BIOCHEMISTRY
including biophysical chemistry & molecular biology

Current Topic

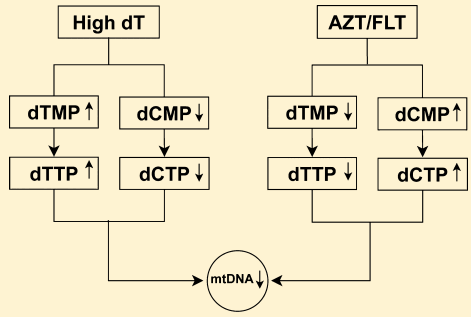
pubs.acs.org/biochemistry

Thymidine Kinase 2 Enzyme Kinetics Elucidate the Mechanism of Thymidine-Induced Mitochondrial DNA Depletion

Ren Sun and Liya Wang*

Department of Anatomy, Physiology and Biochemistry, Swedish University of Agricultural Sciences, Box 7011, SE-750 07 Uppsala, Sweden

ABSTRACT: Mitochondrial thymidine kinase 2 (TK2) is a nuclear gene-encoded protein, synthesized in the cytosol and subsequently translocated into the mitochondrial matrix, where it catalyzes the phosphorylation of thymidine (dT) and deoxycytidine (dC). The kinetics of dT phosphorylation exhibits negative cooperativity, but dC phosphorylation follows hyperbolic Michaelis–Menten kinetics. The two substrates compete with each other in that dT is a competitive inhibitor of dC phosphorylation, while dC acts as a noncompetitive inhibitor of dT phosphorylation. In addition, TK2 is feedback inhibited by dTTP and dCTP. TK2 also phosphorylates a number of pyrimidine nucleoside analogues used in antiviral and anticancer therapy and thus plays an important role in mitochondrial toxicities caused by nucleoside analogues. Deficiency in TK2 activity due to genetic alterations causes devastating mitochondrial diseases, which are characterized by mitochondrial DNA (mtDNA) depletion or multiple deletions in the affected tissues. Severe TK2 deficiency is associated with early-onset fatal mitochondrial DNA depletion syndrome, while less severe deficiencies result in late-onset phenotypes. In this review, studies of the enzyme kinetic behavior of TK2 enzyme variants are used to explain the mechanism of mtDNA depletion caused by TK2 mutations, thymidine overload due to thymidine phosphorylase deficiency, and mitochondrial toxicity caused by antiviral thymidine analogues.



Mitochondria are dynamic organelles that undergo constant changes, e.g., fission and fusion, during the lifetime of the cell. Mitochondria contain multiple copies of circular mitochondrial DNA (mtDNA) molecules, which encode 13 essential subunits of the mitochondrial respiratory chain complexes. To maintain a sustainable mtDNA copy number, a constant supply of precursors, i.e., dNTPs, is essential because mtDNA replicates in a manner that is independent of the cell cycle. A limitation of one or more dNTPs will stall mtDNA synthesis and result in mtDNA depletion, which leads to mitochondrial diseases.[1] In dividing cells and/or tissues, mitochondrial dNTPs are synthesized via the cytosolic *de novo* pathway and the salvage pathway; however, in nondividing cells, the dNTPs required for mtDNA replication are mainly generated *in organello* via thymidine kinase 2 (TK2) and deoxyguanosine kinase (dGK). TK2 and dGK catalyze the phosphorylation of all four natural deoxynucleosides to their respective monophosphates using ATP or other nucleoside triphosphates as phosphate donors. Both enzymes are essential for mtDNA precursor synthesis and mitochondrial function in many cell and tissue types.[2]

Thymidine kinase 2 (TK2, EC 27.1.21) catalyzes the transfer of the γ-phosphate group from ATP to the 5′-hydroxyl group of thymidine (dT), deoxycytidine (dC), or deoxyuridine (dU) to form their corresponding monophosphates. TK2 also phosphorylates a number of pyrimidine nucleoside analogues, such as zidovudine (AZT) used in anti-HIV therapy, and thus may play an important role in the mitochondrial toxicity observed in antiviral and anticancer therapies using nucleoside analogues.[3−5] TK2 is present in all cells that contain mitochondria, and the levels of TK2 correlated to the mitochondrial content of the cells or tissues.[2] A recent study showed that TK2 is upregulated during the stationary phase growth of cultured cells.[6]

Deficiency in TK2 activity due to genetic mutations causes devastating mitochondrial diseases, which are characterized as tissue-specific mtDNA depletion and/or deletion. Mitochondrial DNA depletion syndrome (MDS) is characterized by a severe and tissue-specific reduction in the mtDNA copy number in the absence of qualitative defects in mtDNA.[7] MDS caused by TK2 mutations affected mainly liver and skeletal muscle, but in some cases, multiple tissues were involved.[8−10] Mutations in the TK2 gene are also the genetic causes of late-onset autosomal recessive progressive external ophthalmoplegia (arPEO).[11] The subject of this review is the substrate specificity, enzyme kinetics, and regulation of the TK2 enzyme, as well as the mechanism of thymidine overload-induced mtDNA depletion.

TK2 ENZYME KINETICS

The first extensive kinetic analysis was performed using the purified enzyme obtained from human leukemic spleen.[12] It was shown that with dC and dU as substrates, the reactions

Received: June 4, 2014
Revised: September 11, 2014
Published: September 12, 2014

   dx.doi.org/10.1021/bi5006877 | *Biochemistry* 2014, 53, 6142–6150

followed hyperbolic Michaelis–Menten kinetics, with K_m (micromolar) and V_{max}/K_m (units per milligram per micromolar) values of 36 and 25 for dC and 6 and 115 for dU, respectively. However, the phosphorylation of dT exhibited negative cooperativity with Hill coefficients in the range of 0.3–0.5, indicating that the affinity of dT for TK2 decreases with an increase in concentration as shown by the biphasic Hofstee plot. The K_m value was 0.3 μM when the dT concentration was <8 μM, while at higher concentrations, the K_m value for dT was 16 μM, which gives a k_{cat}/K_m value of ~2.6 × 10^4 s^{-1} M^{-1} at high dT concentrations and a k_{cat}/K_m value of 2.7 × 10^5 s^{-1} M^{-1} at low dT concentrations. Thus, the efficiency of dT phosphorylation is lower at high dT concentrations (>8 μM) than at low dT concentrations (Figure 1). The specific activity

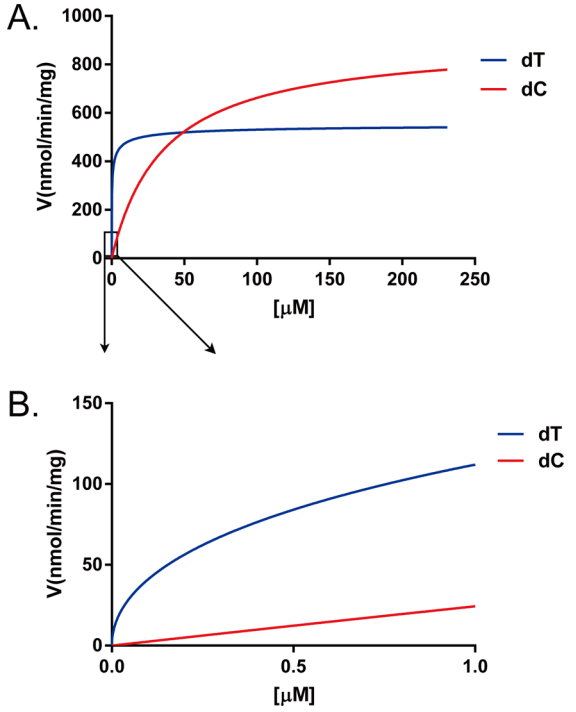


Figure 1. (A) TK2 substrate saturation curves of V (velocity) vs S (substrate concentration). The curves were drawn by using K_m, V_{max}, $S_{0.5}$, and n values from ref 12. (B) Substrate saturation curves at low concentrations.

with dT phosphorylation is significantly higher than that with dC phosphorylation at physiologically relevant substrate concentrations (<0.5 μM) because of the negative cooperativity with dT (Figure 1B). AZT phosphorylation also showed negative cooperativity.[12] Similar results were obtained with recombinant human TK2.[13]

In organello studies with mitochondria isolated from rat liver, heart, and brain showed that the phosphorylation of dT also exhibited negative cooperativity, and the catalytic efficiencies differed in mitochondria isolated from different tissues. Negative cooperative kinetic behavior was also observed with AZT phosphorylation in heart and liver mitochondria, but not in brain mitochondria. Instead, in brain mitochondria, AZT phosphorylation followed hyperbolic Michaelis–Menten kinetics, but negative cooperativity was observed with dCyd phosphorylation.[14−18] Thus, TK2 enzyme kinetics are complex and tissue-specific.

TK2 belongs to the enzyme family that includes mitochondrial deoxyguanosine kinase (dGK), cytosolic deoxycytidine kinase (dCK), and *Drosophila melanogaster* deoxynucleoside kinase (Dm-dNK). The three-dimensional (3D) structures of dGK, dCK, and Dm-dNK have been determined but not the TK2 structure. Substrate and inhibitor binding sites have been studied in detail with dGK, dCK, and Dm-dNK.[19−22] However, there is a fundamental lack of structural information about TK2; hence, the number of substrate binding sites per active enzyme is not known, which is a real barrier to achieving a clear understanding of its biological function.

REGULATION OF TK2 ACTIVITY BY SUBSTRATES, SUBSTRATE ANALOGUES, AND FEEDBACK INHIBITORS

Substrate dT and dC phosphorylations are regulated by a different kinetic mechanisms, and thus, the enzyme has different catalytic efficiencies for dT and dC, as described above. The negative cooperative kinetics with dT suggest that under physiological conditions, e.g., low dT concentrations (<0.05 μM), the TK2 activity will be several hundred-fold higher than with a noncooperative enzyme with the same K_m value (Figure 1B).[23] In cells, both substrates coexist and a competition between dT and dC for the same enzyme is unavoidable. In the presence of both substrates, dC acted as a noncompetitive inhibitor of dT phosphorylation with a K_i value of 40 μM and dT behaved as a competitive inhibitor toward dC phosphorylation with a K_i value of 4.9 μM,[24] resulting in changes in apparent K_m or k_{cat} values for dT and dC in the presence of alternative substrates (Figure 2).

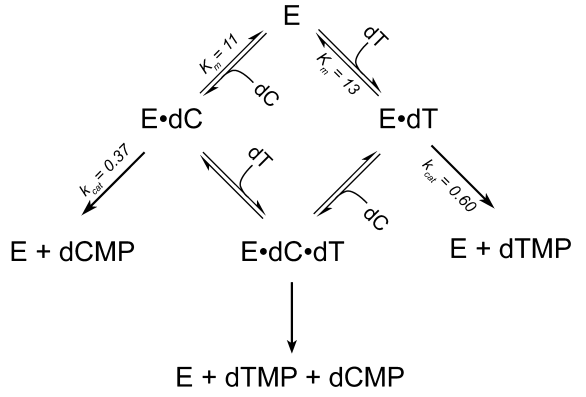
Thymidine analogues such as AZT and FLT strongly inhibited dT phosphorylation with K_i values of 3 and 1.4 μM, respectively. AZT and FLT bind to the enzyme in the same way as dT and thus acted as competitive inhibitors of dT phosphorylation. In the absence of these analogues, recombinant human TK2 phosphorylates dC with a K_m value of 11 ± 1 μM and a k_{cat} value of 0.37 ± 0.01 s^{-1}.[24] However, addition of AZT or FLT resulted in an increased level of dC phosphorylation; for instance, in the presence of 1.2 μM AZT, the K_m and k_{cat} values for dC were changed to 7.9 ± 0.7 μM and 0.71 ± 0.03 s^{-1}, respectively, and in the presence of 2.0 μM FLT, the K_m and k_{cat} values for dC were changed to 6.9 ± 0.5 μM and 0.43 ± 0.02 s^{-1}, respectively (Figure 2). This stimulatory effect was shown to be due to the presence of a feedback inhibitor, e.g., dTTP in the TK2 enzyme.[25]

TK2 exists in monomer, dimer, or tetramer forms.[12,24,26−28] Each monomer is likely to have one nucleoside binding site and one nucleotide (phosphate donor) binding site, as predicted by the structural model of TK2, which was built on the basis of amino acid sequence homology to and the 3D structure of Dm-dNK.[4] Dm-dNK strictly follows Michaelis–Menten kinetics with all of its substrates[29] but not TK2. Therefore, the TK2 kinetic behavior may involve a third molecule (inhibitor or substrate) that acts through one of the following mechanisms: (1) a protein–protein interaction in a multimeric state, (2) a covalent phosphoprotein intermediate that transfers the phosphate group to the nucleoside, or (3) an uncharacterized effector binding site on each monomer.

Protein–protein or subunits interactions are most likely because the kinetic studies with natural substrates and inhibitors described above suggest that the binding of the first substrate/inhibitor to one subunit induced conformational changes in the other subunit, leading to changes in the binding

dx.doi.org/10.1021/bi5006877 | *Biochemistry* 2014, 53, 6142–6150

A.



B.

Inhibitor (μM)	Substrate	Mode of inhibition	$K_{m,\,app}$ (μM)	$k_{cat,\,app}$ (s^{-1})
-	dT	-	13	0.60
-	dC	-	11	0.37
dC (10)	dT	Non-competitive	13	0.48
dT (10)	dC	competitive	33.4	0.37
AZT (1.2)	dT	competitive	18.2	0.60
FLT (2.0)	dT	competitive	31.6	0.60
AZT (1.2)	dC	stimulatory	7.9	0.71
FLT (2.0)	dC	stimulatory	6.9	0.43

Figure 2. (A) Reaction pathways of TK2 (E) with dT and dC as substrates. In the proposed reaction pathways, TK2 acts as a dimer or multimer. Each monomer (subunit) has one catalytically active center and is capable of converting one substrate (dT or dC) to its product (dTMP or dCMP) in the presence of ATP or other nucleoside triphosphates (omitted for the sake of simplicity). The binding of an alternative substrate or inhibitor to the other subunit affects either the apparent K_m, the apparent k_{cat}, or both. The K_m and k_{cat} values for dT and dC are from ref 24. (B) Apparent K_m and k_{cat} values ($K_{m,app}$ and $k_{cat,app}$) for dT and dC in the presence of inhibitors were calculated using data from refs 24 and 25.

affinity of the next substrate or inhibitor. Thus, TK2 most likely exists as a dimer or an oligomer. The interaction between the two subunits is probably weak, and therefore, the enzyme can be eluted as a monomer via size-exclusion chromatography in some cases. A covalent phosphorprotein intermediate is very unlikely because we have not been able to isolate such an intermediate by using radiolabeled ATP or nucleosides and purified TK2. However, we cannot exclude the possibility of an effector binding site in addition to the nucleoside and phosphate binding sites on the TK2 enzyme as the cause of the cooperative behavior of TK2. Thus, further structural information regarding the identity and locations of the effector binding sites and their relation to the oligomeric states of TK2 is needed to elucidate the kinetic mechanisms of TK2.

For the competitive inhibition mode, the percent inhibition is dependent on both substrate and inhibitor concentrations and can be calculated using eq I. For the noncompetitive inhibition mode, the percent inhibition is dependent only on the concentration of the inhibitor and can be calculated using eq II

$$i_{\%} = (100[\mathrm{I}])/[[\mathrm{I}] + K_i(1 + [\mathrm{S}]/K_m)] \quad \text{(I)}$$

$$i_{\%} = 100[[\mathrm{I}]/(K_i + [\mathrm{I}])] \quad \text{(II)}$$

where [S] is the substrate concentration, [I] is the inhibitor concentration, and $i_{\%}$ is the percent inhibition.[30]

Using these two equations and the K_m and K_i values for dT and dC, the extent of substrate inhibition can be plotted (Figure 3A,B). Over a physiologically relevant concentration range (0.05−0.5 μM), the phosphorylation of dC is very sensitive to the levels of dT, and when the concentration of dT increases, the level of dC phosphorylation decreases dramatically (Figure 3A). However, the phosphorylation of dT is not sensitive to the changes in the concentration of dC (Figure 3B). Thus, the outcome of substrate competition under normal physiological conditions is a relative higher level of dTMP and a lower level of dCMP, resulting in higher dTTP and lower dCTP levels. In the case of metabolic alterations, for example, in thymidine phosphorylase (TP) deficiency, elevated levels of dT will strongly inhibit the phosphorylation of dC, which results in high levels of dTMP and very low levels of dCMP and eventually high dTTP and very low dCTP levels (Figure 4). This has been demonstrated by recent studies using cell culture and animal models that are deficient in TP activity.[31,32]

Using K_i values for AZT and FLT, the percent inhibition by various concentrations of AZT or FLT was plotted according to eq II. As shown in panels C and D of Figure 3, with increasing concentrations of the analogues, the extent of inhibition also increased. The inhibitory effect on dT and the stimulatory effect on dC phosphorylation will result in low dTMP levels and relatively high dCMP levels and eventually low dTTP and higher dCTP levels (Figure 4).[25] The consequence of the effect of AZT and FLT on dT and dC phosphorylation by TK2 will be discussed more in detail below.

TK2 is also feedback inhibited by dTTP and dCTP.[25,33] Both dTTP and dCTP competitively inhibited dT phosphorylation with K_i values of 2.0 and 0.8 μM, respectively. However, inhibition of dC phosphorylation by dTTP was noncompetitive with a K_i value of 2.5 μM, and dCTP competitively inhibited dC phosphorylation with a K_i value of 0.9 μM.[26] Furthermore, purified recombinant human TK2 and native rat liver TK2 contain a tightly bound dTTP at a stoichiometry of 1:1.[25] Thus, dT and dC would have to compete with dTTP for binding to the active site. It has been previously shown that dT competes more efficiently than dC with enzyme-bound dTTP, and this may contribute to a level of dT phosphorylation higher than that of dC phosphorylation.[25] The implications of this kinetic behavior will be further discussed in later sections.

RECENT DEVELOPMENT OF TK2-SPECIFIC INHIBITORS

Pyrimidine analogues have long been used as antimetabolites in antiviral and/or anticancer therapy. Many of them are substrates or inhibitors of TK2 and/or cytosolic thymidine kinase 1 (TK1). Long-term use of these analogues is associated with mitochondrial toxicities that involve TK2. In this respect, potent and selective TK2 inhibitors are valuable tools for the study of the role of TK2 in the maintenance of mitochondrial dNTP pools and the contribution of TK2 in mitochondrial toxicities of certain nucleoside analogues.[34,35] Two types of modifications, i.e., position 5 of the base moiety and position 3′ on the sugar moiety, are well-tolerated by TK2. For instance, (*E*)-5-(2-bromovinyl)deoxyuridine (BvdU) is a much better substrate for TK2 than for TK1. Thus, it has been suggested as a selective substrate to distinguish between TK2 and TK1 activity in cell extracts.[36,37] AZT, araT (arabinofuranosyl thymine), and FLT can be phosphorylated by TK2, but at much lower rates, while 2′,3′-didehydrodideoxythymidine is not a substrate for TK2.[12,13] TK2 also phosphorylates several dU

dx.doi.org/10.1021/bi5006877 | *Biochemistry* 2014, 53, 6142−6150

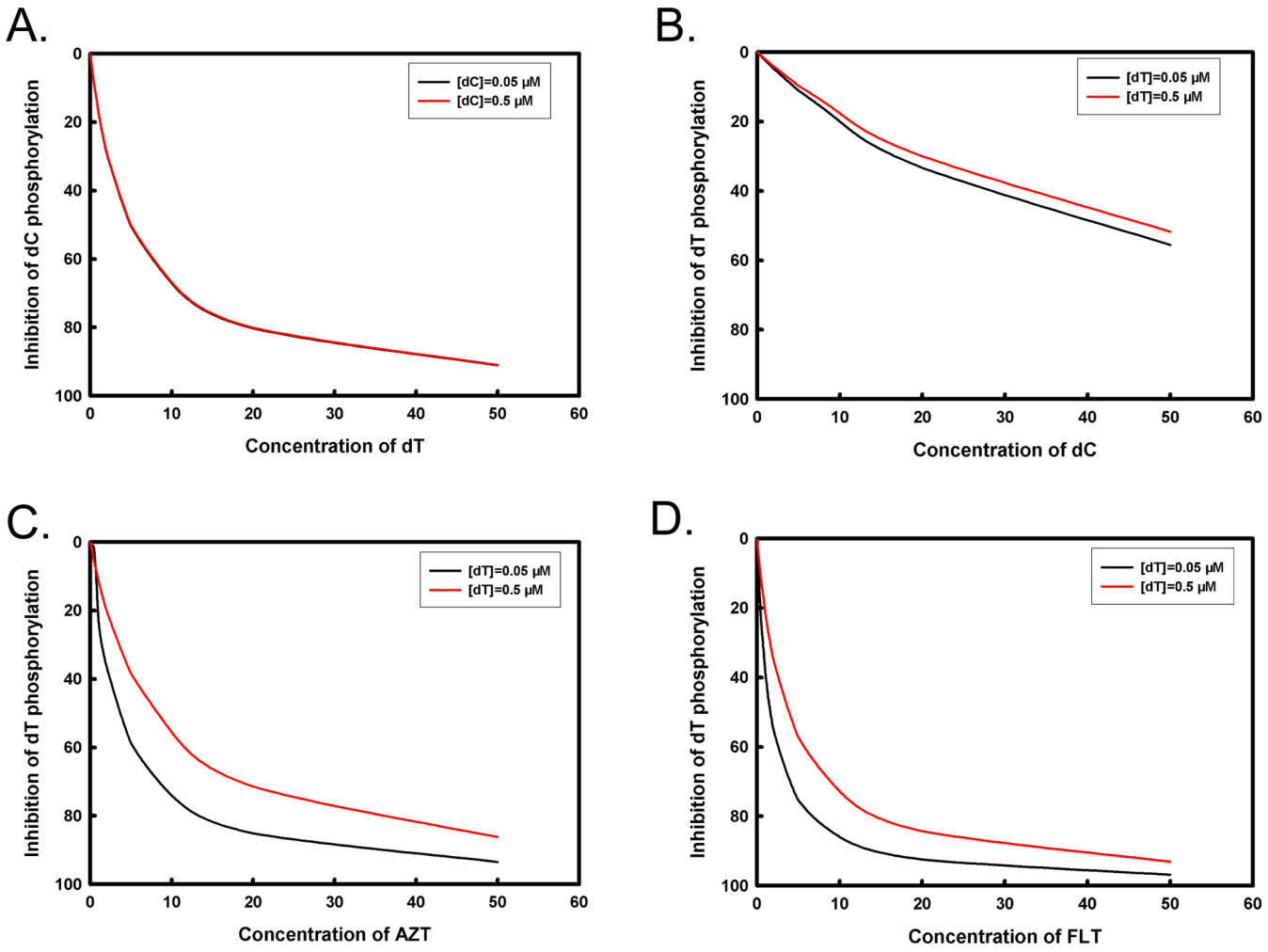


Figure 3. (A) Percent inhibition of dC phosphorylation by dT. (B) Percent inhibition of dT phosphorylation by dC. (C) Percent inhibition of dT phosphorylation by AZT. (D) Percent inhibition of dT phosphorylation by FLT.

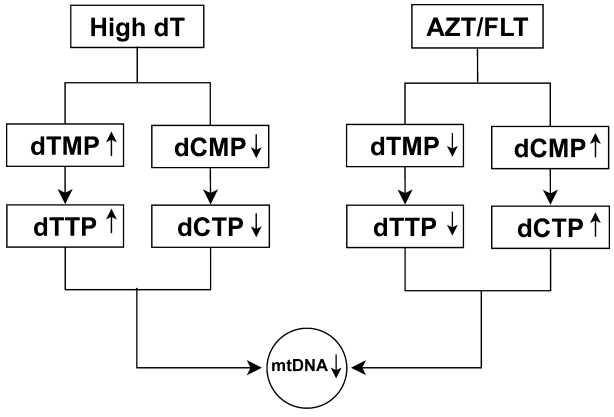


Figure 4. Consequences of a high level of dT or the presence of AZT or FLT with respect to mitochondrial dTTP and dCTP levels, and ultimate effect on mtDNA synthesis: ↑, upregulation; ↓, downregulation.

and dC analogues, including 5-fluorodeoxyuridine, 1-(2′-deoxy-2′-fluoro-1-β-D-arabinofuranosyl)-5-iodouracil, 2′,2′-difluorodeoxyuridine, 2′,2′-difluorodeoxycytidine, and arabinofuranosyl cytosine.[13,38,39]

Many of the thymidine analogues are poor substrates of TK2 but may act as inhibitors of dT or dC phosphorylation. For instance, AZT and FLT are potent inhibitors of dT phosphorylation with K_i values of 3.0 and 1.4 μM, respectively.[25] Several potent TK2-specific inhibitors have been designed, synthesized, and characterized recently with modifications on both the base (at position 5) and the sugar moiety (at position 2′ or 3′) by using molecular docking approaches.[34,35,40,41] Compounds with either an arylthiourea derivative or a 1,4-disubstituted 1,2,3-triazole motif substituted at position 3′ showed a potent and selective inhibitory effect on TK2. Two of these derivatives, 5-(3′-amino-3′-deoxy-β-D-thymidin-3′N-yl)-1-(4-chloro-3-trifluoromethylphenyl)-tetrazole and 5-(3′-amino-3′-deoxy-β-(*E*)-5-(2-bromovinyl)-2′-deoxyuridin-3′N-yl)-1-(4-chloro-3-trifluoromethylphenyl)-tetrazole, showed IC_{50} values of 35 and 14 nM, respectively.[35] Through structural analysis of the TK2 model docked with various inhibitors, the authors pinpointed that a pronounced flexibility of the enzyme around the substrate binding pocket and a hydrophobic channel at the interface between the α-helices that connects the thymidine binding pocket largely accommodate bulk substitutions at position 3′. A closed loop conformation further stabilized the binding of inhibitors via the amino group bridging the tetrazole ring to the sugar moiety. For detailed analysis of the structural function−activity relationship, see the recent publications.[34,41]

dx.doi.org/10.1021/bi5006877 | *Biochemistry* 2014, 53, 6142−6150

Table 1. Clinical and Biochemical Data of TK2 Mutations Identified in Patients with Mitochondrial Diseases[a]

TK2 mutation (reported)	NM_004614.4	age at onset (months)	age at death (months)	mtDNA content (%)	residual TK2 activity	disease(s)	ref
<H90N/H90N	H121N/H121N	0	4	22%	45%	severe myopathy	8
I181N/I181N	I212N/I212N	8	19	16−20%	14−23%	severe myopathy	8
H90N/T77M	H121N/T108M	12	40	6−14%	36%	encephalopathy/ nephropathy	62
I22M/I22M	I53M/I53M	1	24	6−14%	28%	encephalopathy/ myopathy	62
T77M/T77M	T108M/T108M	12	23−40	10−20%	*b*	myopathy	63
R183G/R254X	R183G/R254X	2	10	60%	*b*	infantile encephalomyopathy	64
E48fsX149	E48fsX149	5	15	37%	*b*	infantile encephalomyopathy	64
R152G/K171del	R183G/K202del	36	alive at 14 years	normal	1%	myopathy	49
T77M/R161 K	T108M/R192K	28	alive at 12 years	8%	7%	mitochondrial myopathy	48
T64M/R183W	T64M/R183W	11	24−28	<10%	*b*	severe myopathy	65
A181V/A181V	A139V/A139V	24	alive at 5 years	5, 70%	14.8−25.9%	myopathy	66
C108W/L257P	C66W/L215P	7	6 years	8−9%	19.7%	myopathy	66
V113fs20X/I212N	V113fs20X/I212N	24	16 years	*b*	*b*	myopathy	67
V113fs20X/N93S	V113fs20X/N93S	24	alive at 9 years	21%	*b*	myopathy	67
R120R/H121N	R120R/H121N	24	6 years	25%	*b*	myopathy	67
T108M/Q125X	T108M/Q125X	7	19	18%	*b*	myopathy	67
Y154N/R172Q	Y112N/R130Q	*b*	*b*	*b*	*b*	mitochondrial myopathy	68
R225W/R225W	R183W/R183W	18	38	5−10%	*b*	mitochondrial myopathy	46
R172W/R172W	R130W/R130W	2	7	10−12%	*b*	mitochondrial myopathy	46
Q87X/N100S	Q45X/N58S	3	18	<5%	*b*	encephalomyopathy	69
K85NfsX9/R172Q	K43NfsX9/R130Q	12	alive at 13 years	24%	*b*	myopathy, hearing loss	70
A73fs.X230/R130W	A73fsX230/R130W	1	3	20%	4%	encephalomyopathy	71
K244del/S93IfsX99	K202del/S51IfsX57	24	alive at 6 years	<10%	*b*	myopathy, hearing loss	72
T108M/T108M	T108M/T108M	0	alive at 44 years	34%	40%	severe myopathy	47
W4V.fsX40/R90C	W4V.fsX40/R90C	13 years	alive at 31 years	39%	*b*	severe myopathy	47
R183W/R183W	R183W/R183W	9	alive at 4 years	25%	*b*	encephalomyopathy	45
N58S/N58S	N58S/N58S	9 years	alive at 28 years	86%	*b*	encephalomyopathy	45
R130Q/K44Nfsx9	R130Q/K44Nfsx9	24	alive at 15 years	24%	*b*	encephalomyopathy	45
c.157_2A> G/R196S	c.157_2A>G/R196S	48	alive at 10 years	73%	*b*	encephalomyopathy	45
I212N/5.8 kb del	I212N/5.8 kb del	3	8	10−20%	*b*	encephalomyopathy	45
S135L/R183W	S135L/R183W	5	22	*b*	*b*	encephalomyopathy	45
S135L/N58S	S135L/N58S	9	alive at 1.2 years	22%	*b*	encephalomyopathy	45
T108M/L233P	T108M/L233P	5	3 years	5%	*b*	encephalomyopathy	45
T230A/R225W	T230A/R225W	40−50 years	55−60 years	multiple deletion	22−40%	ophthalmoplegia	11

[a]TK2 mutations reported in the original publication (column 1) and amino acid numbering normalized to the GenBank database (column 2). Age for disease onset and death (columns 3 and 4) is given in months and, in some cases, years. Residual TK2 activity is given as relative activity (with dT as the substrate) compared with healthy controls. fsX*x*, frame shift and stop after amino acid number *x*. [b]Data not available.

■ ROLE OF TK2 IN MITOCHONDRIAL DTTP AND DCTP POOL MAINTENANCE AND MTDNA DEPLETION AND DELETION

In mitochondria, the levels of DNA precursors, e.g., dNTPs, play a crucial role in mtDNA replication. A limitation of one or more of the four dNTPs will stall mtDNA replication and result in mtDNA depletion or deletion[1] and lead to mitochondrial diseases. Currently, 12 of >200 nuclear genes encoding proteins involved in mtDNA replication and mtDNA precursor metabolism are associated with mitochondrial diseases, including *TK2*, *DGUOK* (deoxyguanosine kinase), and *TYMP* (thymidine phosphorylase).[10,42]

Progressive external ophthalmoplegia (PEO) manifests as multiple mtDNA deletions, which accumulate with age and cause late-onset symptoms in postmitotic tissues, such as the nervous system and skeletal muscle. These symptoms usually are weakness of the external eye muscles, bilateral ptosis, exercise intolerance, proximal muscle weakness, wasting, hearing loss, hypogonadism, optic atrophy, and Parkinsonism. Mutations in *DGUOK* and *TK2* are linked to autosomal recessive PEO (arPEO).[11,43,44]

TK2 Kinetics Elucidate the Mechanism of MDS Caused by TK2 Mutations. MDS was initially described by Moraes et al. in 1991, and 10 years later, the first report assigning TK2 mutations to MDS was published.[7,8] The two missense mutations in the TK2 gene, His90Asn and Ile181Asn, were found in four individuals with devastating isolated skeletal myopathy, mtDNA depletion, and death at an early age.[8] Since then, more than 50 individuals suffering from MDS with both a molecularly confirmed diagnosis and histological or biochemical analysis have been linked to a variety of genetic alternations in the *TK2* gene.[45] In addition to myopathy, neurological phenotypes and multitissue pathology have been discovered in patients carrying TK2 mutations[46] (Table 1) (see recent articles for more details[5,10,45,47]).

The first kinetic characterization of TK2 mutations identified in MDS patients showed that the His90Asn mutant exhibited

dx.doi.org/10.1021/bi5006877 | *Biochemistry* 2014, 53, 6142−6150

normal activity with dT but strongly reduced activity with dC (65% reduction) under conditions of excess substrates. The His90Asn mutant also lost negative cooperativity with dT, which indicated that at physiological dT concentrations (<0.05 μM), the His90Asn mutant would only have <7% of activity with dT compared to that with the wild-type enzyme. This difference would cause a severe reduction in the size of the dTTP and/or dCTP pool and thus a reduced level of mtDNA synthesis. The Ile181Asn mutant enzyme had only 0.6% activity with dT and 0.01% activity with dC compared with that of the wild-type enzyme, which would ultimately result in a depletion of dTTP and dCTP pools and depletion of mtDNA.[24] The characterization of two other mutations in TK2 found in patients, e.g., T77M, showed not only drastically reduced overall efficiency with dT and dC but also altered cooperativity for dT and dC; i.e., the presence of dT enhanced dC phosphorylation, while dC functions as a strong inhibitor of dT phosphorylation. Such an alteration exaggerated the effects of reduced TK2 activity and thus, severe mtDNA depletion.[48] The two TK2 mutations, e.g., T230A and R225W, causing late-onset arPEO, showed significantly reduced activity with both dT and dC and an altered ratio of dC/dT phosphorylation.[11] These studies suggested that a severe reduction in TK2 activity or an alteration of substrate specificity or kinetic behavior resulted in a disruption and limitation of mitochondrial dTTP and dCTP production, which caused mtDNA depletion and/or deletion.

Many of the TK2 mutations identified in MDS patients have not been characterized; however, from the data available to date, we concluded that the level of residual TK2 activity in the affected tissues is vital for survival (Table 1). However, the residual TK2 levels measured using a single substrate cannot provide adequate information regarding mtDNA depletion caused by the TK2 mutations, as was exemplified with the His90Asn mutant. Thus, kinetic studies of mutant TK2 are important for understanding the mechanism underlying mtDNA depletion. In most cases, the severity of TK2 deficiency is correlated with the patient phenotype, namely, severe impairment of TK2 resulting in encephalomyopathy with multiple-organ involvement, whereas partial reduction of TK2 was associated with myopathy.[5] Thus, relatively high levels of residual TK2 activity are associated with late-onset disease. However, in one case, the patient survived longer with severe TK2 deficiency (1% of controls),[49] and it was suggested that an alternative compensatory mechanism could be involved.

TK2 Kinetics Underlie Thymidine Overload-Induced mtDNA Depletion. High plasma dT levels were found in patients with defective thymidine phosphorylase (TP), which presented itself as mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) with multiple mtDNA deletions and depletions.[50,51] TP is an enzyme that catalyzes the reversible phosphorolysis of dT to thymine and deoxyribose 1-phosphate and plays an important role in maintaining a low plasma level of dT and dU.[52] Deficiency in TP activity resulted in an accumulation of dT and dU in patients, which has been suggested to be the cause of the mtDNA deletion and depletion observed in MNGIE patients.[24,26] In the plasma of healthy individuals, dT is undetectable; however, in TP patients, the plasma level of dT and dU ranged from 3.9 to 17.7 μM,[51,53] which indicates several hundred-fold increases in dT and dU concentrations. According to eq I, dT alone will inhibit dC phosphorylation of up to 80% (Figure 3A). In mitochondria, elevated dT levels will eventually result in an increased level of dTTP and a diminished dCTP pool, as illustrated in Figure 4, and ultimately to mtDNA deletion or depletion. It was demonstrated experimentally that high levels of dT and dU, because of TP deficiency, resulted in higher levels of dTTP but lower levels of dCTP in TP-knockout mouse brain mitochondria, which resulted in mtDNA instability, e.g., mtDNA deletion and depletion.[54] *In vitro* studies also showed that limited dCTP levels due to dT overload could cause mtDNA depletion.[31] On the basis of the TK2 kinetics described above, mtDNA depletion was prevented by deoxynucleoside (dC) supplementation or inhibition of dC catabolism in a cell culture model and a mouse model with defective TP. Taken together, these findings point to a potential treatment for mtDNA depletion caused by defects in dNTP metabolism.[32]

TK2 Kinetics Shed Light on Thymidine Analogue-Induced mtDNA Depletion. In early chemotherapy against HIV, AZT was administered alone at a relatively large dose and often resulted in severe mitochondrial dysfunction. In addition, mtDNA depletion was observed in muscles, peripheral blood, and other tissues of treated patients.[55] Although the doses of AZT are much smaller in a modern HAART regime, long-term exposure to this analogue still causes adverse effects, including anemia, lactic acidosis, neutropenia, skeletal muscle myopathy, and cardiomyopathy with mtDNA depletion.[56] These side effects were attributed to AZT-mediated mitochondrial toxicity, which was thought to be a class-wide major adverse effect associated with nucleoside analogues. Previously, the mechanism of AZT toxicity had been identified as the inhibition of mtDNA polymerase-γ, i.e., the DNA pol-γ hypothesis.[57] However, to reach 50% inhibition of pol-γ, at least 100 μM AZT-TP is required, which is far greater than the concentration that can be clinically achieved using the plasma concentration of AZT.[18]

Thus, an alternative mechanism underlying AZT-associated mitochondrial toxicity has been suggested; AZT is a potent inhibitor of dT phosphorylation catalyzed by TK2, which may result in a depleted mitochondrial dTTP pool and limit mtDNA replication, thereby ultimately resulting in mtDNA depletion.[15,17] Compared with proliferating tissues, most postmitotic tissues, such as skeletal muscle, mainly rely on the TK2-catalyzed dT/dC phosphorylation for the maintenance of dTTP and dCTP pools and, thus, exhibit greater sensitivity to AZT and other pyrimidine analogues. As shown in panels C and D of Figure 3, dT phosphorylation by TK2 is strongly inhibited by AZT or FLT. Assuming that the mitochondrial AZT concentration is within the same range of plasma concentration, i.e., ~3 μM,[18] AZT would inhibit dT phosphorylation by >50% (Figure 3C), and FLT at a concentration of 3 μM will inhibit dT phosphorylation by 70% (Figure 3D). On the other hand, dC phosphorylation will be stimulated. Such opposite effects will alter the ratio of dT and dC phosphorylation and result in a significantly decreased level of dTMP and an increased level of dCMP and eventually small dTTP and relatively large dCTP pools[25] (Figure 4). A >50% reduction of the dTTP pool has been reported in perfused rat hearts with short-term AZT exposure.[58] Recent studies using peripheral blood mononuclear cells from individuals who were infected with HIV and have been treated with antiviral therapy showed significant depletions of both ribonucleotide and deoxynucleotide pools.[59] Thus, these findings strongly suggest that perturbation of intramitochondrial dT and dC phosphorylation by TK2 contributes to the mitochondrial toxicity of AZT and other related pyrimidine

dx.doi.org/10.1021/bi5006877 | *Biochemistry* 2014, 53, 6142−6150

nucleoside analogue drugs in a manner independent of mtDNA polymerase-γ inhibition.

CONCLUSION

Mitochondrial diseases are multifactorial, and interactions of defective gene(s) or proteins with other factors are involved in disease development and contribute to the tissue specificity of the disease. However, the kinetics and tissue-specific distribution of TK2 have helped to explain the mechanism of tissue-specific mtDNA deletion in many patients with TK2 mutations. TK2 kinetic studies have also been used to design therapy for the treatment of TP deficiency and may be used to weaken the side effects of nucleoside analogues and to develop novel nucleoside analogue drugs. In some patients, mtDNA depletion has become less apparent as the disease progresses, despite clinical and biochemical deterioration, and in a few cases, an increase or lack of change in mtDNA copy number has been reported.[49,60,61] Thus, further biochemical characterization of mutant TK2 enzymes and their interactions with other factors and/or proteins are required to fully understand the mechanism of mtDNA copy number alteration and to develop therapeutic strategies for the treatment of mitochondrial diseases caused by defects in dNTP metabolism.

AUTHOR INFORMATION

Corresponding Author

*Department of Anatomy, Physiology and Biochemistry, Swedish University of Agricultural Sciences, Box 7011, SE-750 07 Uppsala, Sweden. E-mail: liya.wang@slu.se. Telephone: +46 18 672820.

Funding

This work was supported by a grant from the Swedish Research Council.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Prof. Staffan Eriksson for valuable discussions.

ABBREVIATIONS

TK2, thymidine kinase 2; mtDNA, mitochondrial DNA; MDS, mtDNA depletion syndrome; dT, 2′-deoxythymidine; dC, 2′-deoxycytidine; dU, 2′-deoxyuridine; AZT, 3′-azido-2′,3′-dideoxythymidine; FLT, 3′-fluoro-2′,3′-dideoxythymidine; dNTP, deoxynucleoside triphosphate; TK1, thymidine kinase 1; TP, thymidine phosphorylase.

REFERENCES

(1) Taanman, J.-W., Muddle, J. R., and Muntau, A. C. (2003) Mitochondrial DNA depletion can be prevented by dGMP and dAMP supplementation in a resting culture of deoxyguanosine kinase-deficient fibroblasts. *Hum. Mol. Genet. 12*, 1839−1845.

(2) Arnér, E. S. J., and Eriksson, S. (1995) Mammalian deoxyribonucleoside kinases. *Pharmacol. Ther. 67*, 155−186.

(3) Eriksson, S., and Wang, L. (2002) The role of the cellular deoxynucleoside kinases in activation of nucleoside analogs used in chemotherapy. *Recent Advances in Nucleosides: Chemistry and Chemotherapy* (Chu, C. K., Ed.) pp 455−475, Elsevier, Amsterdam.

(4) Eriksson, S., Munch-Petersen, B., Johansson, K., and Eklund, H. (2002) Structure and function of cellular deoxynucleoside kinases. *Cell. Mol. Life Sci. 59*, 1327−1346.

(5) Wang, L., and Eriksson, S. (2010) Tissue specific distribution of pyrimidine deoxynucleoside salvage enzymes shed light on the mechanism of mitochondrial DNA depletion. *Nucleosides, Nucleotides Nucleic Acids 29*, 400−403.

(6) Sun, R., Eriksson, S., and Wang, L. (2014) Mitochondrial thymidine kinase 2 but not deoxyguanosine kinase is up-regulated during stationary growth phase of the cultured cells. *Nucleosides, Nucleotides Nucleic Acids 33*, 282−286.

(7) Moraes, C., Shanske, S., Tritschler, H., Aprille, J., Andreetta, F., Bonilla, E., Schon, E., and DiMauro, S. (1991) mtDNA depletion with variable tissue expression: A novel genetic abnormality in mitochondrial diseases. *Am. J. Hum. Genet. 48*, 492−501.

(8) Saada, A., Shaag, A., Mandel, H., Nevo, Y., Eriksson, S., and Elpeleg, O. (2001) Mutant mitochondrial thymidine kinase in mitochondrial DNA depletion myopathy. *Nat. Genet. 29*, 342−344.

(9) Eriksson, S., and Wang, L. (2008) Molecular mechanisms of mitochondrial DNA depletion diseases caused by deficiencies in enzymes in purine and pyrimidine metabolism. *Nucleosides, Nucleotides Nucleic Acids 27*, 800−808.

(10) Wang, L. (2010) Deoxynucleoside salvage enzymes and tissue specific mitochondrial DNA depletion. *Nucleosides, Nucleotides Nucleic Acids. 29*, 370−381.

(11) Tyynismaa, H., Sun, R., Ahola-Erkkilä, S., Almusa, H., Pöyhönen, R., Korpela, M., Honkaniemi, J., Isohanni, P., Paetau, A., Wang, L., and Suomalainen, A. (2012) Thymidine kinase 2 mutations in autosomal recessive progressive external ophthalmoplegia with multiple mitochondrial DNA deletions. *Hum. Mol. Genet. 21*, 66−75.

(12) Munch-Petersen, B., Cloos, L., Tyrsted, G., and Eriksson, S. (1991) Diverging substrate specificity of pure human thymidine kinases 1 and 2 against antiviral dideoxynucleosides. *J. Biol. Chem. 266*, 9032−9038.

(13) Wang, L., Munch-Petersen, B., Herrström Sjöberg, A., Hellman, U., Bergman, T., Jörnvall, H., and Eriksson, S. (1999) Human thymidine kinase 2: Molecular cloning and characterisation of the enzyme activity with antiviral and cytostatic nucleoside substrates. *FEBS Lett. 443*, 170−174.

(14) McKee, E. E., Bentley, A. T., Hatch, M., Gingerich, J., and Susan-Resiga, D. (2004) Phosphorylation of thymidine and AZT in heart mitochondria: Elucidation of a novel mechanism of AZT cardiotoxicity. *Cardiovasc. Toxicol. 4*, 155−167.

(15) Lynx, M. D., Bentley, A. T., and McKee, E. E. (2006) 3′-Azido-3′-deoxythymidine (AZT) inhibits thymidine phosphorylation in isolated rat liver mitochondria: A possible mechanism of AZT hepatotoxicity. *Biochem. Pharmacol. 71*, 1342−1348.

(16) Lynx, M. D., and McKee, E. E. (2006) 3′-Azido-3′-deoxythymidine (AZT) is a competitive inhibitor of thymidine phosphorylation in isolated rat heart and liver mitochondria. *Biochem. Pharmacol. 72*, 239−243.

(17) Susan-Resiga, D., Bentley, A. T., Lynx, M. D., Laclair, D. D., and McKee, E. E. (2007) Zidovudine inhibits thymidine phosphorylation in the isolated perfused rat heart. *Antimicrob. Agents Chemother. 51*, 1142−1149.

(18) McCann, K., Williams, D., and McKee, E. E. (2012) Metabolism of deoxypyrimidines and deoxypyrimidine antiviral analogs in isolated brain mitochondria. *J. Neurochem. 122*, 126−137.

(19) Johansson, K., Ramaswamy, S., Ljungcrantz, C., Knecht, W., Piskur, J., Munch-Petersen, B., Eriksson, S., and Eklund, H. (2001) Structural basis for substrate specificities of cellular deoxyribonucleoside kinases. *Nat. Struct. Biol. 8*, 616−620.

(20) Sabini, E., Ort, S., Monnerjahn, C., Konrad, M., and Lavie, A. (2003) Structure of human dCK suggests strategies to improve anticancer and antiviral therapy. *Nat. Struct. Biol. 10*, 513−519.

(21) Mikkelsen, N. E., Johansson, K., Karlsson, A., Knecht, W., Andersen, G., Piskur, J., Munch-Petersen, B., and Eklund, H. (2003) Structural basis for feedback inhibition of the deoxyribonucleoside salvage pathway: Studies of the *Drosophila* deoxyribonucleoside kinase. *Biochemistry 42*, 5706−5712.

(22) Sabini, E., Hazra, S., Ort, S., Konrad, M., and Lavie, A. (2008) Structural basis for substrate promiscuity of dCK. *J. Mol. Biol. 378*, 607−621.

(23) Munch-Petersen, B. (2010) Enzymatic regulation of cytosolic thymidine kinase 1 and mitochondrial thymidine kinase 2: A mini review. *Nucleosides, Nucleotides Nucleic Acids 29*, 363−369.
(24) Wang, L., Saada, A., and Eriksson, S. (2003) Kinetic properties of mutant human thymidine kinase 2 suggest a mechanism for mitochondrial DNA depletion myopathy. *J. Biol. Chem. 278*, 6963−6968.
(25) Wang, L., Sun, R., and Eriksson, S. (2011) The kinetic effects on thymidine kinase 2 by enzyme bound dTTP may explain the mitochondrial side effects of antiviral nucleoside analogs. *Antimicrob. Agents Chemother. 55*, 2552−2558.
(26) Wang, L., and Eriksson, S. (2000) Cloning and characterization of full length mouse thymidine kinase 2: The N-terminal sequence directs import of the precursor protein into mitochondria. *Biochem. J. 351*, 469−476.
(27) Barroso, J. F., Carvalho, R. N., and Flatmark, T. (2005) Kinetic analysis and ligand-induced conformational changes in dimeric and tetrameric forms of human thymidine kinase 2. *Biochemistry 44*, 4886−4896.
(28) Radivoyevitch, T., Munch-Petersen, B., Wang, L., and Eriksson, S. (2011) A mathematical model of human thymdine kinase 2. *Nucleosides, Nucleotides Nucleic Acids 30*, 203−209.
(29) Munch-Petersen, B., Knecht, W., Lenz, C., Sondergaard, L., and Piskur, J. (2000) Functional expression of a multisubstrate deoxyribonucleoside kinase from *Drosophila melanogaster* and its C-terminal deletion mutants. *J. Biol. Chem. 275*, 6673−6679.
(30) Segel, I. (1993) *Enzyme Kinetics*, Wiley Classics Library Edition, pp 100−132, John Wiley & Sons, Inc., New York.
(31) Gonzalez-Vioque, E., Torres-Torronteras, J., Andreu, A., and Marti, R. (2011) Limited dCTP availability accounts for mitochondrial DNA depletion in mitochondrial neurogastrointestinal encephalomyopathy (MNGIE). *PLoS Genet. 7*, e1002035.
(32) Camara, Y., Gonzalez-Vioque, E., Scarpelli, M., Torres-Torronteras, J., Caballero, A., Hirano, M., and Ramon, M. (2013) Administration of deoxyribonucleosides or inhibition of their catabolism as a pharmacological approach for mitochondrial DNA depletion syndrome. *Hum. Mol. Genet. 23*, 2459−2467.
(33) Barroso, J. F., Elholm, M., and Flatmark, T. (2003) Tight binding of deoxyribonucleotide triphosphates to human thymidine kinase 2 expressed in *Escherichia coli*. Purification and partial characterization of its dimeric and tetrameric forms. *Biochemistry 42*, 15158−15169.
(34) Perez-Perez, M., Priego, E., Hernandez, A., Familiar, O., Camarsa, M., Negri, A., Gago, F., and Balzarini, J. (2008) Structure, physiological role, and specific inhibitors of human thymidine kinase 2 (TK2): Present and future. *Med. Res. Rev. 28*, 797−820.
(35) Van Poecke, S., Negri, A., Janssen, J., Solaroli, N., Karlsson, A., Gago, F., Balzarini, J., and Van Calenbergh, S. (2011) Synthesis, modeling and evaluation of 3′-(1-aryl-1H-tetrazol-5-ylamino)-substituted 3′-deoxythymidine derivaties as potent and selective human mitochondrial thymidine kinase inhibitors. *Org. Biomol. Chem. 9*, 892−901.
(36) Franzolin, E., Rampazzo, C., -Pérez, M. J., Hernández, A. I., Balzarini, J., and Bianchi, V. (2006) Bromovinyl-deoxyuridine: A selective substrate for mitochondrial thymidine kinase in cell extracts. *Biochem. Biophys. Res. Commun. 344*, 30−36.
(37) Wang, L., and Eriksson, S. (2008) 5-Bromovinyl 2′-deoxyuridine phosphorylation by mitochondrial and cytosolic thymidine kinase (TK2 and TK1) and its use in selective measurement of TK2 activity in crude extracts. *Nucleosides, Nucleotides Nucleic Acids 27*, 858−862.
(38) Wang, J., and Eriksson, S. (1996) Phosphorylation of the anti-hepatitis B nucleoside analog 1-(2′-deoxy-2′-fluoro-1-β-D-arabinofuranosyl)-5-iodouracil (FIAU) by human cytosolic and mitochondrial thymidine kinase and implication for cytotoxicity. *Antimicrob. Agents Chemother. 40*, 1555−1557.
(39) Eriksson, S., and Wang, L. (1997) Substrate specificities, expression, and primary sequences of deoxynucleoside kinases; implications for chemotherapy. *Nucleosides Nucleotides 16*, 653−659.
(40) Ciliberti, N., Manfredini, S., Angusti, A., Durini, E., Solaroli, N., Vertuani, S., Buzzoni, L., Bonache, M., Ben-Shalom, E., Karlsson, A., Saada, A., and Balzarini, J. (2007) Novel selective human mitochondrial kinase inhibitors: Design, synthesis and enzymatic activity. *Bioorg. Med. Chem. 15*, 3065−3081.
(41) Priego, E., Karlsson, A., Gago, F., Camarasa, M., Balzarini, J., and Perez-Perez, M. (2012) Recent advances in thymidine kinase 2 (TK2) inhibitors and new perspectives for potential applications. *Curr. Pharm. Des. 18*, 2981−2994.
(42) Suomalainen, A., and Isohanni, P. (2010) Mitochondrial DNA depletion syndromes: Many genes, common mechanisms. *Neuromuscular Disorders 20*, 429−437.
(43) Copeland, W. C. (2012) Defects in mitochondrial DNA replication and human disease. *Crit. Rev. Biochem. Mol. Biol. 47*, 64−74.
(44) Ronchi, D., Garone, C., Bordoni, A., Rios, P., Calvo, S., Ripolone, M., Ranieri, M., Rizzuti, M., Villa, L., Magri, F., Corti, S., Bresolin, N., Mootha, V., Moggio, M., DiMauro, S., Comi, G., and Sciacco, M. (2012) Next-generation sequencing reveals DGUOK mutations in adult patients with mitochondrial DNA multiple deletions. *Brain 135*, 3404−3415.
(45) Chanprasert, S., Wang, J., Weng, S., Enns, G., Boue, D., Wong, B., Mendell, J., Perry, D., Sahenk, Z., Craigen, W., Alcala, F., Pascual, J., Melancon, S., Zhang, V., Scaglia, F., and Wong, L. (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*, 153−161.
(46) Götz, A., Isohanni, P., Pihko, H., Paetau, A., Herva, R., Saarenpää-Heikkilä, O., Valanne, L., Marjavaara, S., and Suomalainen, A. (2008) Thymidine kinase 2 defects can cause multi-tissue mtDNA depletion syndrome. *Brain 131*, 2841−2850.
(47) Behin, A., Jardel, C., Claeys, K., Faggart, J., Louha, M., Romero, N., Laforet, P., Eymard, B., and Lombes, A. (2012) Adult cases of mitochondrial DNA depletion due to TK2 defect: An expanding spectrum. *Neurology 78*, 644−648.
(48) Wang, L., Limongelli, A., Vila, M., Carrara, F., Zeviani, M., and Eriksson, S. (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.
(49) Vila, M., Segovia-Silvestre, T., Gamez, J., Marina, A., Naini, A., Meseguer, A., Lombes, A., Bonilla, E., DiMauro, S., Hirano, M., and Andreu, A. (2003) Reversion of mtDNA depletion in a patient with TK2 deficiency. *Neurology 60*, 1203−1205.
(50) Nishino, I., Spinazzola, A., and Hirano, M. (1999) Thymidine phosphorylase gene mutations in MNGIE, a human mitochondrial disorder. *Science 283*, 689−692.
(51) Valentino, M., Marti, R., Tadesse, S., Lopez, L., Manes, J., Lyzak, J., Hahn, A., Carelli, V., and Hirano, M. (2007) Thymidine and deoxyuridine accumulation in tissues of patients with mitochondrial neurogastrointestinal encephalomyopathy (MNGIE). *FEBS Lett. 581*, 3410−3414.
(52) Spinazzola, A., Marti, R., Nishino, I., Andreu, A., Naini, A., Tadesse, S., Pela, I., Zammarchi, E., Donati, M., Oliver, J., and Hirano, M. (2002) Altered thymidine metabolism due to defects of thymidine phosphorylase. *J. Biol. Chem. 277*, 4128−4133.
(53) Marti, R., Nishigaki, Y., and Hirano, M. (2003) Elevated plasma deoxyurindine in patients with thymidine phosphorylase deficiency. *Biochem. Biophys. Res. Commun. 303*, 14−18.
(54) Lopez, L., Akman, H., Garcia-Cazola, A., Dorado, B., Marti, R., Nishino, I., Tadesse, S., Pizzorno, G., Shungu, D., Bonilla, E., Tanji, K., and Hirano, M. (2009) Unbalanced deoxynucleotide pools cause mitochondrial DNA instability in thymidine phosphorylase-deficient mice. *Hum. Mol. Genet. 18*, 714−722.
(55) Dalakas, M., Illa, I., Pezeshkpour, G., Laukaitis, J., Cohen, B., and Griffin, J. (1990) Mitochondrial myopathy caused by long-term zidovudine therapy. *N. Engl. J. Med. 322*, 1098−1105.
(56) Scruggs, E., and Dirks Naylor, A. (2008) Mechanisms of zidovudine-induced mitochondrial toxicity and myopathy. *Pharmacology 82*, 83−88.

(57) Lewis, W., and Dalakas, M. C. (1995) Mitochondrial toxicity of antiviral drugs. *Nat. Med. 1*, 417−422.
(58) Morris, G. W., Iams, T. A., Slepchenko, K. G., and McKee, E. E. (2009) Origin of pyrimidine deoxynucleotide pools in perfused rat heart implications for 3′-azido-3′-deoxythymidine-dependent cardiotoxicity. *Biochem. J. 422*, 513−520.
(59) Selvaraj, S., Ghebremichael, M., Li, M., Foli, Y., Langs-Barlow, A., Ogbuagu, A., Barakat, L., Tubridy, E., Edifor, R., Lam, W., Cheng, Y.-C., and Paintsil, E. (2014) Antiretroviral therapy-induced mitochondrial toxicity: Potential mechanisms beyond polymerase-γ inhibition. *Clin. Pharmacol. Ther. 96*, 110−120.
(60) Leshinsky-Silver, E., Michelson, M., Cohen, S., Ginsberg, M., Sadeh, M., Barash, V., Lerman-Sagie, T., and Lev, D. (2008) A defect in the thymidine kinase 2 gene causing isolated mitochondrial myopathy without mtDNA depletion. *European Journal of Paediatric Neurology 12*, 309−313.
(61) Vila, M., Villarroya, J., Garcia-Arumi, E., Castellote, A., Meseguer, A., Hirano, M., and Roig, M. (2008) Selective muscle fiber loss and molecular compensation in mitochondrial myopathy due to TK2 deficiency. *J. Neurol. Sci. 267*, 137−141.
(62) Mancuso, M., Salviati, L., Sacconi, S., Otaegui, D., Camano, P., Marina, A., Bacman, S., Moraes, C., Carlo, J., Garcia, M., Garcia-Alvarez, M., Monzon, L., Naini, A., Hirano, M., Bonilla, E., Taratuto, A., DiMauro, S., and Vu, T. (2002) Mitochondrial DNA depletion: Mutations in thymidine kinase gene with myopathy and SMA. *Neurology 59*, 1197−1202.
(63) Mancuso, M., Filosto, M., Bonilla, E., Hirano, M., Shanske, S., Vu, T., and DiMauro, S. (2003) Mitochondrial myopathy of childhood associated with mitochondrial DNA depletion and a homozygous mutation (T77M) in the TK2 gene. *Arch. Neurol. 60*, 1007−1009.
(64) Carrozzo, R., Bornstein, B., Lucioli, S., Campos, Y., de la Pena, P., Petit, N., Dionisi-Vici, C., Vilarinho, L., Rizza, T., Bertini, E., Garesse, R., Santorelli, F., and Arenas, J. (2003) Mutation analysis in 16 patients with mtDNA depletion. *Hum. Mutat. 21*, 453−454.
(65) Tulinius, M., Moslemi, A., Darin, N., Holme, E., and Oldfors, A. (2005) Novel mutations in the thymidine kinase 2 gene (TK2) associated with fatal mitochondrial myopathy and mitochondrial DNA depletion. *Neuromuscular Disorders 15*, 412−415.
(66) Galbiati, S., Bordoni, A., Papadimitriou, D., Toscano, A., Rodolico, C., Katsarrou, E., Sciacco, M., Garufi, A., Prelle, A., Aguennouz, M., Bonsignore, M., Crimi, M., Martinuzzi, A., Bresolin, N., Papadimitriou, A., and Comi, G. (2006) New mutations in TK2 gene associated with mitochondrial DNA depletion. *Paediatric Neurology 34*, 177−185.
(67) Oskoui, M., Davidzon, G., Pascual, J., Erazo, R., Gurgel-Giannetti, J., Krishna, S., Bonilla, E., De Vivo, D., Shanske, S., and DiMauro, S. (2006) Clinical spectrum of mitochondrial DNA depletion due to mutations in the thymidine kinase 2 gene. *Arch. Neurol. 63*, 1122−1126.
(68) Alberio, S., Mineri, R., Tiranti, V., and Zeviani, M. (2007) Depeltion of mtDNA: Syndromes and genes. *Mitochondrion 7*, 6−12.
(69) Blakely, E., He, L., Gardner, J., Hudson, G., Walter, J., Hughes, I., Turnbull, D., and Taylor, R. (2008) Novel mutations in the TK2 gene associated with fatal mitochondrial DNA depletion myopathy. *Neuromuscular Disorders 18*, 557−560.
(70) Collins, J., Bove, K., Dimmock, D., Morehart, P., Wong, L., and Wong, B. (2009) Progressive myofiber loss with extensive fibro-fatty replacement in a child with mitochondrial DNA depeltion syndrome nd novel thymidine kinase 2 gene mutations. *Neuromuscular Disorders 19*, 784−787.
(71) Lesko, N., Naess, K., Wibom, R., Solaroli, N., Nennesmo, I., von Döbeln, U., Karlsson, A., and Larsson, N. G. (2010) Two novel mutations in thymidine kinase 2 cause early onset fatal encephalomyopathy and severe mtDNA depletion. *Neuromuscular Disorders 20*, 198−203.
(72) Marti, R., Nascimento, A., Colomer, J., Lara, M., Lopez-Gallardo, E., Ruiz-Pesini, E., Montoya, J., Andreu, A., Briones, P., and Pineda, M. (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*, 151−154.