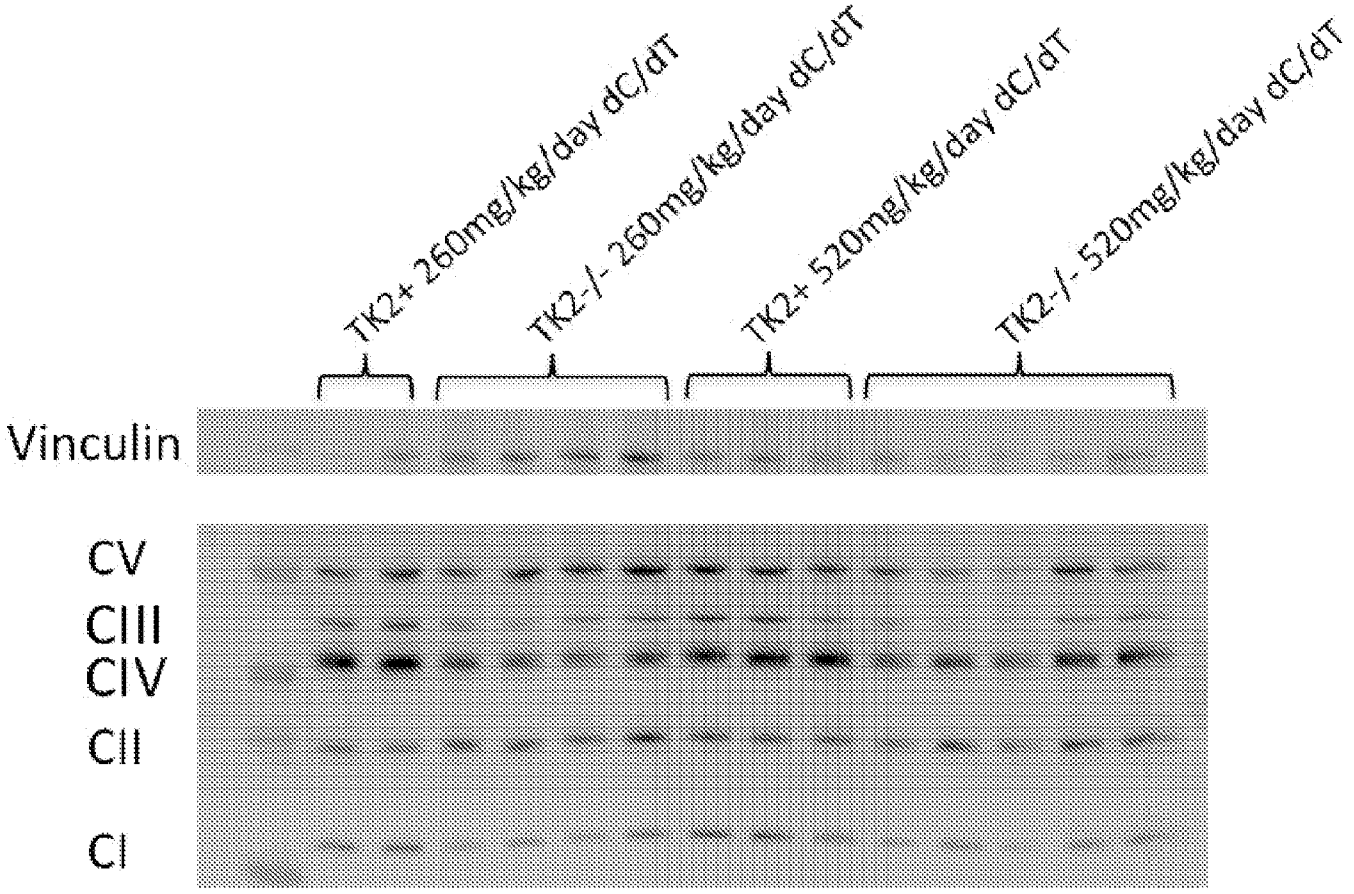

CS/Protein
CI/Protein
CII/Protein
CII+III/Protein
CIV/Protein
Enzyme activity (μmol/min)
TK2-/- 400mg/kg/day dCMP+dTMP+THU 13 days
TK2-/- 260mg/kg/day dCdT 13 days
TK2-/- 520mg/kg/day dC/dT29 days
*

Figure 6

Figure 7A



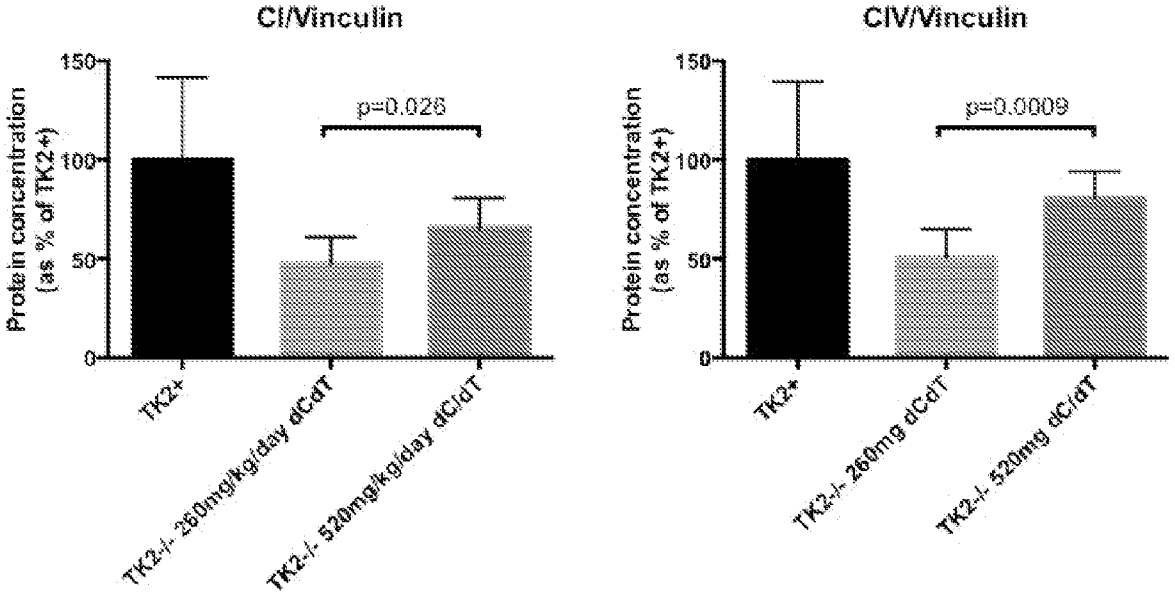


Figure 7B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 16/38110

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 31/7068, 31/7064, 31/7072; C07D 403/06 (2016.01)
CPC - A61K 31/7068, 31/7064, 31/7072; C12Q 1/6869; C07D 403/06
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8): A61K 31/7068, 31/7064, 31/7072; C07D 403/06 (2016.01)
CPC: A61K 31/7068, 31/7064, 31/7072; C12Q 1/6869; C07D 403/06

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
CPC: A61K 31/7068, 31/7064, 31/7072; C12Q 1/6869; C07D 403/06 (text search)
USPC: 514/49(text search)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Electronic data bases: PatBase; Google Patents; Google Scholar
Search terms: Mitochondrial DNA depletion syndrome (MDS), thymidine kinase 2 (TK2) deficiency, mutation TK2 gene, sequence TK2 gene, administer deoxypyrimidine (deoxythymidine or deoxycytidine),

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X — Y	GARONE et al. Deoxypyrimidine monophosphate bypass therapy for thymidine kinase 2 deficiency. EMBO Mol Med 26 June 2014 Vol 6 No 8 Pages 1016-1027. Especially abstract, pg 1016 col 2, pg 1017 col 1 para 2, para 2, pg 1024 col 1 para 1, pg 1024 col 1 para 5	1-4, 6, 7, 9, 10, 13-15, 22-25, 29-31 --- 26-28, 32-39
Y	MedChem Express. Tipiracil hydrochloride (online) 2014 [retrieved 26 October 2016] Available on the internet: <ULR: https://www.medchemexpress.com/Tipiracil-hydrochloride.html?gclid=Cj0KEQjwqMHABRDVl6_hqKGDyNIBEiQAN-O9hEkNyE4wS-bWnZLDeUwtN_gQ5bhgZHaeJwqW6uzbxVkaAsPf8P8HAQ>. Especially pg 1	26
Y	CAMARA et al. Feeding the deoxyribonucleoside salvage pathway to rescue mitochondrial DNA. Drug Discov Today October 2013 Vol 18 No 19-20 Pages 950-957. Especially Pg 955 col 2 para 3-4.	27, 28
Y	WO 2012/125848 A2 (Baylor College of Medicine) 30 September 2012 (30.09.2012). Especially para [0044], [0090]	32-39
Y	GARONE et al. Clinical and genetic spectrum of mitochondrial neurogastrointestinal encephalomyopathy. Brain November 2011 Vol 134 Pt 11 pages 3326-3332. Especially pg 3328 col 1 para 3	38, 39

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 October 2016	16 NOV 2016
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 16/38110

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
----Go to Extra Sheet for continuation------

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-4, 6, 7, 9, 10, 13-15, 22-39 limited to TK2 defects and therapeutical deoxycytidine

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 16/38110

----continuation of Box III (Lack of Unity of Invention)----

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I+: Claims 1-39, drawn to a method of treating a disease or disorder characterized by unbalanced nucleotide pools in a subject comprising administering at least one deoxynucleoside. The method will be searched to the extent that the DNA depletion syndrome encompasses the defect in the first named gene, thymidine kinase 2 (TK2) (claims 3-4), and the first named therapeutical deoxynucleotide, deoxycytidine (dC) (claim 10). It is believed that claims 1-4, 6, 7, 9, 10, 13-15, 22-39 read on this first named invention and thus these claims will be searched without fee to the extent that they encompass TK2 defects and therapeutical deoxycytidine (dC). Additional depletion syndromes, mutant genes, and therapeutic deoxynucleosides will be searched upon payment of additional fees. Applicant must specify the claims that encompass any additional elected depletion syndromes, mutant genes, and therapeutic deoxynucleosides. Applicants must further indicate, if applicable, the claims which read on the first named invention if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined. An exemplary election would be deoxyguanosine kinase (dGK), DGUOK and deoxyguanosine (dG): (Claims 1, 2, 5-8, 11-24, 29-31).

The inventions listed as Group I+ do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features:

Among the inventions listed as Groups I+ are the specific nucleotide deficiencies and mutant genes recited therein. The inventions do not share a special technical feature, because no significant structural similarities can readily be ascertained among the nucleotide deficiencies or genes containing a mutation.

Common Technical Features:

Group I+ shares the common technical feature of independent claims 1 and 32.

However, said common technical features do not represent a contribution over the prior art and is obvious over the technical publication titled "Deoxypyrimidine monophosphate bypass therapy for thymidine kinase 2 deficiency" by Garone et al. (hereinafter "Garone") [published 26 June 2014 EMBO Mol Med Vol 6 No 8 Pages 1016-1027.], in view of WO 2012/125848 A2 to Baylor College of Medicine (hereinafter "Baylor).

As to claim 1, Garone teaches a method of treating a disease or disorder characterized by unbalanced nucleotide pools in a subject in need thereof comprising administering to the subject a therapeutically effective amount of a composition comprising at least one deoxynucleoside or a physiologically functional derivative thereof (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 phenotypically normal, but biochemically affected. Assessment of 13-day-old Tk2-/- mice treated with dCMP+dTMP 200 mg/kg/day each (Tk2-/-200dCMP/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)").

As to claim 32, Baylor teaches a method for the treatment of TK deficiency in a subject comprising:
a. obtaining a sample from the subject, said sample comprising nucleic acid (para [0090]);
b. performing sequence analysis of the TK2 gene in the nucleic acid of the subject (para [0090]; The Depletion Panel is a panel that may be performed using the deep sequencing technique described above. It contains 14 nuclear genes (C10ORF2, DGUOK, MPV17, OPA1, OP A3, POLG, POLG2, RRM2B, SLC25A4, SUCLA2, SUCLG1, SUCLG2, TK2 and TYMP) that are involved in the maintenance of mtDNA integrity and deoxynucleotide salvage pathway. These genes are analyzed by the "deep sequencing technique" by the application of Massive Parallel Sequencing (MPS) utility to the clinical diagnosis")
c. determining the subject has TK2 deficiency when a homozygous mutation or compound heterozygous mutations in the TK2 gene is detected (Para [0044]; For the identification of mutations in nuclear genes, coverage of greater than 30X sequence reads would usually be considered adequate for making homozygous or heterozygous base calls and the detection of small indel variations for research purposes"). Baylor does not teach d. administering a therapeutically effective amount of a composition comprising deoxycytidine (dC), deoxythymidine (dT), and mixtures thereof to the subject. However, Garone teaches administering a therapeutically effective amount of a composition comprising deoxycytidine (dC), deoxythymidine (dT), and mixtures thereof to the subject. It would have been obvious to combine claim limitations (a), (b), and (c), as taught by Baylor, with claim limitation (d), as taught by Garone because it would have enabled a combination of diagnosis and treatment in a subject suffering from TK deficiency.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 16/38110

-----continued from prevous sheet------

As the common technical features were known in the art at the time of the invention, they cannot be considered common special technical features that would otherwise unify the groups. The inventions lack unity with one another.

Therefore, Groups I+ lacks unity of invention under PCT Rule 13 because they do not share a same or corresponding special technical feature.

Note concerning claim 34. Claim 34 is written to depend from claim 3, is objected, because claim 3 does not have required antecedent "monitoring the subject". For the purposes of the International Search & Opinion, claim 34 is interpreted to depend from claim 32.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 16/38110

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 31/7068, 31/7064, 31/7072; C07D 403/06 (2016.01)
CPC - A61K 31/7068, 31/7064, 31/7072; C12Q 1/6869; C07D 403/06
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8): A61K 31/7068, 31/7064, 31/7072; C07D 403/06 (2016.01)
CPC: A61K 31/7068, 31/7064, 31/7072; C12Q 1/6869; C07D 403/06

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
CPC: A61K 31/7068, 31/7064, 31/7072; C12Q 1/6869; C07D 403/06 (text search)
USPC: 514/49(text search)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Electronic data bases: PatBase; Google Patents; Google Scholar
Search terms: Mitochondrial DNA depletion syndrome (MDS), thymidine kinase 2 (TK2) deficiency, mutation TK2 gene, sequence TK2 gene, administer deoxypyrimidine (deoxythymidine or deoxycytidine),

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X — Y	GARONE et al. Deoxypyrimidine monophosphate bypass therapy for thymidine kinase 2 deficiency. EMBO Mol Med 26 June 2014 Vol 6 No 8 Pages 1016-1027. Especially abstract, pg 1016 col 2, pg 1017 col 1 para 2, para 2, pg 1024 col 1 para 1, pg 1024 col 1 para 5	1-4, 6, 7, 9, 10, 13-15, 22-25, 29-31 ---------- 26-28, 32-39
Y	MedChem Express. Tipiracil hydrochloride (online) 2014 [retrieved 26 October 2016] Available on the internet: <ULR: https://www.medchemexpress.com/Tipiracil-hydrochloride.html?gclid=Cj0KEQjwqMHABRDVl6_hqKGDyNIBEiQAN-O9hEkNyE4wS-bWnZLDeUwtN_gQ5bhgZHaeJwqW6uzbxVkaAsPf8P8HAQ>. Especially pg 1	26
Y	CAMARA et al. Feeding the deoxyribonucleoside salvage pathway to rescue mitochondrial DNA. Drug Discov Today October 2013 Vol 18 No 19-20 Pages 950-957. Especially Pg 955 col 2 para 3-4.	27, 28
Y	WO 2012/125848 A2 (Baylor College of Medicine) 30 September 2012 (30.09.2012). Especially para [0044], [0090]	32-39
Y	GARONE et al. Clinical and genetic spectrum of mitochondrial neurogastrointestinal encephalomyopathy. Brain November 2011 Vol 134 Pt 11 pages 3326-3332. Especially pg 3328 col 1 para 3	38, 39

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 October 2016	16 NOV 2016
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 16/38110

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
----Go to Extra Sheet for continuation------

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
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Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

----continuation of Box III (Lack of Unity of Invention)----

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However, said common technical features do not represent a contribution over the prior art and is obvious over the technical publication titled "Deoxypyrimidine monophosphate bypass therapy for thymidine kinase 2 deficiency" by Garone et al. (hereinafter "Garone") [published 26 June 2014 EMBO Mol Med Vol 6 No 8 Pages 1016-1027.], in view of WO 2012/125848 A2 to Baylor College of Medicine (hereinafter "Baylor).

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b. performing sequence analysis of the TK2 gene in the nucleic acid of the subject (para [0090]; The Depletion Panel is a panel that may be performed using the deep sequencing technique described above. It contains 14 nuclear genes (C10ORF2, DGUOK, MPV17, OPA1, OP A3, POLG, POLG2, RRM2B, SLC25A4, SUCLA2, SUCLG1, SUCLG2, TK2 and TYMP) that are involved in the maintenance of mtDNA integrity and deoxynucleotide salvage pathway. These genes are analyzed by the "deep sequencing technique" by the application of Massive Parallel Sequencing (MPS) utility to the clinical diagnosis")
c. determining the subject has TK2 deficiency when a homozygous mutation or compound heterozygous mutations in the TK2 gene is detected (Para [0044]; For the identification of mutations in nuclear genes, coverage of greater than 30X sequence reads would usually be considered adequate for making homozygous or heterozygous base calls and the detection of small indel variations for research purposes"). Baylor does not teach d. administering a therapeutically effective amount of a composition comprising deoxycytidine (dC), deoxythymidine (dT), and mixtures thereof to the subject. However, Garone teaches administering a therapeutically effective amount of a composition comprising deoxycytidine (dC), deoxythymidine (dT), and mixtures thereof to the subject. It would have been obvious to combine claim limitations (a), (b), and (c), as taught by Baylor, with claim limitation (d), as taught by Garone because it would have enabled a combination of diagnosis and treatment in a subject suffering from TK deficiency.

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As the common technical features were known in the art at the time of the invention, they cannot be considered common special technical features that would otherwise unify the groups. The inventions lack unity with one another.

Therefore, Groups I+ lacks unity of invention under PCT Rule 13 because they do not share a same or corresponding special technical feature.

Note concerning claim 34. Claim 34 is written to depend from claim 3, is objected, because claim 3 does not have required antecedent "monitoring the subject". For the purposes of the International Search & Opinion, claim 34 is interpreted to depend from claim 32.

(19)中华人民共和国国家知识产权局

(12)发明专利申请

(10)申请公布号 CN 107847512 A
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(71)申请人 纽约市哥伦比亚大学理事会
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代理人 王建秀 郗名悦

(51)Int.Cl.
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C07D 403/06(2006.01)
A61K 31/7064(2006.01)
A61K 31/7072(2006.01)

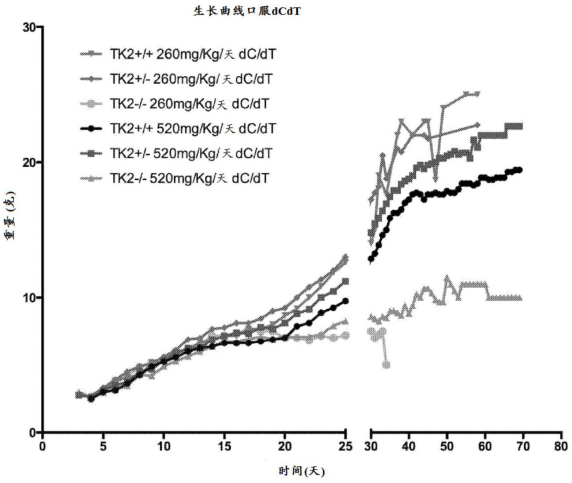
权利要求书3页 说明书18页 附图7页

(54)发明名称

用于包括线粒体DNA耗竭综合症在内的由不平衡的核苷酸库引起的疾病的脱氧核苷疗法

(57)摘要

本发明通常涉及人类遗传疾病的药理学疗法，所述人类遗传疾病具体地是以不平衡的核苷酸库为特征的那些，更具体地是线粒体DNA耗竭综合症，且更具体地是胸苷激酶2(TK2)缺乏症。所述药理学疗法涉及施用至少一种脱氧核苷或其混合物。对于治疗TK2缺乏症，所述药理学疗法涉及施用脱氧胸苷(dT)或脱氧胞苷(dC)或其混合物。脱氧核苷的这种施用适用于不平衡的核苷酸库的其它病症，特别是见于线粒体DNA耗竭综合症的那些。



CN 107847512 A

1.一种治疗有需要的受试者的以不平衡的核苷酸库为特征的疾病或病症的方法，所述方法包括对所述受试者施用治疗有效量的包含至少一种脱氧核苷或其生理学功能衍生物的组合物。

2.如权利要求1所述的方法，其中所述以不平衡的核苷酸库为特征的疾病或病症是线粒体DNA耗竭综合症。

3.如权利要求2所述的方法，其中所述线粒体DNA耗竭综合症是胸苷激酶2(TK2)缺乏症。

4.如权利要求1所述的方法，其中所述以不平衡的核苷酸库为特征的疾病或病症的特征在于选自以下的基因中的至少一种突变：TK2；DGUOK；TYMP；RRM2B；SUCLA2；SUCLG1；和MPV17。

5.如权利要求2所述的方法，其中所述线粒体DNA耗竭综合症选自脱氧鸟苷激酶(dGK)缺乏症、胸苷磷酸化酶(TP)缺乏症以及选自DGUOK、TYMP、RRM2B、POLG和MPV17基因的基因中的至少一种突变。

6.如权利要求1所述的方法，其中所述受试者是哺乳动物。

7.如权利要求1所述的方法，其中所述受试者是人。

8.如权利要求1所述的方法，其中所述组合物包含两种或更多种脱氧核苷。

9.如权利要求1所述的方法，其中所述脱氧核苷是脱氧嘧啶。

10.如权利要求9所述的方法，其中所述脱氧嘧啶选自脱氧胞苷(dC)、脱氧胸苷(dT)及其混合物。

11.如权利要求1所述的方法，其中所述脱氧核苷是脱氧嘌呤。

12.如权利要求19所述的方法，其中所述脱氧嘌呤选自脱氧腺苷(dA)、脱氧鸟苷(dG)及其混合物。

13.如权利要求1所述的方法，其中所述治疗有效量介于约100mg/kg/天与约1000mg/kg/天之间。

14.如权利要求1所述的方法，其中所述治疗有效量介于约200mg/kg/天与约800mg/kg/天之间。

15.如权利要求1所述的方法，其中所述治疗有效量介于约250mg/kg/天与约400mg/kg/天之间。

16.如权利要求13所述的方法，其中所述组合物包含不只一种脱氧核苷，且所述治疗有效量是介于约100mg/kg/天与约1000mg/kg/天之间的所述组合物中的每种脱氧核苷。

17.如权利要求13所述的方法，其中所述组合物包含不只一种脱氧核苷，且所述治疗有效量是介于约100mg/kg/天与约1000mg/kg/天之间的所述组合物中的总脱氧核苷。

18.如权利要求14所述的方法，其中所述组合物包含不只一种脱氧核苷，且所述治疗有效量是介于约200mg/kg/天与约800mg/kg/天之间的所述组合物中的每种脱氧核苷。

19.如权利要求14所述的方法，其中所述组合物包含不只一种脱氧核苷，且所述治疗有效量是介于约200mg/kg/天与约800mg/kg/天之间的所述组合物中的总脱氧核苷。

20.如权利要求15所述的方法，其中所述组合物包含不只一种脱氧核苷，且所述治疗有效量是介于约250mg/kg/天与约400mg/kg/天之间的所述组合物中的每种脱氧核苷。

21.如权利要求15所述的方法，其中所述组合物包含不只一种脱氧核苷，且所述治疗有

效量是介于约250mg/kg/天与约400mg/kg/天之间的所述组合物中的总脱氧核苷。

22.如权利要求1所述的方法，其中每日一次、每日两次、每日三次、每日四次、每日五次或每日六次施用所述组合物。

23.如权利要求1所述的方法，其中口服、鞘内、经肠或静脉内施用所述组合物。

24.如权利要求23所述的方法，其中所述组合物是口服施用的，并且包含与牛乳、人乳、婴幼儿配方奶或水混合的脱氧核苷。

25.如权利要求1所述的方法，进一步包括对所述受试者施用胸苷磷酸化酶的抑制剂。

26.如权利要求25所述的方法，其中所述胸苷磷酸化酶的抑制剂是地匹福林。

27.如权利要求1所述的方法，进一步包括对所述受试者施用胞苷脱氨酶的抑制剂。

28.如权利要求27所述的方法，其中所述胞苷脱氨酶的抑制剂是四氢尿苷[THU]。

29.如权利要求1所述的方法，其中随着时间的推移增加对所述受试者施用的所述组合物的所述治疗有效量。

30.如权利要求29所述的方法，其中对所述受试者施用的所述组合物的第一治疗有效量是约100mg/kg/天的组合物，且其中随着时间的推移将所述组合物的所述治疗有效量增加到200mg/kg/天、400mg/kg/天、800mg/kg/天、最多1000mg/kg/天。

31.如权利要求1所述的方法，其中所述组合物包含药学上可接受的载体。

32.一种治疗受试者的TK缺乏症的方法，其包括：

a.从所述受试者获得样品，所述样品包括核酸；

b.对所述受试者的所述核酸中的TK2基因进行序列分析；

c.当检测到所述TK2基因中的纯合突变或复合杂合突变时确定所述受试者患有TK2缺乏症；以及

d.对所述受试者施用治疗有效量的包含脱氧胞苷(dC)、脱氧胸苷(dT)及其混合物的组合物。

33.如权利要求32所述的方法，其进一步包括：

a.检测来自所述受试者的样品中的肌酸激酶浓度的水平；

b.对所述受试者的骨骼肌进行活检；

c.测量所述受试者的骨骼肌中的线粒体DNA计数；以及

d.如果检测到以下中的一项或多项，则进一步确定和/或证实所述受试者患有TK2缺乏症：与健康的对照者相比所述肌酸激酶浓度的水平增加或升高；所述受试者的骨骼肌包含纤维尺寸上的显著差异、可变的肌浆液泡、可变增多的结缔组织、破碎样红纤维和细胞色素c氧化酶(COX)缺乏的纤维；以及与健康的对照者相比线粒体DNA水平降低。

34.如权利要求3所述的方法，其进一步包括在施用所述组合物后监测所述受试者，包括：

a.观察肌肉力量和控制；

b.观察身高和体重的差异；

c.观察行动能力；以及

d.如果在施用所述组合物后，观察结果(a)-(c)中任何有所增加的话，则确定所述受试者的病状有改善，且如果在施用所述组合物后，观察结果(a)-(c)中任何都无变化或有所减少的话，则确定没有改善。

35.如权利要求34所述的方法,其中如果在步骤(d)中确定出没有改善,则增加所述组合物的所述治疗有效量。

36.如权利要求1所述的方法,其进一步包括在施用所述组合物后监测所述受试者中的副作用,其中如果观察到副作用的话,则减少所述组合物的所述治疗有效量。

37.如权利要求36所述的方法,进一步包括在减少所述组合物的所述治疗有效量后监测所述受试者中所观察到的副作用,其中如果不再观察到所述副作用的话,则增加所述组合物的所述治疗有效量。

38.如权利要求36所述的方法,其中副作用是胃肠不耐受。

39.如权利要求36所述的方法,其中所述副作用选自腹泻和腹胀。

用于包括线粒体DNA耗竭综合症在内的由不平衡的核苷酸库引起的疾病的脱氧核苷疗法

[0001] 政府支持

[0002] 本发明是按照由NIH给予的HD080642在政府的支持下完成的。美国政府对本发明享有一定的权利。

[0003] 相关申请的交叉引用

[0004] 本申请要求享有2015年6月17日提交的系列号为62/180,194的美国临时专利申请的优先权,该临时专利申请据此以引用的方式并入。

发明领域

[0005] 本发明通常涉及人类遗传性疾病的药理学治疗,所述人类遗传性疾病具体地说是以不平衡的核苷酸库为特征的疾病,例如线粒体DNA耗竭综合症,并且更具体地,胸苷激酶2(TK2)缺乏症。药理学治疗涉及施用至少一种脱氧核苷或其混合物。对于治疗TK2缺乏症,药理学治疗涉及施用脱氧胸苷(dT)或脱氧胞苷(dC)或其混合物。一种或多种脱氧核苷的这种施用适用于不平衡的核苷库的其它病症,特别是见于线粒体DNA耗竭综合症中的那些。

[0006] 发明背景

[0007] 线粒体疾病是由于线粒体呼吸链(RC)和氧化磷酸化的缺陷引起的临床异质性疾病,所述线粒体呼吸链(RC)和氧化磷酸化是将电子中的能量转化到三磷酸腺苷(ATP)当中的生化途径。呼吸链由转移电子以产生跨越线粒体的内膜的质子梯度的四种多亚基酶(复合物I-IV)组成,并且质子流过复合物V驱动ATP合成(DiMauro和Schon 2003;DiMauro和Hirano 2005)。辅酶Q_{10}(CoQ_{10})是使电子从复合物I和II穿梭到复合物III的必要分子。呼吸链由于受线粒体DNA(mtDNA)及核DNA(nDNA)两个基因组控制而在真核(例如,哺乳动物)细胞中是独特的。因而,任一基因组中的突变都可能引起线粒体疾病。大多数线粒体疾病影响多种身体器官,并且在儿童期或早期成年生活中通常是致命的。对于线粒体疾病没有经证实的有效治疗方法,只有支持疗法,如施用CoQ_{10}及其类似物以增强呼吸链活性并解毒作为功能失调的呼吸链酶的有毒副产物的活性氧物质(ROS)。

[0008] 线粒体DNA耗竭综合症(MDS)是线粒体疾病的一个亚组,其是严重儿童期脑肌病变的常见病因,其分子特征在于组织中的线粒体DNA(mtDNA)拷贝数减少以及线粒体RC复合物的合成不足(Hirano等人,2001)。若干核基因中的突变已被确认为婴幼儿MDS的病因,所述基因包括:TK2、DGUOK、POLG、POLG2、SCLA25A4、MPV17、RRM2B、SUCLA2、SUCLG1、TYMP、OPA1和C10orf2(PEO1)。(Bourdon等人,2007;Copeland 2008;Elpeleg等人,2005;Mandel等人,2001;Naviaux和Nguyen 2004;Ostergaard等人,2007;Saada等人,2003;Sarzi等人,2007;Spinazzola等人,2006)。此外,这些核基因中的突变也可引起伴有或没有mtDNA耗竭的mtDNA的多重缺失(Béhin等人,2012;Garone等人,2012;Longley等人,2006;Nishino等人,1999;Paradas等人,2012;Ronchi等人,2012;Spelbrink等人,2001;Tyynismaa等人,2009;Tyynismaa等人,2012;Van Goethem等人,2001)。

[0009] 这些基因之一是TK2,其编码胸苷激酶(TK2),后者是嘧啶核苷(胸苷和脱氧胞苷)

磷酸化以产生单磷酸脱氧胸苷(dTMP)和单磷酸脱氧胞苷(dCMP)所需的线粒体酶(Saada等人,2001)。TK2中的突变损害合成三磷酸脱氧核苷酸(dNTP)所需的线粒体核苷/核苷酸补救途径,三磷酸脱氧核苷酸是用于mDNA复制和修复的构建块(building block)。

[0010] Saada及同事(Saada等人,2001)于2001年首次描述了来自罹患严重破坏性肌病变的四个不同家庭的四名受感染儿童的TK2缺乏症。在平稳的早期发育之后,在6-36月龄时患者出现高CK血症、严重肌肉张力减退,随后丧失自发活动性。该疾病进展迅速,并且两名患者在3岁时接受机械通气,而另两名患者在报告之时已经死亡。

[0011] 在首次描述之后,文献中已报道了另外六十名患者,并且进一步有至少二十六名患者已被确诊但未被报道(Alston等人,2013;Bartesaghi等人,2010;Béhin等人,2012;Blakely等人,2008;Carrozzo等人,2003;Chanprasert等人,2013;Collins等人,2009;Galbiati等人,2006;Gotz等人,2008;Leshinsky-Silver等人,2008;Lesko等人,2010;Mancuso等人,2002;Mancuso等人,2003;Marti等人,2010;Oskoui等人,2006;Paradas等人,2012;Roos等人,2014;Tulinius等人,2005;Tyynismaa等人,2012;Vilà等人,2003;Wang等人,2005),结果是有九十名患者,53名男性和37名女性。

[0012] 近期诊断的二十六名患者通过下一代DNA测序得到确认。这种大量新确认的病例表明,TK2缺乏症是一种诊断不足的病症。

[0013] TK2缺乏症表现出广泛的临床和分子遗传谱,大多数患者在儿童早期表现出破坏性的临床过程,而其他患者具有经数十年缓慢进展的虚弱。

[0014] 治疗TK2缺乏症,像大多数MDS和线粒体病症一样,一直是限于支持疗法。虽然施用单磷酸脱氧胸苷(dTMP)和单磷酸脱氧胞苷(dCMP)改善了TK2敲入突变小鼠和患有TK2缺乏症的人类患者的病状(系列号为15/082,207的美国申请,其整体并入本文),但仍需要TK2缺乏症的治疗性干预。

[0015] 另外,需要治疗其它形式的MDS和以不平衡的核苷酸库为特征的其它疾病。例如,伴有mtDNA耗竭或多重缺失或兼而有之的若干孟德尔病症(mendelian disorder)的特征在于不平衡的三磷酸脱氧核苷酸库,其导致mtDNA复制的缺陷。一种这样的病症DGUOK突变损害线粒体内酶脱氧鸟苷激酶,后者通常使脱氧嘌呤核苷脱氧鸟苷和脱氧胞苷磷酸化以产生单磷酸脱氧鸟苷(dGMP)和单磷酸脱氧胞苷(dCMP)。破坏线粒体dNTP库的其它核基因包括TYMP、RRM2B、SUCLA2、SUCLG1和MPV17。恢复dNTP库平衡的疗法同样将可适用于治疗这些病症。

发明内容

[0016] 在某些实施方案中,本发明涉及治疗以不平衡的核苷酸库为特征的疾病或病症的方法,包括对有需要的受试者施用治疗有效量的包含一种或多种脱氧核苷的组合物。

[0017] 可通过本发明的方法治疗的以不平衡的核苷酸库为特征的疾病或病症包括但不限于特征在于以下基因中的突变的那些:TK2;DGUOK;TYMP;RRM2B;SUCLA2;SUCLG1;和MPV17。

[0018] 在优选的实施方案中,所述病症是线粒体DNA耗竭综合症(MDS)。在更优选的实施方案中,MDS包括以TK2中的突变为特征的肌病形式、以SUCLA2中的突变为特征的脑肌病形式、以TYMP中的突变为特征的神经胃肠脑病形式和以DGUOK、POLG及MPV17中的突变为特征

的肝病形式的病症。在最优选的实施方案中，所述病症是以TK2基因中的突变为特征的胸苷激酶2缺乏症。

[0019] 可用包括施用脱氧核苷的本发明的方法治疗所有的线粒体DNA耗竭综合症。可通过本发明的方法治疗的MDS的实例包括但不限于以下基因中的缺陷：编码脱氧鸟苷激酶dGK的DGUOK基因；编码核糖核苷酸还原酶RNR的p53诱导型小亚基p53R2的RRM2B基因；以及编码胸苷磷酸化酶TP的TYMP基因。

[0020] 在优选的实施方案中，脱氧核苷是脱氧胸苷(dT)或脱氧胞苷(dC)或其混合物。脱氧腺苷(dA)和脱氧鸟苷(dG)单独或一起也可用在本发明的方法中。一种脱氧核苷(即，dT、dC、dA或dG)以及任意四种脱氧核苷中的两种或更多种的混合物可用在本发明的方法中。

[0021] 脱氧核苷的优选剂量介于约100与约1,000mg/kg/天之间，更优选介于约300与约800mg/kg/天之间，且最优选介于约250与约600mg/kg/天之间。如果组合物包含单一脱氧核苷，则剂量是单一脱氧核苷的剂量。如果组合物包含不只一种脱氧核苷，则剂量可以是组合物中每种脱氧核苷的剂量或总脱氧核苷的剂量。

[0022] 脱氧核苷的施用可以是每日一次、每日两次、每日三次、每日四次、每日五次、最多每日六次，优选按定时间隔施用。

[0023] 优选的施用方法是口服、鞘内、静脉内和经肠施用。

[0024] 刚在有以不平衡的核苷酸库为特征的病症(例如，MDS)的嫌疑之时就应开始施用脱氧核苷，并且施用持续贯穿患者的生命周期。包括TK2缺乏症在内的这类病症的诊断测试是本领域中已知的。

[0025] 附图简述

[0026] 为了说明本发明的目的，在附图中描述了本发明的某些实施方案。然而，本发明不限于附图中描述的实施方案的确切安排及手段方式。

[0027] 图1描述从出生后第4天用260mg/kg/天或520mg/kg/天的脱氧胞苷(dC)和脱氧胸苷(dT)处理的野生型($Tk2^{+/+}$和$Tk2^{+/-}$)以及$Tk2^{-/-}$小鼠的生长曲线。每个符号表示在每个时间点的体重的平均值。每个组的N示于图中。

[0028] 图2描述采用以下处理的野生型($Tk2^{+/+}$)以及$Tk2^{-/-}$小鼠的存活曲线：$Tk2^{-/-乳汁}$对比$Tk2^{-/-200mg/kg/天dCMP+dTMP}$，$p=0.0013$；$Tk2^{-/-乳汁}$对比$Tk2^{-/-260mg/kg/天dC+dT}$，$p=0.0006$；$Tk2^{-/-乳汁}$对比$Tk2^{-/-520mg/kg/天dC+dT}$，$p<0.0001$；$Tk2^{-/-260mg/kg/天dC=dT}$对比$Tk2^{-/-520mg/kg/天dCdT}$，$p=0.0009$，均在出生后第4天。每个组的N示于图中。由Mantel-Cox检验确定p值。

[0029] 图3是从野生型($Tk2^{+/+}$)以及$Tk2^{-/-}$的脑和肝组织中分离的线粒体中的dNTP的相对比例的图示，上述的小鼠是在出生后第13日龄(上图)和出生后第29日龄(下图)时未处理的或用200mg/kg/天的dCMP和dAMP或者260mg/kg/天或520mg/kg/天的脱氧胞苷(dC)和脱氧胸苷(dT)处理的。

[0030] 图4是显示与$Tk2^{-/-}$小鼠相比，野生型Tk2小鼠($Tk2^{+/+}$)(左手侧条)的脑、肝、肠和肌肉中的mtDNA/nDNA的比率的图示，上述的小鼠是在出生后第13和29日龄时未处理的或用260mg/kg/天或520mg/kg/天的脱氧胞苷(dC)和脱氧胸苷(dT)处理的。数据被表示为mtDNA拷贝相对于$Tk2^{+}$的百分比的平均值±标准偏差(SD)。由Mann-Whitney检验估定p值。(*$p<0.05$，**$p<0.01$，***$p<0.001$，****$p<0.0001$)。

[0031] 图5是描述测量小鼠的血浆中的dT和尿嘧啶的HPLC的结果的图示，所述小鼠是未

处理的野生型($Tk2^{+/+}$)小鼠、用260mg/kg/天的脱氧胞苷(dC)和脱氧胸苷(dT)处理的野生型($Tk2^{+/+}$)小鼠、用260mg/kg/天的脱氧胞苷(dC)和脱氧胸苷(dT)处理的$Tk2^{-/-}$小鼠以及用200mg/kg/天的dCMP和dTMP处理的$Tk2^{-/-}$小鼠，上述经处理的小鼠是处理后30分钟的。数据被表示为平均值±SD。

[0032] 图6是在出生后13天用400mg/kg/天的dCMP和dTMP及THU处理、在出生后13和29天用260mg/kg/天的脱氧胞苷(dC)和脱氧胸苷(dT)处理或者在出生后29天用520mg/kg/天的脱氧胞苷(dC)和脱氧胸苷(dT)处理的$Tk2^{-/-}$小鼠中的呼吸链酶活性的水平的图示。对于各处理，数据被表示为$Tk2^{-/-}$小鼠组织中的RCE活性的百分比，其是针对蛋白质水平标准化且相对于$Tk2^{+}$的。由Mann-Whitney检验确定p值。$^{*}p<0.05$。

[0033] 图7A是在出生后29天用260mg/kg/天或520mg/kg/天的脱氧胞苷(dC)和脱氧胸苷(dT)处理的野生型小鼠以及用260mg/kg/天或520mg/kg/天的脱氧胞苷(dC)和脱氧胸苷(dT)处理的$Tk2^{-/-}$小鼠中的呼吸链蛋白的免疫印迹。图7B是显示针对复合物II标准化的RCE水平的图示，表示为$TK2^{+/+}$小鼠中的RCE水平的百分比。由Mann-Whitney检验估计p值。

[0034] 缩写词:CS＝柠檬酸合酶;CI＝NADH-脱氢酶;CII＝琥珀酸脱氢酶;CIII＝细胞色素c还原酶;CIV＝细胞色素c氧化酶(COX);CI+III＝NADH-细胞色素c还原酶;CII+III＝琥珀酸脱氢酶-细胞色素c还原酶。

具体实施方式

[0035] 本发明基于意外的发现，即可以用脱氧核苷治疗线粒体DNA耗竭综合症，包括TK2缺乏症。如本文的结果所示，脱氧核苷的施用极大地改善了TK2缺乏症的小鼠模型和患有TK2缺乏症的人类患者中的病状。

[0036] 定义

[0037] 本说明书中所用的术语在本发明的上下文以及使用每个术语的具体上下文内具有它们在所属领域中的普通含义。下文或在说明书的别处讨论了某些术语以对执业医生提供描述本发明的方法以及如何加以使用的附加指导。此外，将要理解的是，可以按不只一种方式叙述同样的事情。因此，替代性语言和同义词可用于本文讨论的任何一个或多个术语，本文是否详尽阐述或讨论术语也不被赋予任何特殊意义。提供了某些术语的同义词。引述一个或多个同义词不排除使用其它同义词。说明书中任何地方的实例(包括本文讨论的任何术语的实例)的使用仅是说明性的，并且绝不是要限制本发明或任何示例的术语的范围及含义。同样，本发明不限于其优选的实施方案。

[0038] 如本申请中所用的术语“受试者”意指哺乳动物。哺乳动物包括犬科动物、猫科动物、啮齿类动物、牛科动物、马科动物、猪科动物、绵羊科动物和灵长类动物。因此，本发明可用在兽医学中(例如)以治疗伴侣动物、农场动物、动物园中的实验动物和野外中的动物。本发明对于人类医学应用是特别理想的。

[0039] 如本申请中所用的术语“患者”意指人类受试者。在本发明的一些实施方案中，“患者”已知或疑似患有以不平衡的核苷酸库为特征的疾病或病症、线粒体疾病、线粒体DNA耗竭综合症或TK2缺乏症。

[0040] 短语“治疗有效量”用在本文中意指足以引起受试者中的临床显著病状的改善或者使与疾病或病症相关的一种或多种症状延迟或最小化或减轻或者在受试者中导致所需

的生理学有益变化的量。

[0041] 术语“治疗”(treat/treatment)等是指减慢、解除、改善或缓解疾病或病症的至少一种症状或在疾病或病症发作后将其逆转的方法。

[0042] 术语“预防”(prevent/prevention)等是指在明显的疾病或病症发作之前起作用以防止疾病或病症发展或使疾病或病症的程度最小化或减缓其发展进程。

[0043] 术语“有需要的”是已知或疑似患有或有风险患以不平衡的核苷酸库为特征的疾病或病症、线粒体疾病、线粒体DNA耗竭综合症或TK2缺乏症的受试者。

[0044] 如本文所用的术语“药剂”意指产生或能够产生疗效的物质,并且将包括但不限于化学品、药品、生物制品、有机小分子、抗体、核酸、肽和蛋白质。

[0045] 如本文所用的术语“脱氧核苷”意指脱氧胸苷或dT、脱氧胞苷或dC、脱氧腺苷或dA和脱氧鸟苷或dG。每一者的全长名称和常用缩写将可互换使用。这类脱氧核苷还包括脱氧核苷的生理学功能衍生物。

[0046] 如本文所用,术语“生理学功能衍生物”是指在体内转化以产生脱氧核苷的化合物(例如,药物前体)。转化可通过各种机制(例如,通过代谢或化学过程)发生,例如经过在血液中水解。前药是这类衍生物,并且T.Higuchi和W.Stella在“Pro-drugs as Novel Delivery Systems”,A.C.S.Symposium Series的第14卷中以及Bioreversible Carriers in Drug Design,编著Edward B.Roche,American Pharmaceutical Association and Pergamon Press,1987中给出了前药的用途的讨论。

[0047] 如本文所用,“副作用”是通过施用药物引起的不想要的反应。在大多数情况下,施用脱氧核苷不会引起副作用。最意料之中的副作用将是轻微的胃肠道不耐受。

[0048] 术语“约”或“大约”意指对于由本领域普通技术人员确定的特定值在可接受的误差范围内,所述可接受的误差范围将部分地取决于如何测量或确定该值,即测量系统的限制,即对于特定目的(如药物制剂)所需的精确度。例如,“约”可意指按本领域中的惯例在1或多于1个标准偏差以内。或者,“约”可意指给定值的最多20%、优选最多10%、更优选最多5%及还更优选最多1%的范围。或者,特别是关于生物系统或过程,该术语可意指在一个数量级以内,优选在值的5倍以内,且更优选在值的2倍以内。在申请和权利要求中描述特定值的情况下,除另有说明外,应假定术语“约”意指对于特定的值在可接受的误差范围内。

[0049] 施用脱氧核苷用以治疗线粒体DNA耗竭综合症

[0050] 线粒体DNA(mtDNA)耗竭综合症(MDS)包括以感染组织中的mtDNA拷贝数减少为特征的若干严重的常染色体疾病。大多数MDS致病性核基因编码属于mtDNA复制机制的蛋白质或参与三磷酸脱氧核糖核苷(dNTP)代谢。

[0051] MDS的一种形式是胸苷激酶缺乏症或TK2。由核基因TK2编码的TK2是使胸苷和脱氧胞苷核苷磷酸化以产生单磷酸脱氧胸苷(dTMP)和单磷酸脱氧胞苷(dCMP)的线粒体基质蛋白,单磷酸脱氧胸苷和单磷酸脱氧胞苷继而被转化为线粒体DNA合成所需的三磷酸脱氧核苷酸(dNTP)。如在背景部分中所讨论,常染色体隐性TK2突变在婴幼儿和儿童中引起伴有线粒体DNA(mtDNA)的严重耗竭的破坏性神经肌肉虚弱,以及在成人中引起伴有mtDNA多重缺失的渐进性外部眼肌麻痹。许多患者不能行走,并且需要一些类型的机械通气和饲管。中枢神经系统以可变的方式被牵涉进这些病症,伴有的症状包括癫痫发作、脑病变、认知损害和听力丧失。生存超过42年的患者不到7%。

[0052] 基于如此诊断的患者的临床及分子遗传学调查结果，三种疾病表现得到确认：i)婴幼儿发作的(≤1岁)肌病变，在生命的第一年开始虚弱，伴有严重的mtDNA耗竭和早期死亡；ii)儿童期发作的(>1-11岁)肌病变，伴有严重的mtDNA耗竭；和iii)晚发性肌病变(≥12岁)，发作时轻度虚弱，并缓慢进展为行走力丧失、呼吸功能不全或兼而有之，青春期或成年期中往往伴有与mtDNA多重缺失、mtDNA拷贝数减少或这两者关联的慢性渐进性外部眼肌瘫痪。准备中一般参见Garone等人，(2016)。

[0053] 使用来自患者培养的成纤维细胞研究TK2缺乏症的发病机制及测试其疗法的尝试一直是不成功的，因为复制细胞未能表现出mtDNA耗竭。相比之下，纯合Tk2H126N敲入突变($Tk2^{-/-}$)小鼠模型表现出与由TK2突变引起的人类婴幼儿脑肌病变惊人相似的表型，其特征为在10日龄时发作，伴随行走力下降、步态不稳、不雅震颤、生长迟缓，并且线粒体DNA(mtDNA)的耗竭迅速进展为在14至16日龄时的早期死亡，这是类似于人类婴幼儿发作的疾病的时间段(Akman等人，2008；Dorado等人，2011)。

[0054] 本文阐述的采用Tk2敲入小鼠进行的研究已显示，口服dC/dT的施用延长了TK2缺乏症的临床症状的延迟发作，并且将小鼠的寿命延长两到三倍(实施例2)。

[0055] 另外的实验显示出组织特异性效果。测量线粒体提取物中的dNTP库水平显示，dCTP在脑中被拯救，并且dTTP在肝中被拯救(实施例3)。测量mtDNA耗竭显示，dCMP+dTMP和dC+dT疗法都拯救了肝、肌肉和组织中的mtDNA拷贝数(实施例4)。以前推测的是，血脑屏障的形成可能损害脑中的治疗生物利用度。然而，HPLC测量显示，在单磷酸核苷酸和脱氧核苷处理后均以较高的浓度发现这些化合物的催化产物，表明它们能够跨越血脑屏障。mtDNA耗竭测量也显示出肠中的mtDNA拷贝数的完全拯救。

[0056] 因此，本文阐述的使用Tk2缺乏症的小鼠模型的实验显示，施用脱氧核苷对于治疗疾病是有效和安全的。另外，如实施例5中所示，施用dT和dC极大地改善了患者的TK2缺乏症的症状。

[0057] 因此，本发明包括对有需要的患者施用至少一种脱氧核苷。在一个实施方案中，本发明包括施用至少一种脱氧嘧啶。在进一步的实施方案中，脱氧嘧啶选自dC、dT及其混合物。在又一实施方案中，本发明包括施用至少一种脱氧嘌呤。在进一步的实施方案中，脱氧嘌呤选自dA、dG及其混合物。

[0058] 会受益于施用脱氧核苷的患者将是被诊断为患有TK2缺乏症的那些。在这些患者中，将施用至少一种脱氧嘧啶dC或dT或其混合物。

[0059] 由于DGUOK中的常染色体隐性突变所致的脱氧鸟苷激酶(dGK)的并行缺陷(伴有dGMP和dAMP缺乏)引起mtDNA耗竭，通常表现为儿童早期发作的肝脑疾病(Mandel等人，2001)。这些患者将会受益于施用至少一种脱氧嘌呤dG或dA或其混合物。

[0060] 可通过施用特定的脱氧核苷(即dA、dG、dC或dT或其混合物)来治疗其它形式的MDS以及与不平衡的核苷酸库有关的其它病症。这些病症将包括但不限于与RRM2B(编码核糖核苷酸还原酶RNR的p53诱导型小亚基p53R2)有关的缺陷和TYMP(编码胸苷磷酸化酶TP)中的突变，后者引起线粒体神经胃肠脑肌病变(MNGIE)。破坏线粒体dNTP库的另外的核基因包括但不限于SUCLA2、SUCLG1和MPV17。也可以通过施用一种或多种脱氧核苷来治疗与这些基因有关的病症。

[0061] 另外，由于其它形式的MDS及其它病症的机制得到了阐明，因而可由熟练的从业人

员来确定用于治疗的适当脱氧核苷。

[0062] 可以对显示出上文针对TK2缺乏症(包括以一般性张力减退、近端肌肉无力、先前获得的运动技能丧失、喂养不良和呼吸困难为特征的渐进性肌肉疾病的最典型表现)讨论的表型的患者进行测试以明确地诊断疾病。

[0063] 如果临床表现高度疑似mtDNA耗竭综合症,则应使用已知引起mtDNA耗竭综合症的一组基因进行分子遗传测试(Chanprasert等人,2012)。TK2基因是已知其中的突变引起TK2相关性线粒体DNA耗竭综合症的唯一基因。这种测试可包括对TK2的整个编码和外显子/内含子接合区进行的序列变异和缺失/重复的序列分析。如果在序列分析中确认出复合杂合或纯合有害的突变,则TK2缺乏症的诊断得到证实,并且因此,受试者将受益于脱氧核苷疗法。如果序列分析没有确认出两种复合杂合或纯合有害的突变,则应考虑缺失/重复分析以确定和/或证实TK2缺乏症诊断。

[0064] 用以确定和/或证实TK2缺乏症诊断的进一步测试可包括测试血清肌酸激酶(CK)浓度、肌电图、骨骼肌上的组织病理学、线粒体DNA(mtDNA)含量(拷贝数)和骨骼肌中的电子传递链(ETC)活性。如果在这些测试中发现以下中的一项或多项,则TK2缺乏症得到确定和/或证实。与健康的对照相比升高的CK浓度可指示TK2缺乏症。可进行骨骼肌活检,然后进行骨骼肌中的mtDNA含量分析。如果骨骼肌活检显示出纤维尺寸上的显著差异、可变的肌浆液泡、可变增多的结缔组织和破碎样红纤维以及增加的琥珀酸脱氢酶(SDH)活性且细胞色素c氧化酶(COX)活性低至没有活性,并且mtDNA拷贝数严重减少(通常不到年龄和组织匹配的健康对照的20%),则TK2缺乏症的诊断可得到确定和/或证实(Chanprasert等人,2012)。

[0065] 另外,TK2缺乏症是以常染色体隐性方式遗传的。因此,可在感染患者的同胞出生后尽早对其进行测试以诊断疾病。

[0066] 在所有这些实例中,在诊断TK2缺乏症后应尽快开始脱氧核苷疗法。

[0067] 药物组合物、施用的方法和剂量

[0068] 本发明包括施用脱氧核苷,更具体地是施用一种或多种脱氧核苷。

[0069] 最优选的施用方法是口服、鞘内和亲本(parental)施用,包括静脉内施用。脱氧核苷必须呈适于所选择的施用的形式。

[0070] 脱氧核苷容易溶于液体容易溶于液体(如水、配方奶或乳汁),而游离酸形式不易溶于液体。

[0071] 包含一种或多种用于施用的脱氧核苷的这类药物组合物可包含治疗有效量的脱氧核苷和药学上可接受的载体。短语"药学上可接受的"是指这样的分子实体及组合物,当对人施用时其是生理上可耐受的,且通常不会产生过敏或类似的不良反应,如胃不适、眩晕等,并且经过联邦或州政府的管理机构批准,或者在美国药典或其它公认的药典中列出供在动物中且尤其是在人类中使用。"载体"是指用以施用治疗剂的稀释剂、佐剂、赋形剂或媒介物。这类药物载体可以是无菌液体,如水中的盐水溶液以及油,包括石油、动物、植物或合成来源的那些油,如花生油、大豆油、矿物油、芝麻油等。当静脉内施用药物组合物时,盐水溶液是优选的载体。盐水溶液和葡萄糖水溶液及甘油溶液也可用作液体载体,特别是用于可注射溶液。合适的药物赋形剂包括淀粉、葡萄糖、乳糖、蔗糖、明胶、麦芽、大米、面粉、白垩、硅胶、硬脂酸钠、单硬脂酸甘油酯、滑石、氯化钠、脱脂乳粉、甘油、丙烯、二醇、水、乙醇等。如果需要的话,组合物还可含有少量的润湿剂或乳化剂或pH缓冲剂。

[0072] 口服施用是优选的施用方法。可将脱氧核苷添加到患者将摄取的任何形式的液体中，包括但不限于乳汁(牛乳和人乳)、婴幼儿配方奶和水。

[0073] 另外，适于口服施用的药物组合物可以是胶囊、片剂、粉末、颗粒、溶液、糖浆、混悬液(在非水或水性液体中)或乳液。片剂或硬明胶胶囊可包含乳糖、淀粉或其衍生物、硬脂酸镁、糖精钠、纤维素、碳酸镁、硬脂酸或其盐。软明胶胶囊可包含植物油、蜡、脂肪、半固体或液体多元醇。溶液和糖浆可包含水、多元醇和糖。可将旨在用于口服施用的活性剂用延迟活性剂在胃肠道中的崩解和/或吸收的物质包衣或与之混合。如此，可实现经许多小时的持续释放，并且如果必要的话，可保护活性剂免于胃内降解。可将用于口服施用的药物组合物配制成由于特定的pH或酶促条件而便于在特定的胃肠位置释放活性剂。

[0074] 为了克服脱氧核苷跨越血/脑屏障的任何问题，鞘内施用是进一步优选的施用形式(Galbiati等人，2006;Gotz等人，2008)。鞘内施用涉及将药物注射到椎管里，更具体地是注射到蛛网膜下隙里，使其抵及脑脊液。这种方法常用于脊髓麻醉、化学疗法和疼痛药物治疗。可通过腰椎穿刺(推注)或通过药盒导管系统(推注或输注)进行鞘内施用。最常将导管插在腰椎的椎板之间，并将尖端向上穿过鞘隙至所需水平(通常为L3-L4)。鞘内制剂最常使用水和盐水作为赋形剂，但也已使用过EDTA和脂质。

[0075] 进一步优选的施用形式是肠胃外施用，包括静脉内施用。适于肠胃外施用(包括静脉内施用)的药物组合物包括水性及非水无菌可注射溶液或混悬液，其可含有抗氧化剂、缓冲剂、抑菌剂和使组合物与受试者的血液基本上等渗的溶质。可存在于这类组合物中的其它组分包括水、醇、多元醇、甘油和植物油。可将适于亲本施用的组合物提供在单位剂量或多剂量容器中，如密封的安瓿和小瓶中，并且可储存在冷冻干燥(冻干)条件下，临使用之前仅需要添加无菌载体。可由无菌粉末、颗粒和片剂制备临时注射溶液和混悬液。可用于提供本发明的肠胃外剂型的合适媒介物是本领域技术人员熟知的。实例包括：注射用水USP；水性媒介物，如氯化钠注射液、林格氏注射液、葡萄糖注射液、葡萄糖和氯化钠注射液以及乳酸化林格氏注射液；水混溶性媒介物，如乙醇、聚乙二醇和聚丙二醇；以及非水媒介物，如玉米油、棉籽油、花生油、芝麻油、油酸乙酯、肉豆蔻酸异丙酯和苯甲酸苄酯。

[0076] 另外，由于一些患者在脱氧核苷治疗开始的时候可能正在接受肠营养品，则可以通过烹饪饲管或其它肠营养手段施用dN。

[0077] 进一步的施用方法包括经粘膜施用，如经鼻、舌下、经阴道、经颊或经直肠施用；或对受试者透皮施用。

[0078] 适于经鼻和经肺施用的药物组合物可包含诸如粉末的固体载体，其可借助于通过鼻子快速吸入来施用。用于经鼻施用的组合物可包含液体载体，如喷雾剂或滴剂。或者，可借助于深度吸入或通过吹口的装置来完成直接吸入到肺里。这些组合物可包含活性成分的水溶液或油溶液。可在特别适应的装置中提供用于吸入的组合物，这些装置包括但不限于加压喷雾器、雾化器或吹入器，可将它们构造成提供预定剂量的活性成分。

[0079] 可将适于经直肠施用的药物组合物提供为栓剂或灌肠剂。可将适于经阴道施用的药物组合物提供为阴道栓、棉塞、乳膏剂、凝胶剂、糊剂、泡沫剂或喷雾制剂。

[0080] 可将适于透皮施用的药物组合物提供为分立的贴剂，其旨在经长时间段与接受者的表皮保持紧密接触。

[0081] 脱氧核苷疗法包括施用选自脱氧胸苷(dT)、脱氧胞苷(dC)、脱氧腺苷(dA)和脱氧

鸟苷(dG)的一种或多种脱氧核苷。

[0082] 熟练的执业医生可根据所述缺乏症确定哪种脱氧核苷是有益的。执业医生确定是否应该以及按何种比例施用脱氧核苷的混合物也是本领域技术范围之内的事情。如果要施用两种脱氧核苷,则它们可以按各脱氧核苷(例如,dC和dT)为50/50的比例,或者按约5/95、10/90、15/85、20/80、25/75、30/70、35/65、40/60、45/55、55/45、60/40、65/35、70/30、75/25、80/20、85/15、90/10和95/5的比例。

[0083] 举例来说,对于TK2缺乏症,以等量的混合物施用dT和dC。

[0084] 本领域技术人员将考虑若干因素来确定治疗有效剂量的选择,这对本领域普通技术人员来说是已知的。这类因素包括脱氧核苷的特定形式及其药代动力学参数,如生物利用度、代谢和半衰期,这些将是在通常用来获得药物化合物的监管批准的常规开发程序期间已确立好的。考虑剂量的进一步因素包括要治疗的病状或疾病或要在正常个体中取得的有益效果、患者的体重、施用途径、是短期还是长期施用、伴随的药物治疗以及影响施用的药剂的疗效的其它众所周知的因素。因此,应该根据本领域技术人员的判断及每一患者的情况并根据标准的临床技术来决定精确的剂量。

[0085] 优选的剂量范围是约100mg/kg/天至约1,000mg/kg/天。进一步优选的剂量范围是约200mg/kg/天至约800mg/kg/天。进一步优选的剂量范围是约250mg/kg/天至约400mg/kg/天。这些剂量是单独的脱氧核苷的量或具有不只一种脱氧核苷(例如,dT和dC)的混合物的组合物的量。例如,一个剂量可单独包含400mg/kg/天的dT。在进一步的实例中,一个剂量可包含200mg/kg/天的dT和200mg/kg/天的dC的混合物。在进一步的实例中,一个剂量可包含400mg/kg/天的dT和dC的混合物。

[0086] 脱氧核苷的施用可以是一天一次、一天两次、一天三次、一天四次、一天五次、最多一天六次,优选按定时间隔施用。例如,当每天四次施用脱氧核苷时,给药将是在8:00AM、12:00PM、4:00PM和8:00PM。

[0087] 如果是静脉内或鞘内施用的话,则也可以降低剂量。这种施用的优选剂量范围是约50mg/kg/天至约500mg/kg/天。

[0088] 如实施例5中所示,可调节剂量以优化在受试者中的效果。例如,可按100mg/kg/天开始施用脱氧核苷,然后随着时间的推移增加到200mg/kg/天、到400mg/kg/天、到800mg/kg/天、最多1000mg/kg/天,这取决于受试者的反应和耐受性。

[0089] 在增加剂量之前可监测受试者的病状改善情况。可通过观察受试者的肌肉力量和控制及行动能力以及身高和体重的变化来监测受试者对脱氧核苷的治疗性施用的反应。如果在施用后这些参数中的一者或多者增加,则可以继续进行治疗。如果这些参数中的一者或多者保持不变或减少,则可以增加脱氧核苷的剂量。

[0090] 如实施例中所示,脱氧核苷是耐受性良好的。任何观察到的副作用都是轻微的,并且主要是腹泻、腹胀及其它胃肠道临床表现。还可监测受试者中的任何副作用,如胃肠不耐受,例如腹泻。如果在施用后观察到一种或多种副作用,则可以减少剂量。如果没有观察到这类副作用,则可以增加剂量。另外,一旦由于观察到副作用而减少剂量,并且不再能观察到副作用时,可以增加剂量。

[0091] 也可以将脱氧核苷与其它药剂共同施用。这类药剂将包括用于治疗特定形式的MDS的症状的治疗剂。特别地,对于TK2缺乏症,可将dT和dC与普遍性核苷分解代谢酶的抑制

剂共同施用，这些抑制剂包括但不限于诸如四氢尿苷(胞苷脱氨酶的抑制剂)和免疫霉素H(嘌呤核苷磷酸化酶的抑制剂)及地匹福林(tipiracil，胸苷磷酸化酶的抑制剂)的酶抑制剂。这类抑制剂是已知的并且用于治疗一些癌症。

[0092] 实施例

[0093] 通过参考以下非限制性实施例可以更好地理解本发明，提供这些实施例是为了更全面地说明本发明的优选实施方案。它们绝不应被解释为限制本发明的宽广范围。

[0094] 实施例1-材料和方法

[0095] TK2缺乏症的小鼠模型

[0096] 先前已报道了纯合Tk2H126N敲入突变($Tk2^{-/-}$)小鼠，其表现出与人类婴幼儿脑肌病变惊人相似的表型(Akman等人，2008)。在出生后第10天与第13天之间，$Tk2^{-/-}$小鼠迅速显露出致命性脑肌病变，其特征为行走力下降、步态不稳、不雅震颤、生长迟缓，并且迅速进展为在14至16日龄时的早期死亡。小鼠模型的分子及生化分析表明，该疾病的发病机制是由于酶活性的丧失和随之而来的dNTP库不平衡，伴随脑中dTTP水平以及肝中dTTP和dCTP两者水平降低，这些继而产生mtDNA耗竭和含有mtDNA编码的亚基的呼吸链酶的缺陷，最突出是在脑和脊髓中。

[0097] 所有的实验都是根据由哥伦比亚大学医学中心的研究机构动物护理与使用委员会(Institutional Animal Care and Use Committee)批准的方案进行的，并且符合国立卫生研究院护理与使用实验动物指南。根据国际标准条件安置和饲养小鼠，采用12小时光照、12小时黑暗循环，并在4、13和29日龄时处死。

[0098] 取出器官(脑、脊髓、肝、心脏、肾、四头肌、肺和胃肠道)并冷冻在异戊烷的液相中，用干冰在其凝固点(-160℃)附近预冷却，或者固定于10％中性缓冲福尔马林中，并采用标准程序包埋在石蜡中。然后将石蜡包埋的组织用苏木精和曙红(H&E)染色进行形态学研究，或者如在补充程序中详述的那样处理以用GFAP、COX I或复合物I亚基进行免疫染色研究。杂合及纯合野生型小鼠均被视为对照组($Tk2^{+}$)，因为先前没有描述过临床和生化差异(Akman等人，2008；Dorado等人，2011)。

[0099] 治疗施用和实验计划

[0100] 对Tk2H126N敲入小鼠($Tk2^{-/-}$)和年龄匹配的对照野生型($Tk2^{+}$)，从出生后第4至29天采用260mg/kg/天和520mg/kg/天这2个剂量，通过每日口服喂饲施用在50μl用于小宠物的Esbilac配方乳汁(Pet-Ag)中的脱氧胞苷(dC)和脱氧胸苷(dT)。在21日龄时，将小鼠与母亲分开，并通过分别采用1.6mM和3.2mM的等摩尔剂量施用饮用水中的dC和dT来继续进行处理。将未处理的Tk2突变体和对照野生型小鼠的阴性对照组称重并仔细观察以进行比较。

[0101] 表型评估

[0102] 每日对体重进行评估，因为先前已经观察到，不能增重是疾病的第一征兆(Akman等人，2008)。

[0103] 为确定dT/dC的安全度和疗效，将经处理和未处理的Tk2小鼠中的存活时间、疾病发作时龄、症状的类型和严重程度、副作用的发生以及由于不良事件而终止处理的比例进行比较。在出生后第4天开始每日评估小鼠的一般行为、存活时间和体重。

[0104] 通过聚合酶延伸测定法测量的dNTP库

[0105] 在10体积(w/v)的冷MTSE缓冲液(210mM甘露醇、70mM蔗糖、10mM Tris-HCl pH

7.5、0.2mM EGTA、0.5％BSA)中将组织在冰上均化，并在4℃下以1000g离心5分钟，接着在4℃下三次以13,000g离心2分钟。将上清液用60％甲醇沉淀，在-80℃下保持2小时，煮沸3分钟，在-80℃下储存(1小时至过夜)并在4℃下以20,800g离心10分钟。将上清液蒸发至干并将沉淀再悬浮于65μl水中，并且在分析以前储存于-80℃下。为使核糖核苷酸干扰最小化，如报道的那样测定总dNTP库(Ferraro等人，2010;Marti等人，2012a)。简言之，通过将5μl样品或标准dNTP与15μl反应缓冲液[0.025U/ml ThermoSequenase DNA聚合酶(GE Healthcare,Piscataway,NJ,USA)或Taq聚合酶(Life Technologies,NY,USA)、0.75μM 3H-dTTP或3H-dATP(Moravek Biochemicals)、0.25μM特定的寡核苷酸、40mM Tris-HCl pH 7.5、10mM MgCl2、5mM DTT]混合产生20μl体积反应物。在48℃下60分钟后，将18ml反应物点样在Whatman DE81过滤器上，风干并用5％Na2HPO4洗涤三次持续10分钟，一次在蒸馏水中且一次在无水乙醇中。通过闪烁计数测定保留的放射性。

[0106] 通过HPLC测量的核苷测量结果

[0107] 通过如先前所述的梯度洗脱HPLC方法(Lopez等人，2009;Marti等人，2012b)稍作修改来评估脱氧胸苷(dT)、脱氧尿苷(dU)、尿嘧啶(U)和胸腺嘧啶(T)水平。简言之，以1.5ml/分钟的恒定流速(除指出的情况外)将脱蛋白样品注入带有Alltima C18NUC反相柱(Alltech)的Alliance HPLC系统(Waters Corporation)，使用四种缓冲液：洗脱液A(20mM磷酸钾，pH 5.6)、洗脱液B(水)和洗脱液C(甲醇)。以如下梯度经60分钟洗脱样品：0-5分钟，100％洗脱液A;5-25分钟，100-71％洗脱液A，29％洗脱液B;25-26分钟，0-100％洗脱液C;26-30分钟，100％洗脱液C;30-31分钟，0-100％洗脱液B;31-35分钟，100％洗脱液B(1.5-2ml/分钟);35-45分钟，100％洗脱液B(2ml/分钟);45-46分钟，100％洗脱液B(2-1.5ml/分钟);46-47分钟，0-100％洗脱液C;47-50分钟，100％洗脱液C;50-51分钟，0-100％洗脱液A;和51-60分钟，100％洗脱液A。

[0108] 在267nm处监测洗脱物的吸光度，并将dThd和dUrd峰定量，方式是将它们的峰面积与用水性标准物获得的校准曲线进行比较。为了明确地确认每个样品的脱氧胸苷、脱氧尿苷、尿嘧啶和胸腺嘧啶峰，将第二等分试样用过量的纯化大肠杆菌TP(Sigma)处理以特异性地消除dT和dU。此方法对于所有核苷的检测限为0.05mmol/l。结果表示为nmol/mg蛋白质。

[0109] RT-qPCR：线粒体DNA定量

[0110] 在Step One Plus实时PCR系统(Applied Biosystems)中采用ddCt方法，如所描述的那样用鼠COX I基因(mtDNA)及小鼠甘油醛-3-磷酸脱氢酶(GAPDH,nDNA)的引物和探针(Applied Biosystems,Invitrogen,Foster City,CA,USA)进行实时PCR(Dorado等人，2011)。将MtDNA值针对nDNA值标准化，并表示为相对于野生型(100％)的百分比。

[0111] 线粒体呼吸链蛋白质水平

[0112] 将三十微克的全脑大脑或小脑提取物在SDS-12％PAGE凝胶中电泳，转移至Immun-Blot™ PVDF膜(Biorad,Hercules,CA,USA)上，并用抗体MitoProfile®总OXPHOS啮齿动物WB混合型抗体(MitoProfile® Total OXPHOS Rodent WB Antibody Cocktail)(MitoSciences,Eugene,OR,USA)进行探测。使用Amersham™ ECL Plus蛋白质印迹检测系统(GE Healthcare Life Sciences,UK)，用过氧化物酶缀合的小鼠抗小鼠IgG抗体(Sigma-Aldrich,St Louis,MO,USA)检测蛋白质-抗体相互作用。使用NIH ImageJ 1.37V软件进行蛋白质的定量。在选定的区域内平均灰度值计算为选择的所有像素的灰度值的总和除以像

素的数量。

[0113] 通过分光光度计分析测量的线粒体呼吸链酶活性

[0114] 如先前描述的那样在大脑组织中进行线粒体RC酶分析(DiMauro等人,1987)。

[0115] 统计方法

[0116] 将数据表示为每组至少3次实验的平均值±SD。采用Gehan-Breslow-Wilcoxon检验比较各组小鼠的存活比例。p值<0.05被视为是统计学显著的。

[0117] 实施例2-对$Tk2^{-/-}$小鼠施用dC/dT延迟TK2缺乏症的临床发作并增加存活者

[0118] 对$Tk2^{-/-}$小鼠施用剂量为260和520mg/kg/天的脱氧核苷(dC/dT)中的每一者。这些剂量的脱氧核苷分别为400和800mg/kg/天的摩尔当量的dCMP+dTMP。

[0119] 用口服dC+dT(从4日龄起260或520mg/kg/天)处理的小鼠直到出生后第21天看起来还是正常的(图1)。在21日龄后,用260mg/kg/天剂量处理的突变小鼠($Tk2^{-/-260mg/kg/天\ dC/dT}$)停止增重并显露出轻微的头部震颤和虚弱,其导致在出生后第31±4.3天死亡(图2)。

[0120] 用520mg/kg/天的dC+dT($Tk2^{-/-520mg/Kg/天\ dC/dT}$)处理的突变小鼠继续再增重一周,但随后表现出与$Tk2^{-/-260mg/Kg/天\ dC/dT}$类似的恶化,并在出生后第43±10天死亡。这些结果与用200或400mg/kg/天的口服dCMP/dTMP处理的$Tk2^{-/-}$小鼠所显示的相当。关注$Tk2^{+260mg/kg/天\ dC/dT}$和$Tk2^{+520mg/kg/天\ dC/dT}$直到出生后第60天。没有观察到副作用。

[0121] 如所示的那样,经处理的$Tk2^{-/-}$的寿命显著增加。未处理的$Tk2^{-/-}$小鼠显示平均寿命为13天,而用260和520mg/kg/天剂量处理的小鼠分别存活了平均31天和40天(图2)。有意思的是,其中一只小鼠存活到出生后第56天,这是迄今为止Tk2敲入小鼠模型的最长寿命。

[0122] 实施例3-口服dC/dT改善脑和肝中的分子异常

[0123] 测量线粒体提取物中的dNTP显示,在出生后第13天,$Tk2^{-/-260mg/Kg/天\ dC/dT}$和$Tk2^{-/-520mg/Kg/天\ dC/dT}$均没有完全纠正线粒体dNTP库不平衡,并且在组织中表现出可变的效果,在脑中有dCTP不足的完全拯救,而dTTP在肝中得到纠正。相比之下,尽管有脱氧核苷补充,但脑中的dTTP和肝中的dCTP的缺乏仍然严重(图3)。

[0124] 在出生后第13天的$Tk2^{-/-260mg/Kg/天\ dC/dT}$和$Tk2^{-/-520mg/Kg/天\ dC/dT}$小鼠中,处理防止了心脏、肝、肾、肠和肌肉中的mtDNA耗竭(图4)。相比之下,mtDNA拷贝数仅在出生后第13天以剂量依赖性方式在脑中部分地得到改善,其中mtDNA/nDNA比值相对于对照脑在260mg/kg/天的dC+dT时达到了39%,且在520mg/kg/天时达到了52%。通过HPLC测量脑中的碱基dT和尿嘧啶显示在用dC+dT处理或用dCMP+dTMP处理的动物中水平较高(图5),进一步表明脱氧核苷和单磷酸脱氧核苷均跨越血脑屏障。在出生后第29天,260和520mg/kg/天的dC+dT疗法在心脏中(40和35%)、肝中(46和45%)、肾中(38和42%)以及肌肉中(24和35%)部分地拯救了mtDNA耗竭,但突出的是在肠中(82和84%)完全拯救(图4)。

[0125] 实施例4-口服dC/dT改善脑中的生化异常

[0126] 呼吸链酶(RCE)活性和蛋白质水平在出生后第13天的$TK2^{-/-260mg/Kg/天\ dC/dT}$的脑中得到完全拯救(图6)。RCE活性在出生后第29天也得到恢复,并且在$TK2^{-/-52\ 0mg/Kg/天\ dC/dT}$中仅能观察到复合物I活性略有降低(图6)。脑中的RCE蛋白质水平在出生后第29天部分地得到恢复,在$TK2^{-/-520mg/Kg/天\ dC/dT}$中比在$TK2^{-/-260mg/Kg/天\ dC/dT}$中水平高(图7)。在出生后第29天的经处理的突变小鼠的脑中,这些蛋白质水平的差异与mtDNA耗竭的差异是一致的,并且有可能解释了在较高剂量时观察到的存活延长。

[0127] 实施例5-在患有TK2缺乏症的患者中施用dC/dT是有效的

[0128] 下面总结了在发明者的监督和控制下已接受脱氧核苷疗法的TK2缺乏症患者的症状、剂量和结果。

[0129] 患者1

[0130] 此患者2011年2月出生于美国。他的症状表现为在12个月时伴有张力减退且头部疲软。他从来没有走过。他还患有呼吸肌无力，并且在19个月时被施以机械通气，他始终是24小时/天依赖于此。他从19个月时也一直依靠饲管。

[0131] 他先前接受100mg/kg/天且然后是200mg/kg/天的dCMP和dTMP。依靠这种疗法，他能够抓握小的物体，并且他的体重从10.4kg增加到19.5kg。

[0132] 在2015年10月，他开始接受260mg/kg/天的dC和dT，且剂量增加到340mg/kg/天的dC和dT。两个月后，他的双手和头部活动得更好，在人的支持下能够站立5分钟，开始咳嗽，并且他的心率更缓慢了(从白天140-170bpm降到白天100-120bpm)。

[0133] 在2016年3月23日，将剂量增加到400mg/kg/天的dC和dT。经此疗法6周后，他显示出进一步的改善：他能够在椅子上坐约5小时/天；在“架子”上站立1.5小时；行将抓起并把持小的毛绒动物玩具；按下电脑按钮；解开他的尿布并瞄准他的阴茎弄湿更换尿布的人；并屈膝几秒钟。

[0134] 治疗期间观察到仅有的副作用是腹泻。

[0135] 患者2

[0136] 此患者1987年出生于西班牙。他在3岁时开始显示出症状，包括近端肌肉无力。他在13岁时丧失了行走能力，并且一天24小时接受通气。他先前以200mg/kg/天服用dAMP和dCMP，并且经通气显示出一天24至22小时的体重增加和减少。

[0137] 他从2015年6月以来一直以400mg/kg/天的dC和dT接受脱氧核苷疗法，并且已显示出肌肉力量的改善，他的体重和通气状况已经稳定，并且他的生活质量有所提高。

[0138] 治疗期间观察到仅有的副作用是腹泻和脱发。

[0139] 患者3

[0140] 此患者1985年出生于西班牙。他的症状开始于6岁，伴有面部、近端和轴向肌肉无力。他在2015年6月开始使用200mg/kg/天的dT和dC，并且迄今为止他的病状已经改善，在6分钟行走测试、起床和走路的时间以及上下攀爬4个台阶方面有改善。

[0141] 治疗期间观察到仅有的副作用是腹泻。

[0142] 患者4

[0143] 此患者2009年2月出生于西班牙。他在六个月时表现出伴随发育不良的症状。他在2015年7月开始接受230mg/kg/天的dC和dT。到2016年1月时，他显示出病状有所改善，并且进食情况更好了。

[0144] 没有观察到副作用。

[0145] 患者5

[0146] 此患者1957年出生于西班牙，并且在50岁时开始出现端坐呼吸和膈肌无力的症状。他在晚上依靠BiPAP。他在2015年11月开始接受200mg/kg/天的dC和dT。

[0147] 没有观察到副作用。

[0148] 患者6

[0149] 此患者2011年10月出生于西班牙，并且在15个月时开始显示出症状，包括张力减退和虚弱。他在22个月时丧失行走力，并且呼吸肌无力。他在16个月时开始机械通气，并且目前一天十二个小时依靠BiPAP。他先前以100mg/kg/天接受dCMP和dAMP，且剂量增加到400mg/kg/天。根据Egen Klassification量表所显示他的力量提高(28/30至13/30)，并且他的体重从9.8kg增加到12.3kg。

[0150] 他在2015年4月以400mg/kg/天的dC和dT开始脱氧核苷疗法。在2015年10月，他在Egen Klassification量表方面从13/30变化到11/30，并且他的体重从12.3kg增加到16.5kg。

[0151] 没有观察到副作用。

[0152] 患者7

[0153] 此患者2012年11月出生于西班牙。他在17个月时开始显示出症状，包括虚弱和张力减退。他在22个月时丧失行走力，并且在29个月时开始机械通气。他先前以100mg/kg/天接受dCMP和dAMP，且剂量增加到400mg/kg/天。根据Egen Klassification量表所显示他的力量提高(30/30至24/30)，并且他的体重从11kg增加到15.7kg。

[0154] 他在2015年4月开始脱氧核苷疗法，剂量为400mg/kg/天的dT和dC。在2015年11月，他在Egen Klassification量表方面从24/30变化到19/30，并且他的体重从15.7kg增加到17kg。

[0155] 没有观察到副作用。

[0156] 患者8

[0157] 此患者1989年9月出生于智利，并且在11个月时开始显示出症状，伴有频繁跌倒和渐进性步态障碍。她在约4岁时丧失了独自行走的能力。她先前曾一直接受核苷酸疗法，并且她在行动能力方面显示出改善，包括不用帮助行走、站立时间更久、攀爬楼梯、参加体育课以及关注个人需求。

[0158] 她在2016年2月转用脱氧核苷疗法，剂量为260mg/kg/天的dC和dT，且然后在2016年5月将剂量增加到400mg/kg/天的dC和dT，并且继续显示出改善。

[0159] 没有观察到副作用。

[0160] 患者9

[0161] 此患者1989年9月出生于危地马拉。他在2015年8月开始使用130mg/kg/天的dC和dT，且剂量在2016年2月增加到260mg/kg/天。他已显示出精力提高。没有观察到副作用。

[0162] 参考文献

[0163] Akman,et al.(2008)Thymidine kinase 2(H126N)knock in mice show the essential role of balanced deoxynucleotide pools for mitochondrial DNA maintenance.Hum.Mol.Genet.17:2433-2440

[0164] Alston.et al.(2013)Late-onset respiratory failure due to TK2 mutations causing multiple mtDNA deletions.Neurology 81:2051-3

[0165] Bartesaghi,et al.(2010)Loss of thymidine kinase 2 alters neuronal bioenergetics and leads to neurodegeneration.Hum.Mol.Genet.19:1669-77

[0166] Béhin,et al.(2012)Adult cases of mitochondrial DNA depletion due to TK2 defect An expanding spectrum.Neurology 78:644-648

[0167] Blakely,et al.(2008)Novel mutations in the TK2 gene associated with fatal mitochondrial DNA depletion myopathy.Neuromuscular Disorders 18:557-560

[0168] Bourdon,em al.(2007)Mutation of RRM2B,encodingp53-controlled ribonucleotide reductase(p53R2),causes severe mitochondrial DNA depletion.Nature Genetics 39:776-780

[0169] Carrozzo,et al.(2003)Matation analysis in 16 patients with mtDNA depletion.Hum.Mutat.21:453-454

[0170] Chanpraserm et al.(2013)Molecular and clinical characterization of the myopathic form of mitochondrial DNA depletion syndrome caused by mutations in the thymidine kinase(TK2)gene.Mol.Genet.Metab,110:153-61

[0171] Chanprasert,et al.(2012)TK2-Related Mitochondrial DNA Depletion Syndrome,Myopathic Form.*GeneReviews®*Internet,December 6,2012

[0172] Collins,et al.(2009)Progressive myofiber loss with extensive fibro-fatty replacement in a child with mitochondrial DNA depletion syndrome and novel thymidine kinsse 2gene mutations.Neuromuscular Disorders 19:784-787

[0173] Copeland(2008)Inherited mitochondrial diseases of DNA replication.Ann.Rev.Med.59:131-146

[0174] DiMauro,et al.(1987)Cytochrome c oxidase deficiency in Leigh syndrome.Ann.Neurol.22:498-506

[0175] DiMauro,Schon.(2003)Mitochondrial respiratory-chain diseases.New England Journal of Medicine 348:2656-2668

[0176] DiMauro,Hirano.(2005)Mitochondrial encephalomyopathies:an update.Neuromuscul.Disord.15:276-286

[0177] Dorado,et al.(2011)Onset and organ specificity of Tk2 deficiency depends on Tk1 down-regulation and transcriptional compensation.Hum.Mol.Genet .20:155-64

[0178] Elpeleg,et al.(2005)Deficiency of the ADP-forming succinyl-CoA synthase activity is associated with encephalomyopathy and mitochondrial DNA depletion.Am.J.Hum.Genet.76:1081-1086

[0179] Ferraro,et al.(2010)Quantitation of cellular deoxynucleoside triphospbates.Nucleic Acids Research 38:e85

[0180] Galbiati,et al.(2006)New mutations in TK2 gene associated with mitochondrial DNA depletion.Pediatr,Neurol.34:177-185

[0181] Garone,et al.(2012).MPV17 Mutations Causing Adult-Onset Multisystemic Disorder With Multiple Mitochondrial DNA Deletions.Arch Neurol 69:1648-1651

[0182] Garone,et al.(2014).Deoxypyrimidine monophosphate bypass therapy for thymidine kinase 2 deficiency.EMBO Mol Med 6:1016-1027

[0183] Garone,et al.(2016)in preparation,“Phenotypic Spectrum and Retrospective Nanural History of Thymidine Kinase 2 Deficiency”

[0184] Gotz.et al.(2008)Thymidine kinase 2 defects can cause multi-tissue mtDNA depletion syndrome.Brain 131:2841-2850

[0185] Hirano,et al.(2001)Defects of intergenomic commumication:autosomal disorders that cause multiple deletions and depletion of mitochondrial DNA.Semin.Cell.Develop.Biol.12:417-427

[0186] Hirano,et al.(2004)MtDNA maintenance and stability genes:MNGIE and mtDNA depletion syndromes.In:Köhler,Bauer,editors.Mitochondrial Function and Biogenetics.Berlin:Springer-Verlag.p.177-200

[0187] Leshinsky-Silver,et al..(2008)A defect in the thymidine kinase 2 gene causing isolated mitochondrial myopathy without mtDNA depletion.Eur.J.Paediatr Neurrol. 12:309-13

[0188] Lesko.et al.(2010)Two novel mutations in thymidine kinase-2 cause early onset fatal encephalomyopathy and severe mtDNA depletion.Neuromuscul.Disord.20:198-203

[0189] Longley,et al.(2006).Mutant POLG2 disrupts DNA polymerase gamma subunits and causes progressive external ophthalmoplegia.Am J Hum Genet.78:1026-1034

[0190] Lopez,et al.(2009)Unbalanced deoxynucleotide pools cause mitochondrial DNA instability in thymidine phosphorylase deficient mice.Human Molecular Genetics 18:714-22

[0191] Mancuso.et al.(2003)Mitochondrial myopathy of childhood associated with mitochondrial DNA depletion and a homozygous mutation(T77M)in the TK2 gene.Arch.Neurol.60:1007-9

[0192] Mancuso,et al.(2002)Mitochondrial DNA depletion:mutations in thymidine kinase gene with myopathy and SMA.Neurology.59:1197-202

[0193] Mandel,et al.(2001)The deoxyguanosine kinase gene is mutated in individuals with depleted hepatocerebral mitochondrial DNA.Nature Genet.29:337-341

[0194] Martí,et al.(2010)Hearing loss in a patient with the myoparhic form of mitochondrial DNA depletion syndrome and a novel mutation in the TK2 gene.Pediatr.Res.68:151-4

[0195] Martí.et al.(2012a)Measurement of mitochondrial dNTP pools.Methods Mol.Biol.837:135-148

[0196] Martí,et al.(2012b)Assessment of thymidine phosphorylase function:measurement of plasma thymidine(and deoxyuridine)and thymidine phosphorylase activity.Methods Mol.Biol.837:121-133

[0197] Naviaux,Nguyen.(2004)POLG mutations associated with Alpers'syndrome and mitochondrial DNA depletion.Ann.Neurol.55:706-712

[0198] Nishino,et al.(1999).Thymidine phosphorylase gene mutations in MNGIE,

a human mitochondrial disorder.Science 283:689-692.

[0199] Oskoui,et al.(2006)Clinical spectrum of mitochondrial DNA depletion due to mutations in the thymidine kinase 2 gene.Arch.Neurol,63:1122-1126.

[0200] Ostergaard,et al.(2007)Deficiency of the alpha subunit of succinate-coenzyme A ligase causes fatal infantile lactic acidosis with mitochondrial DNA depletion.Am.J.Hum.Genet.81:383-387

[0201] Paradas,et al.(2012)TK2 mutation presenting as indolent myopathy.Neurology 29:504-506

[0202] Ronchi,et al.(2012).Next-generation sequencing reveals DGUOK mutations in adult patients with mitochondrial DNA multiple deletions.Brain 135:3404-3415.

[0203] Roos,et al.(2014)Mitochondrial DNA depletion in single fibers in a patient with novel TK2 mutations.Neuromuscul.Disord.24:713-20

[0204] Saada,et al(2001)Mutant mitochondrial thymidine kinase in mitochondrial DNA depletion myopathy.Nature Genet.29:342-344

[0205] Saada.et al.(2003)Mitochondrial deoxyribonucleoside triphosphate pools in thymidine kinase 2 deficiency.Biochem.Biophys.Res.Commun.310:963-966

[0206] Sarzi,et al.(2007)Twinkle helicase(PEO1)gene mutation causes mitochondrial DNA depletion.Ann.Neurol.62:579-587

[0207] Spelbrink,et al.(2001).Human mitochondrial DNA deletions associated with mutations in the gene encoding Twinkle,a phage T7 gene 4-like protein localized in mitochondria.Nature Genet.28:223-231

[0208] Spinazzola,et al.(2006)MPV 17 encodes an inner mitochondrial membrane protein and is mutated in infantile hepatic mitochondrial DNA depletion.Nature Genet.38:570-575

[0209] Tulinius,et al.(2005)Novel mutations in the thymidine kinase 2 gene (TK2)associated with fatal mitochondrial myopathy and mitochondrial DNA depletion.Neuromuscul.Disord.15:412-415

[0210] Tyynismaa,et al.(2012)Thymidine kinase 2 mutations in autosomal recessive progressive external ophthalmoplegia with multiple mitochondrial DNA deletions.Hum.Mol.Genet.21:66-75

[0211] Tyynismaa,et al.(2009).A heterozygous truncating mutation in RRM2B causes autosomal-dominant progressive external ophthalmoplegia with multiple mtDNA deletions.Am.J.Hum.Genet.85:290-295

[0212] Van Goethem,et al.(2001)Mutation of pOLG is associated with progressive external ophthalmoplegia characterized by mtDNA deletions.Nature Genet.28:211-212.

[0213] Vilà,et al.(2003)Reversion of mtDNA depletion in a patient with TK2 deficiency.Neurology 60:1203-1205

[0214] Wang,et al.(2005)Molecular insight into mitochondrial DNA depletion syndrome in two patients with novel mutations in the deoxyguanosine kinase and thymidine kinase 2 genes.Mol.Genet.Metab.84:75-82.

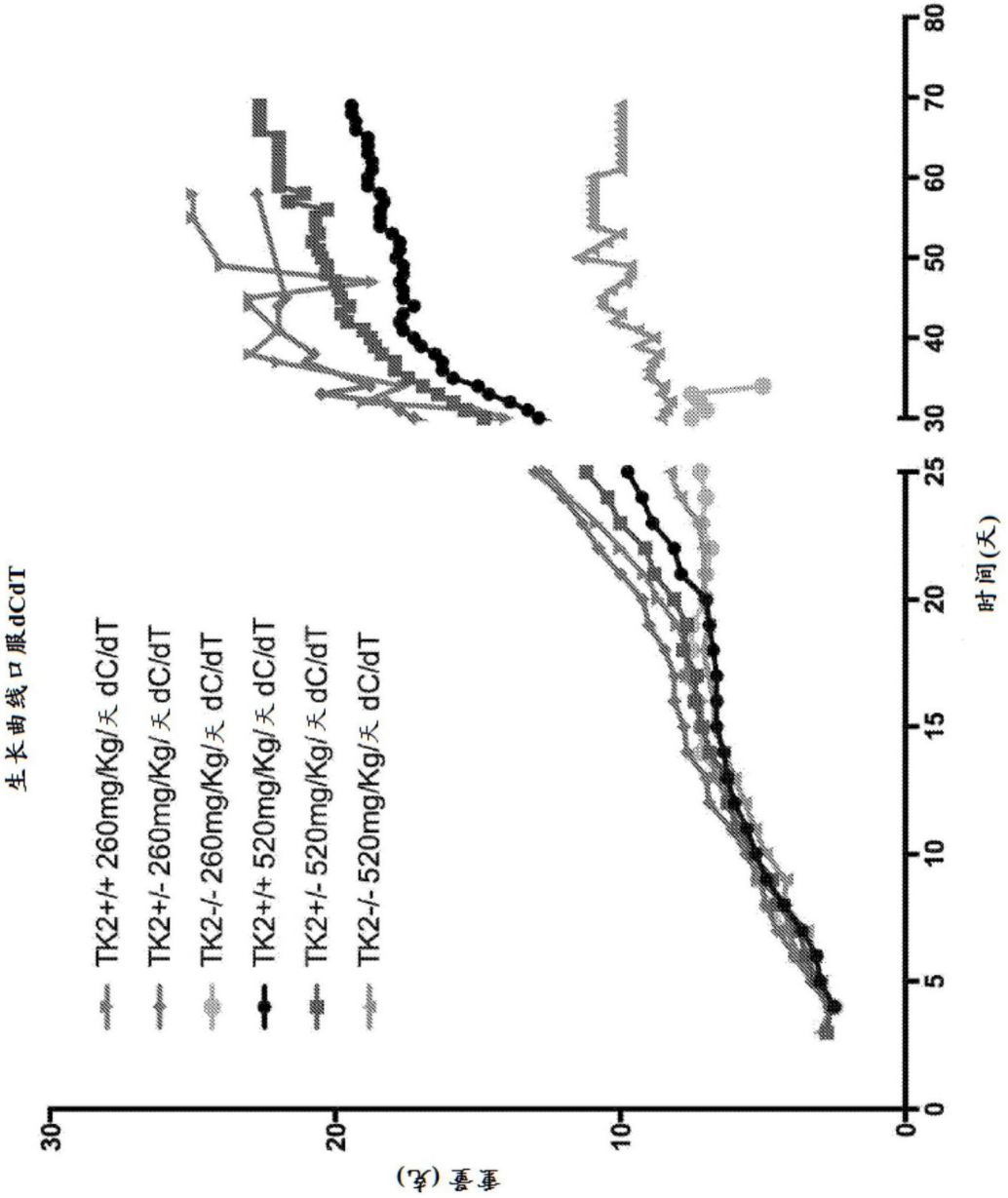

生长曲线口服dCdT
TK2+/+ 260mg/Kg/天 dC/dT
TK2+/- 260mg/Kg/天 dC/dT
TK2-/- 260mg/Kg/天 dC/dT
TK2+/+ 520mg/Kg/天 dC/dT
TK2+/- 520mg/Kg/天 dC/dT
TK2-/- 520mg/Kg/天 dC/dT
重量 (g)
时间(天)
0
10
20
30
0
5
10
15
20
25
30
40
50
60
70
80

图1

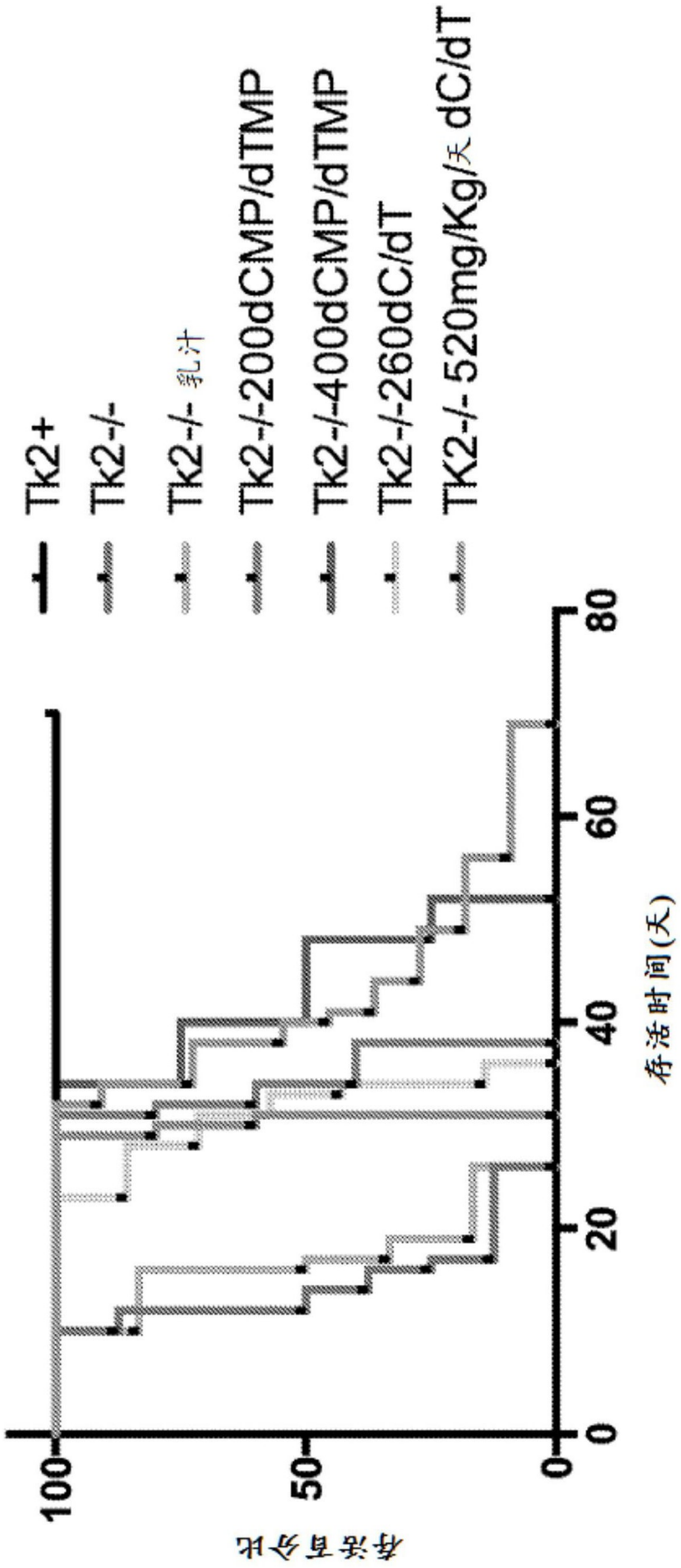

Tk2+
Tk2-/-
Tk2-/- 乳汁
Tk2-/-200dCMP/dTMP
Tk2-/-400dCMP/dTMP
Tk2-/-260dC/dT
TK2-/- 520mg/Kg/天 dC/dT
存活百分比
100
50
0
0
20
40
60
80
存活时间(天)

图2

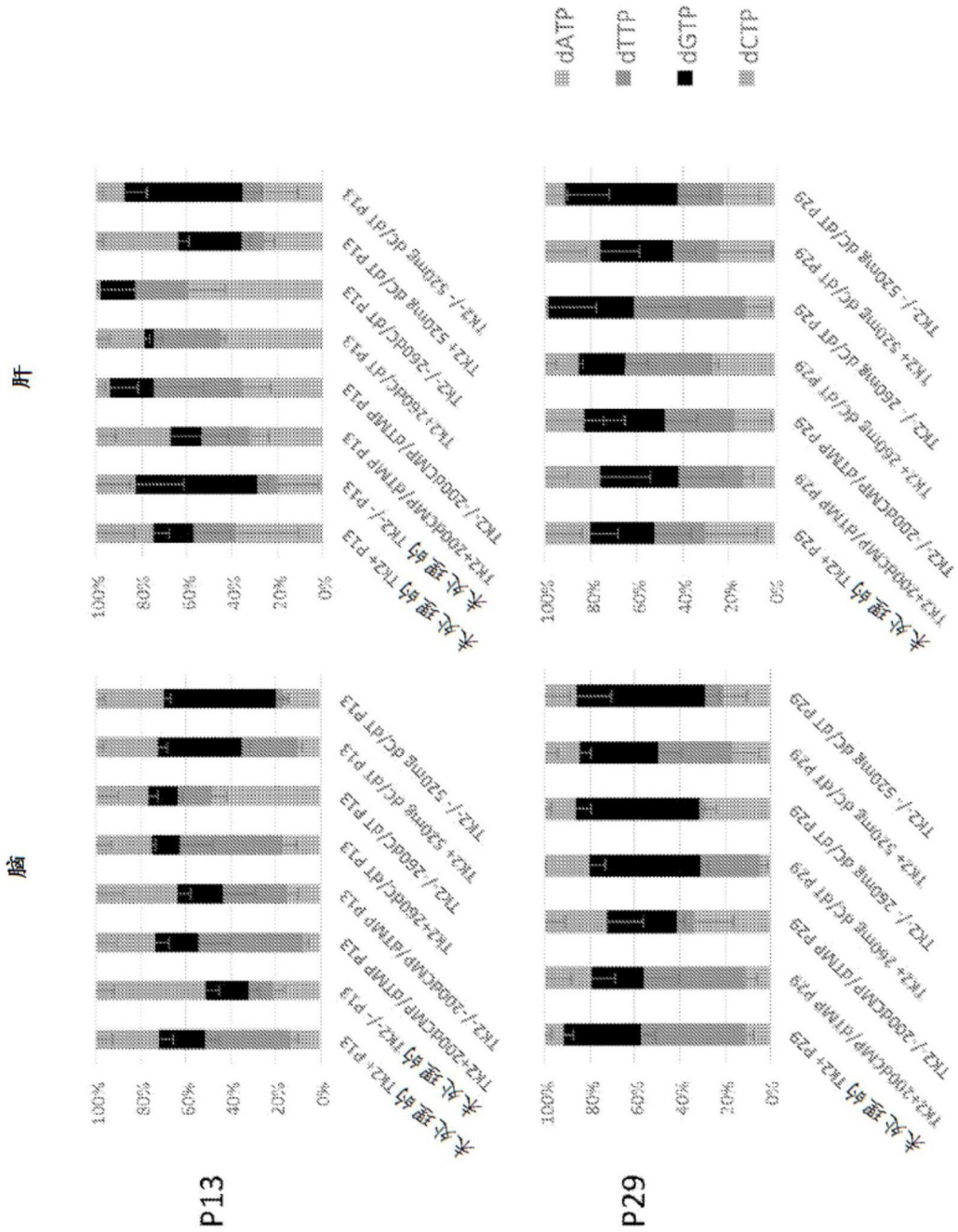

dATP
dTTP
dGTP
dCTP
肝
脑
P13
P29
未处理的 TK2+ P13
TK2+ 200dCMP/dTMP P13
TK2+ 260mg dC/dT P13
TK2+ 520mg dC/dT P13
TK2- 200dCMP/dTMP P13
TK2- 260dC/dT P13
TK2- 520mg dC/dT P13
TK2- 520mg dC/dT P13
未处理的 TK2+ P29
TK2+ 200dCMP/dTMP P29
TK2+ 260mg dC/dT P29
TK2+ 520mg dC/dT P29
TK2- 200dCMP/dTMP P29
TK2- 260mg dC/dT P29
TK2- 520mg dC/dT P29
TK2- 520mg dC/dT P29
100%
80%
60%
40%
20%
0%

图3

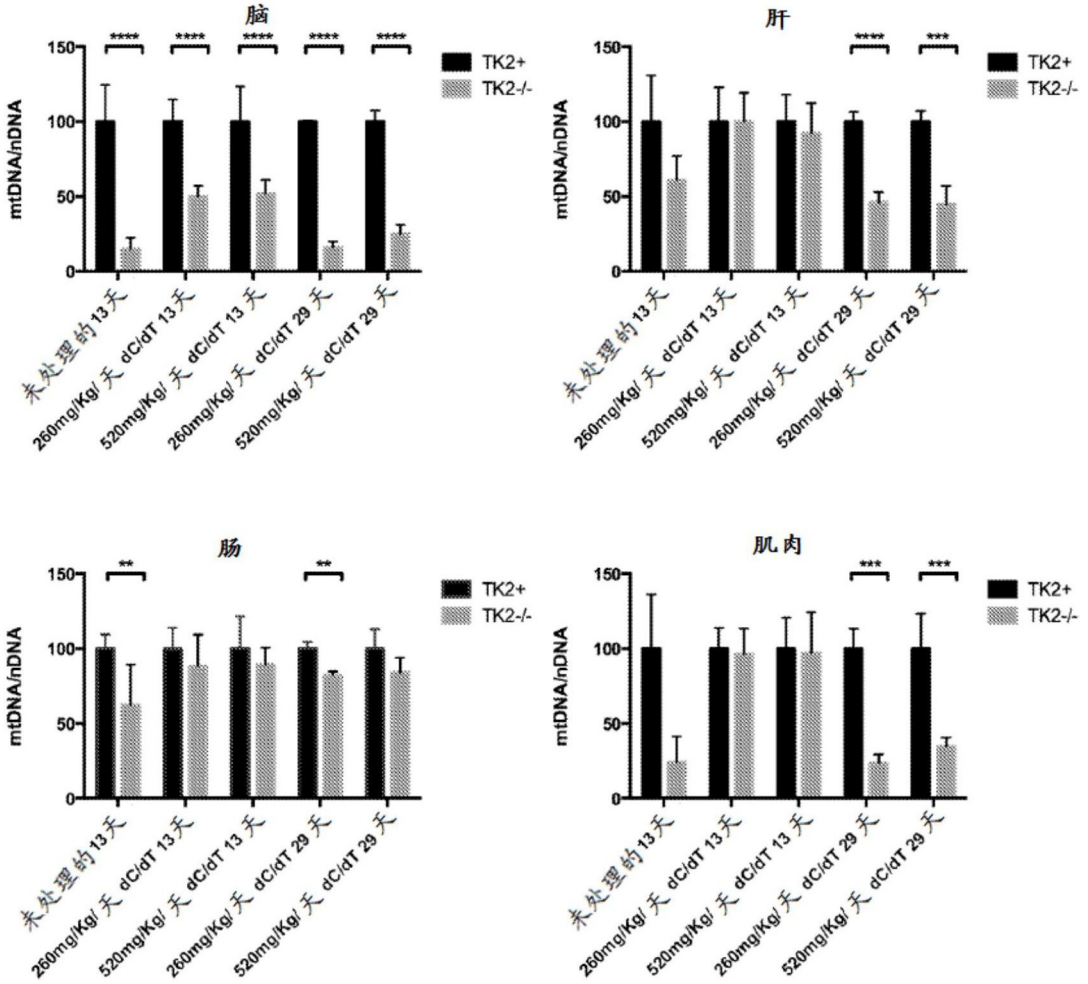

脑
肝
肠
肌肉
mtDNA/nDNA
TK2+
TK2-/-
未处理的13天
260mg/Kg/天 dC/dT 13天
520mg/Kg/天 dC/dT 13天
260mg/Kg/天 dC/dT 29天
520mg/Kg/天 dC/dT 29天

图4

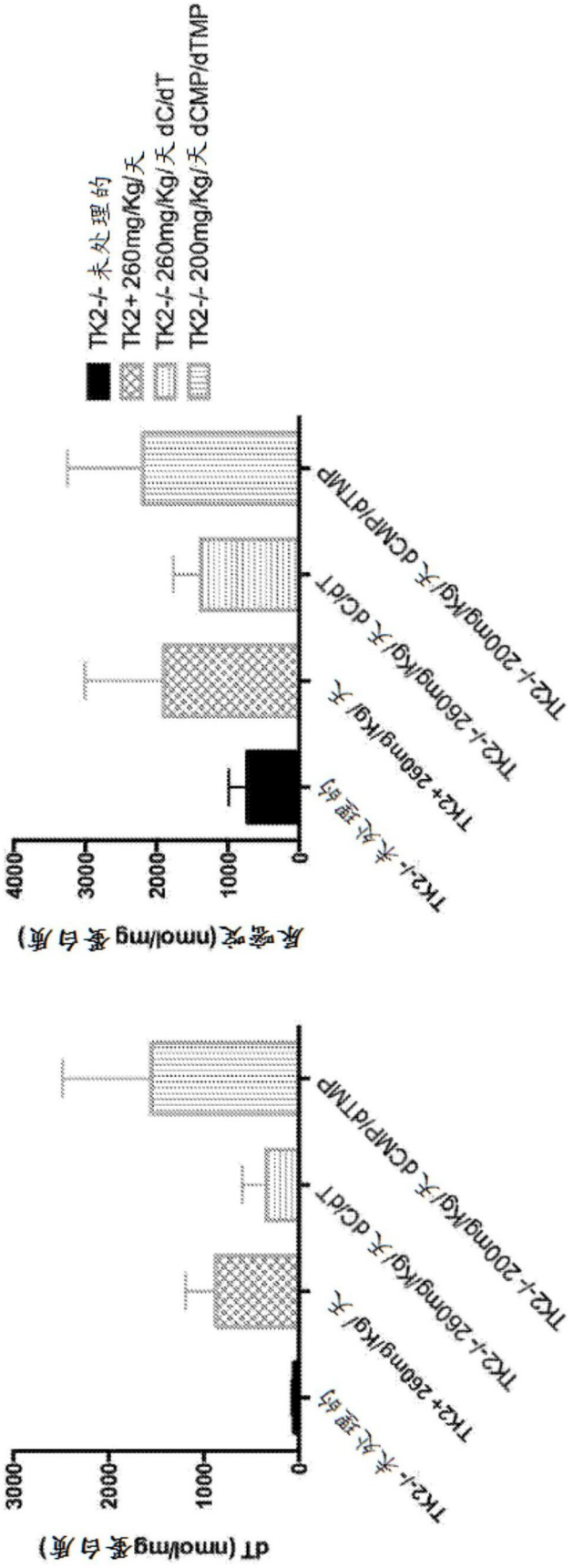

TK2-/- 未处理的
TK2+ 260mg/Kg/天
TK2-/- 260mg/Kg/天 dC/dT
TK2-/- 200mg/Kg/天 dCMP/dTMP
尿嘧啶(nmol/mg 蛋白质)
4000
3000
2000
1000
0
TK2-/- 未处理的
TK2+ 260mg/Kg/天
TK2-/- 260mg/Kg/天 dC/dT
TK2-/- 200mg/Kg/天 dCMP/dTMP
dT (nmol/mg蛋白质)
3000
2000
1000
0

图5

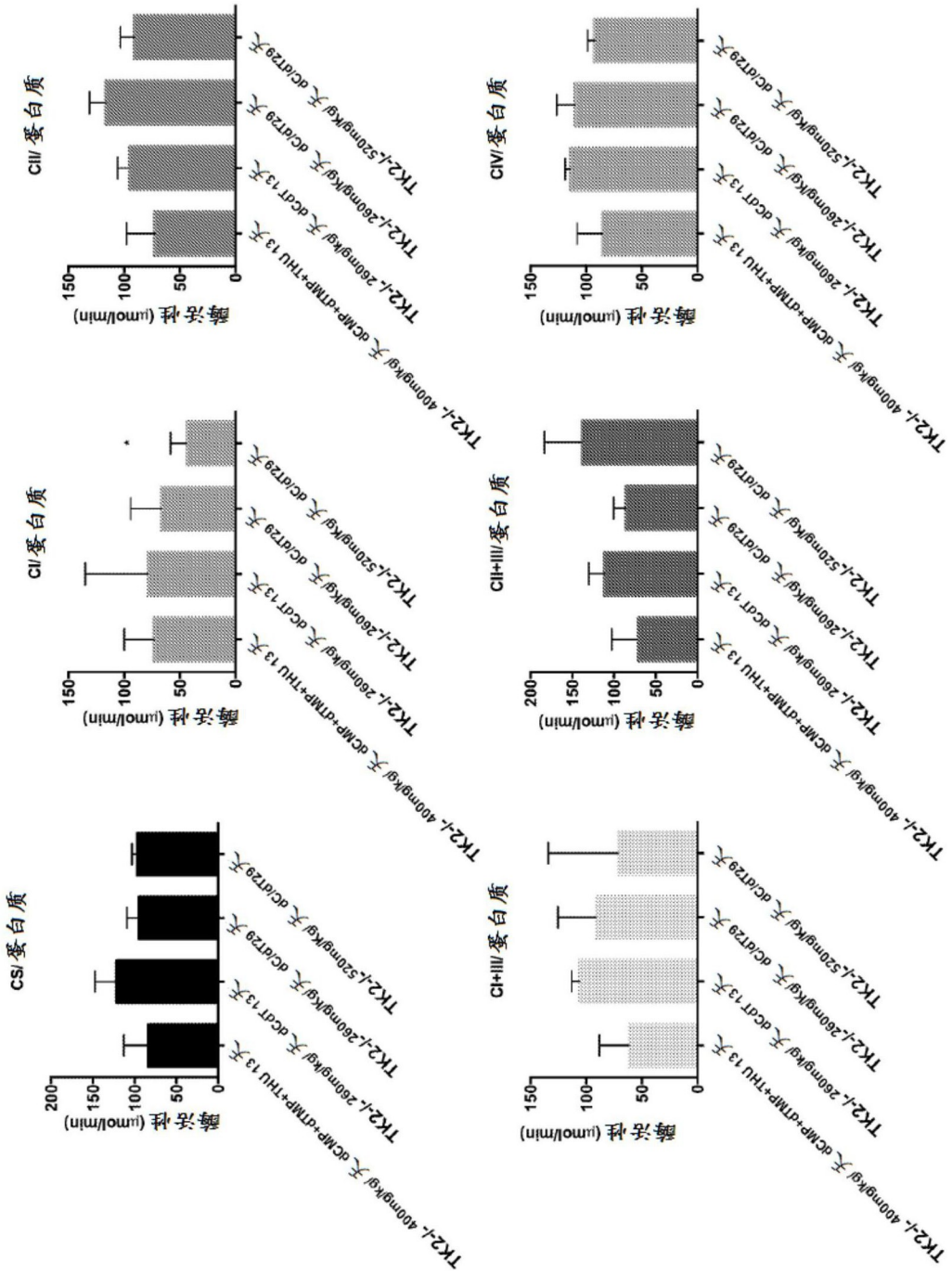

CSI/蛋白质
CI/蛋白质
CII/蛋白质
CIII/蛋白质
CI+III/蛋白质
CII+III/蛋白质
CIV/蛋白质
酶活性 (μmol/min)
TK2-/- 400mg/kg/天 dCMP+dTMP+THU 13天
TK2-/- 260mg/kg/天 dCdT 13天
TK2-/- 260mg/kg/天 dC/dT29天
TK2-/- 520mg/kg/天 dC/dT29天

图6

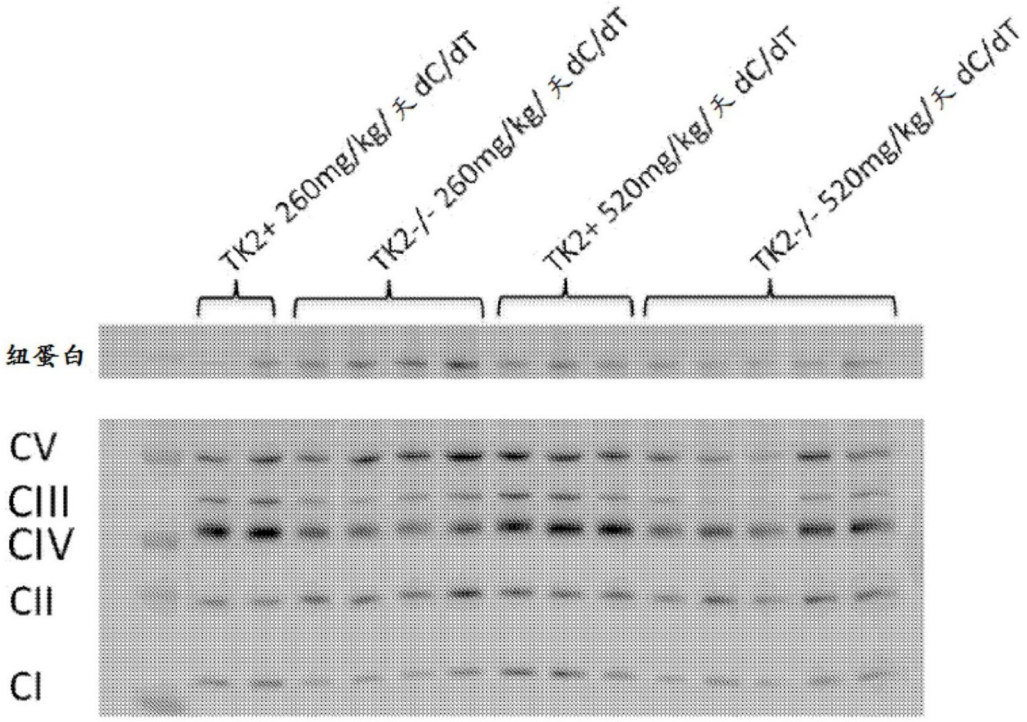

TK2+ 260mg/kg/天 dC/dT
TK2-/- 260mg/kg/天 dC/dT
TK2+ 520mg/kg/天 dC/dT
TK2-/- 520mg/kg/天 dC/dT
纽蛋白
CV
CIII
CIV
CII
CI

图7A

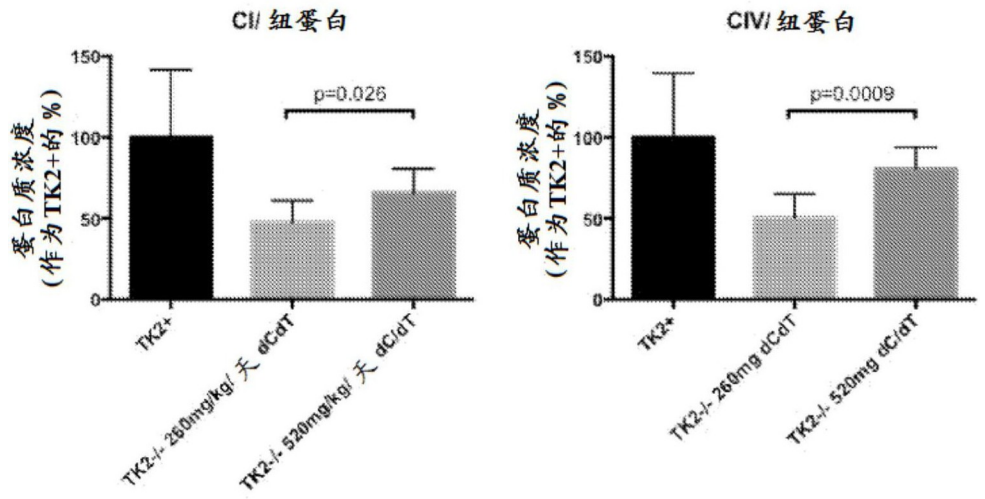

CI/ 纽蛋白
蛋白质浓度
(作为TK2+的%)
150
100
50
0
p=0.026
TK2+
TK2-/- 260mg/kg/天 dC/dT
TK2-/- 520mg/kg/天 dC/dT
CIV/ 纽蛋白
蛋白质浓度
(作为TK2+的%)
150
100
50
0
p=0.0009
TK2+
TK2-/- 260mg dC/dT
TK2-/- 520mg dC/dT

图7B