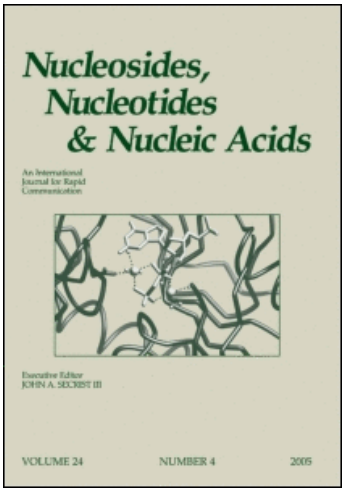

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RETAINED SENSITIVITY TO CYTOTOXIC PYRIMIDINE NUCLEOSIDE ANALOGS IN THYMIDINE KINASE 2 DEFICIENT HUMAN FIBROBLASTS

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□ *Thymidine kinase 2 (TK2) is a mitochondrial deoxyribonucleoside kinase that phosphorylates several nucleoside analogs used in anti-viral and anti-cancer therapy. A fibroblast cell line with decreased TK2 activity was investigated in order to obtain insights in the effects of TK2 deficiency on nucleotide metabolism. The role of TK2 for the sensitivity against cytotoxic nucleoside analogs was also investigated. The TK2 deficient cells retained their sensitivity against all pyrimidine nucleoside analogs tested. This study suggests that nucleoside analog phosphorylation mediated by TK2 may be less important, compared to other deoxyribonucleoside kinases, for the cytotoxic effects of these compounds.*

Keywords Nucleoside kinase; nucleoside analog; mitochondrial DNA

1. INTRODUCTION

Pyrimidine nucleoside analogs are used for treatment of viral infections and as chemotherapy of cancer. The nucleoside analogs are imported from the extracellular space by nucleoside transporter proteins and phosphorylated to the mono-, di-, and tri-phosphate forms within the cells.[1] Phosphorylation of nucleoside analogs is required for pharmacological effects since the phosphorylated nucleoside analogs inhibit enzymes involved in deoxyribonucleotide metabolism or interfere with cellular or viral DNA replication. The rate-limiting step in nucleoside analog phosphorylation is

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catalyzed by nucleoside kinases. Pyrimidine deoxyribonucleoside analogs can be phosphorylated by thymidine kinase 1 (TK1), thymidine kinase 2 (TK2), or deoxycytidine kinase (dCK).[1,2] The substrate specificity of TK1 and dCK does not overlap, whereas TK2 shares several substrates with both these enzymes. The deoxyribonucleoside kinases differ in tissue expression, cell cycle regulation, and subcellular localization. TK1 is cell cycle regulated and only expressed in S-phase cells, whereas TK2 and dCK are constitutively expressed throughout the cell cycle. TK1 and dCK are both located in the cytosol/nuclear compartment and TK2 is located in the mitochondrial matrix.[3–5]

Several pyrimidine nucleoside analogs used in anti-viral therapy such as 3′-azido-2′,3′-dideoxythymidine (AZT) and 2′,3′-dideoxycytidine (ddC), cause severe adverse effects by interfering with mitochondrial DNA (mtDNA) replication.[6] The nucleoside analog triphosphates are incorporated into mtDNA by the mitochondrial DNA polymerase γ and cause DNA chain termination resulting in mtDNA depletion.[7] Although several of these nucleoside analogs are substrates of TK1 or dCK as well as of mitochondrial TK2,[1] the role of mitochondrial TK2 for phosphorylation of these nucleoside analogs has remained unclear.

Inherited TK2 deficiency is a rare autosomal disease that causes mitochondrial DNA depletion syndrome (MDS) with symptoms of severe myopathy.[8,9] The myopathy presents in infancy and is fatal due to respiratory chain dysfunction.[10,11] In the present study a fibroblast cell line with TK2 deficiency has been investigated.[12] The cytotoxicity of several pyrimidine nucleoside analogs was studied to elucidate the importance of TK2 for the cytotoxic effects of these compounds.

2. MATERIALS AND METHODS

2.1. Genotyping

Two pairs of primers were designed to amplify exon 3 and exon 6 of the TK2 deficient fibroblast cell line as well as the two control cell lines. Each forward primer contained the M13 universal sequence in the 5′, while each reverse primer contained M13 reverse sequence. The primers for exon 3 amplification were: 5′-TGTAAAACGACGGCCAGTTGGTTACAT GAGCCCTTTCC-3′ and 5′-CAGGAAACAGCTATGACCAAGCTTTCTCC GCTTCCTTC-3′. The primers for exon 6 amplification were: 5′-TGTAAAA CGACGGCCAGTACATTTCTCAAGGCCTCCTG-3′ and 5′-CAGGAAACA GCTATGACCGAGGATTCGTGGCTGTTTGT-3′. The polymerase chain reaction (PCR) was prepared following the protocol of the AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, CA, USA) with 100 ng of genomic DNA as template. The elongation time was set to 1 minute, and the expected bands (244 bp for exon 3 and 336 bp for exon 6) were excised

from the gel and purified with MiniElute gel extraction kit (Qiagen, Venlo, The Netherlands). The bands were sequenced in both strands using M13 universal and M13 reverse primers.

2.2. Expression and Purification

The wildtype TK2 and the TK2 R130W mutant proteins were expressed in *E. coli* as fusions protein to glutathione S-transferase. The plasmids were transformed into Rosetta (DE3; Novagen, Madison, WI, USA) and single colonies were inoculated into LB medium supplemented with 100 μg/ml ampicillin. The bacteria were grown at 37°C and protein expression was induced at $OD_{600} \approx 0.8$ with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 12 hours at 27°C. The expressed proteins were purified using glutathione-sepharose 4B (Amersham Pharmacia Biotech, Piscataway, NJ, USA) as described by the producer. The purity of the recombinant proteins were verified by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (Phast system, Amersham Pharmacia Biotech) and the protein concentrations were determined with the Bradford Protein Assay (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as the concentration standard.

2.3. Enzyme Assays

The activity of the purified recombinant enzymes was assayed in a 50 μl reaction mixture containing: 50 mM Tris-HCl pH 7.6, 0.1 mg/ml BSA, 2.5 mM ATP, 5 mM $MgCl_2$, 5 mM dithiothreitol and 10 μM [methyl-^{3}H]-thymidine (Moravek Biochemicals, Brea, CA, USA) or 0.2 μM [5′-^{3}H]-5-(Bromovinyl)-2′-deoxyuridine (Moravek Biochemicals), and thymidine (10 μM to 10 mM) or BVDU (10 μM to 500 μM). The samples were incubated 30 minutes at 37°C, and every 10 minutes 10 μl aliquots were spotted on Whatman DE-81 filter paper disks. The filters were dried 1 hour and then washed 3 times for 5 minutes in 5 mM ammonium formate and once in sterile water. The filter bound nucleoside monophosphates were eluted in 500 μl of 0.1 M HCl and 0.1 M KCl and the radioactivity quantified by scintillation counting. The Michaelis-Menten constants were calculated using the GraphPad Prism software (GraphPad Software, La Jolla, CA, USA).

The substrate specificity of the purified enzymes was assayed by thin layer chromatography (TLC). The assay was performed in 50 mM Tris−HCl pH 7.6, 0.5 mg/ml BSA, 5 mM $MgCl_2$, 5 mM DTT, 10 mM ATP, 15 μCi [γ-^{32}P]ATP (Amersham Pharmacia Biotech), 1 mM of each substrate and 2 μg of recombinant human TK2 and TK2 R130W respectively. The samples were incubated for 30 minutes at 37°C. An amount of 2 μl of the reaction mixtures were spotted on polyethyleneimine-cellulose F TLC sheets (Merck, Whitehouse Station, NJ, USA). The nucleoside monophosphates were separated in a buffer containing NH_4OH:isobutyric acid:dH_2O (1:66:33) and

the nucleoside diphosphates were separated in 0.5 M ammonium formate pH 3.5. The sheets were autoradiographed using phosphoimaging plates (BAS-1000, Fujix, Kyoto, Japan).

2.4. Cell Culture

Among a collection of human fibroblast cell lines at our department, one cell line was identified as TK2 deficient whereas two other cell lines with unaltered TK2 activity were selected as control cells. The three cell lines selected for the present study have an unrelated origin. The fibroblast cell lines were cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (Gibco, Life Technologies, Paisley, UK), 120 U/ml penicillin and 0.12 mg/ml streptomycin in a humidified atmosphere with 5% CO_2 at 37°C. Cell culture media were renewed every 3–4 days.

Cell proliferation assays to determine the acute toxicity of nucleoside analogs were performed on $\approx 1.0 \times 10^5$ cells/ml seeded in 200 μl wells of 96 well microtiter plates in the presence of serial dilutions of the test compounds. The cells were cultured at 37°C for 120 hours and the cell number was determined by use of a Coulter counter type ZM (Coulter Electronics, Fullerton, CA, USA). The cells were stained with MitoTracker red (Molecular Probes, Invitrogen, Carlsbad, CA, USA).

2.5. Quantification of mtDNA

To determine the delayed toxicity of the nucleoside analogs 3′-azido-2′,3′-dideoxythymidine (AZT) and 2′,3′-dideoxycytidine (ddC) the cells were cultured for 14 days in the presence of different concentrations of these nucleoside analogs. Cells cultured in 0.25 mg/ml of ethidium bromide (EtBr) were used as control. At day 7 and 14 respectively the cells were harvested and and genomic DNA was extracted from 1×10^6 cells using the Easy DNA extraction kit (Invitrogen, Carlsbad, CA, USA). mtDNA levels were quantified by real-time quantitative PCR as described.[13]

Southern blot analysis of mtDNA was performed on 5 μg DNA digested with BamH1 separated on a 0.8% agarose gel. The gel was blotted onto a Hybond N+ nylon membrane (Amersham). The filter was hybridized with [^{32}P]dCTP-labelled mtDNA probe (a fragment of 1484 bp) (Perkin-Elmer Applied Biosystems, Waltham, MA, USA). Prehybridization, hybridization, and washing were performed following the provider's instructions. The washed membrane was developed and analyzed.

2.6. Phosphorylation Assay

Protein was extracted from 2×10^6 cells by adding 200 μl of an extraction buffer containing 50 mM Tris-HCl pH 7.6, 20% glycerol, 0.5%

NP40, 2 μM DTT, 0.05 mg/ml PMSF, and 5 mM benzamidine. The cells were then frozen/thawed three times. After centrifugation the supernatants were kept at −80°C. The activity of TK2 was measured in the protein extracts. Protein was added to a 100 μl reaction mixture containing: 50 mM Tris-HCl pH 7.6, 0.5 mg/ml BSA, 5 mM ATP, 5 mM $MgCl_2$, 2 mM dithiothreitol and 2.5 μM [5′-^{3}H]BVDU (Amersham Pharmacia Biotech). The samples were incubated for 60 minutes at 37°C and, every 15 minutes 10 μl aliquots were spotted on Whatman DE-81 filter paper disks. The filters were dried for 1 hour and then washed 3 times for 5 minutes in 5 mM ammonium formate. The filter-bound nucleoside monophosphates were eluted in 500 μl of 0.1 N HCl and 0.1 N KCl and the radioactivity quantified by scintillation counting.

2.7. Nucleotide Incorporation Assay

The cells were incubated for 48 hours in DMEM with 0.5% fetal bovine serum to stop cell proliferation. The cells were then treated for 1, 2 and 4 hours with 1μM [methyl-^{3}H]Thymidine (Moravek Biochemicals). They were trypsinized and centrifuged and the pellet was incubated on ice for 1 hour with 10% trichloroacetic acid (TCA). After another centrifugation the pellet were resuspended in 500 μl of 0.5% sodium dodecyl sulphate (SDS) and 0.5 M of NaOH and transferred to a scintillation tube. Thereafter, 3 ml ready safe liquid scintillation reagent was added and the radioactivity was measured by scintillation counting.

3. RESULTS

A recombinant TK2 enzyme was expressed based on the sequence information obtained from the cell line identified with TK2 deficiency described in the methods section. Sequencing of exon 3 of the TK2 gene showed an insertion of 2 bases (C and G) in one allele resulting in a frame shift stop mutation and a truncation of the protein lacking amino acids in the C-terminal end. The remaining truncated polypeptide did not contain the nucleotide binding domain and was subsequently completely devoid of enzymatic activity (data not shown). In exon 6 of the other allele, there was a point mutation (C to T) resulting in a replacement of arginine 130 by tryptophane (R130W) (Figure 1). It was concluded that the remaining TK2 activity in the cell line was derived from the TK2 R130W mutant and thus this enzyme was further characterized. The TK2 R130W mutant enzyme and a wildtype control were expressed in *E. coli* and investigated for substrate recognition and catalytic activity.

The kinetic properties of the recombinant protein were determined, comparing the R130W TK2 mutant to wildtype TK2 (Table 1). The TK2 R130W mutant showed a 5-fold increase of K_m using thymidine as substrate, while there was no significant change in K_m for the nucleoside analog

TABLE 1 Michaelis-Menten kinetic properties (mean ± *SD*, $n = 3$) of thymidine and 5-(2-bromovinyl)-2′-deoxyuridine (BVDU) phosphorylation by TK2 wildtype and R130W recombinant proteins

	K_m (μM)	V_{max} (pmol/mg/min)	V_{max}/K_m
Thymidine			
wt	14.7 ± 2.8	1020 ± 36	69
R130W	75.2 ± 4.8	630 ± 84	8
BVDU			
wt	20.1 ± 3.7	2900 ± 78	144
R130W	21.8 ± 2.8	257 ± 59	12

5-(bromovinyl)-2′-deoxyuridine (BVDU). On the contrary the V_{max} reduction was 11-fold lower when the substrate was BVDU while only slightly reduced for thymidine. The V_{max}/K_m ratio demonstrated a reduction of enzyme efficacy of the TK2 R130W mutant compared to wildtype TK2 of ~8-fold for thymidine and ~12-fold for BVDU. The TK2 R130W mutant enzyme showed a marked decrease in phosphorylation of the nucleoside analogs AZT, BVDU, 1-β-D-arabinofuranosylthymine (araT) and 1-β-D-arabinofuranosylcytosine (araC) as compared to the wildtype TK2 enzyme (Figures 2A and 2B). Accordingly, the R130W mutation in TK2 decreased the ability of the enzyme to phosphorylate dThd as well as several nucleoside analogs (Figure 2C).

The TK2-deficient fibroblast cell line with the TK2 R130W mutant ($TK2^-$ cells) and two human fibroblast control cell lines ($TK2^+$-1 and

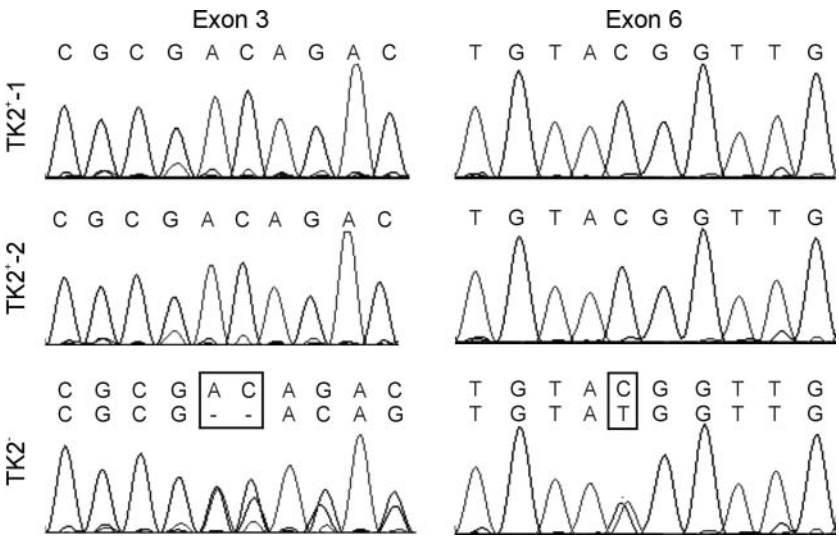

FIGURE 1 Sequences of exon 3 and exon 6 of the TK2 deficient cell line. The sequence of exon 3 shows a frameshift mutation resulting in a truncated protein (left) and exon 6 shows a non synonymous point mutation (C to T), resulting in a replacement of arginine-130 to tryptophane (R130W) (right).

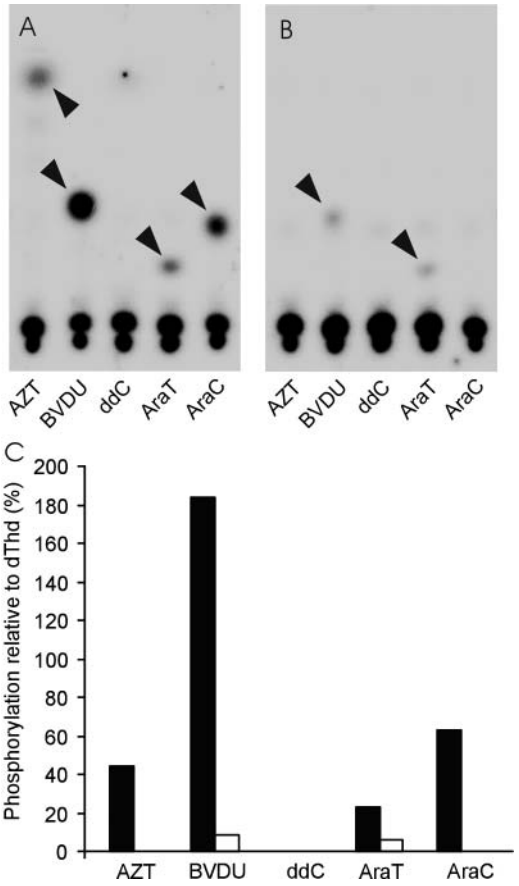

FIGURE 2 (A) Phosphorylation of nucleoside analogs by recombinant wildtype TK2; (B) and mutant R130W TK2 enzyme. The ^{32}P-phosphorylated nucleoside analogs were detected by thin-layer chromatography analysis (arrows indicate nucleoside monophosphate products); (C) Phosphorylation rate of nucleoside analogs relative to dThd phosphorylation catalyzed by wildtype TK2 (black bars) or mutant R130W TK2 (white bars).

TK2$^+$-2 cells) were exponentially grown in culture. The three cell lines had similar morphology and the TK2$^-$ cells exhibited rapid growth with a doubling time that was comparable with one of the two TK2$^+$ cell lines (Figure 3A). The TK2$^-$ fibroblast cells showed a normal distribution of the mitochondria when the cells were stained with Mitotracker (Figures 3B and 3C). The phosphorylation of BVDU in protein extracts from the different cell lines was determined and showed that the BVDU phosphorylation rate was decreased in the TK2$^-$ cells compared to the two TK2$^+$ cell lines (Figure 4A). The TK2 deficiency was confirmed by measuring thymidine incorporation in resting cells. Almost no incorporation of ^{3}H-dThd could be detected in the TK2$^-$ cells as compared to the TK2$^+$ cells (Figure 4B).

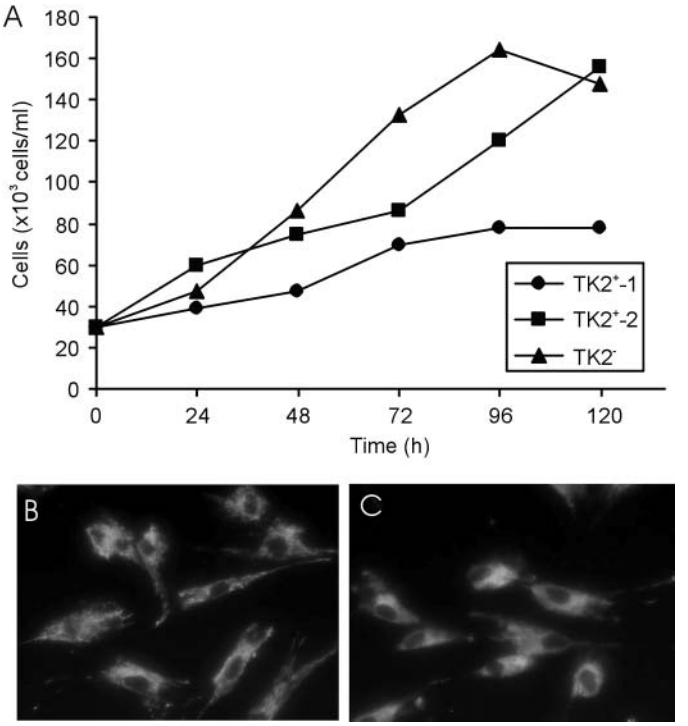

FIGURE 3 (A) Growth rate of $TK2^+$ and $TK2^-$ fibroblasts; mitochondrial DNA stained with Mitotracker in (B) $TK2^+$-1 fibroblast cell line; (C) $TK2^-$ fibroblast cell line.

TK2 deficiency is associated with mtDNA depletion in patients and in a mouse model.[8,9,14] Southern blot analysis of *Bam*H1 digested mtDNA from the cell lines showed a major band of 16.7 kbp corresponding to the expected size of the mitochondrial genome. The analysis also showed that there were no major deletions in the mtDNA (Figure 5A). The intensity of the mtDNA band from the $TK2^-$ cell lines was several-folds stronger than the band from the $TK2^+$ cells, suggesting that the cells may have increased levels of mtDNA compared to the control cells. Real-time PCR analysis was used to quantify the level of mtDNA in the cell lines and these experiments confirmed that the $TK2^-$ cell had a slightly increased mtDNA copy-number compared to the other two cell lines (Figure 5B). The mtDNA levels in the cells cultured in the presence of the nucleoside analogs AZT and ddC were also studied (Figure 6). The level of mtDNA in $TK2^+$-2 and $TK2^-$ remained unchanged both after 7 as well as 14 days when cultured in 100 and 500 μM of AZT respectively. The mtDNA level in the cells cultured in the presence of 20, 50, and 100 μM of ddC, respectively, showed a 90–97% decrease in all the cell lines at day 14. EtBr was used as control (0.25 μg/ml) and showed a 3-fold decrease in mtDNA in all cells lines at day 14.

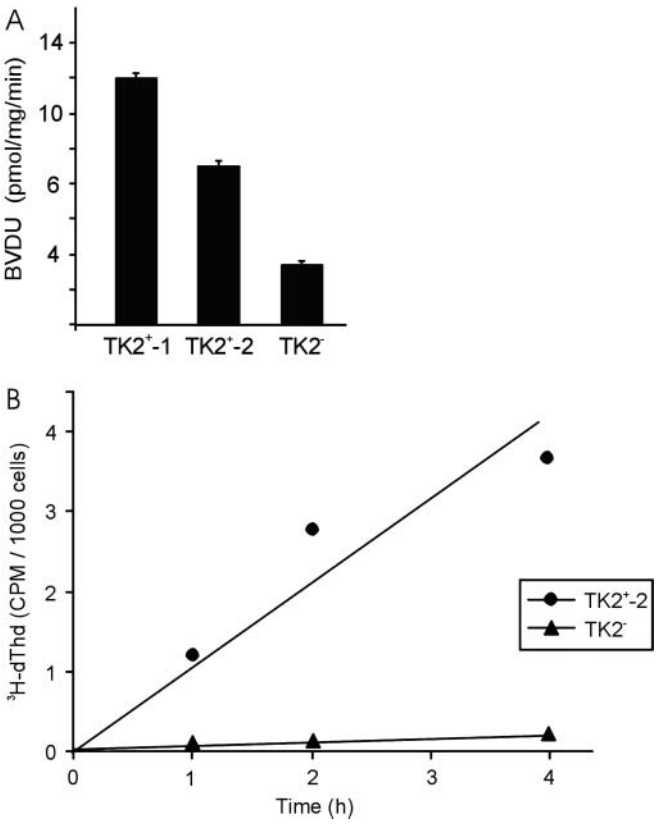

FIGURE 4 (A) Phosphorylation of BVDU in protein extracts from the different cell lines (mean ± *SD;* $p < 0.05$); (B) ^{3}H-dThd incorporation in non-proliferating TK2^{+}-2 and TK2^{-} fibroblast cell lines.

The sensitivity of the TK2^{+} and TK2^{-} cell lines towards several pyrimidine nucleoside analogs was determined (Table 2). All three cell lines were highly sensitive to dFdC and FdUrd suggesting that dCK activity determines the toxicity of dFdC and that TK1 is the major contributing

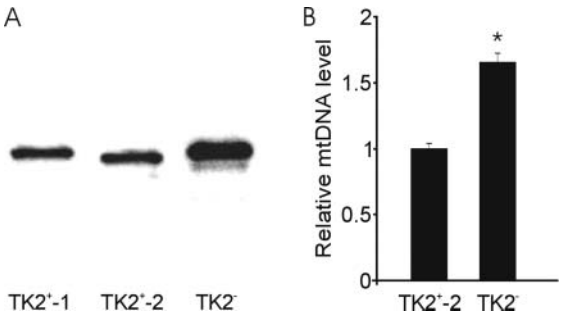

FIGURE 5 (A) Southern blot analysis of *BamH1* digested mtDNA from the cell lines; (B) RT-PCR quantification of mtDNA levels relative to nuclear DNA levels in wildtype TK2^{+}-2 and TK2 deficient fibroblast cell line (mean ± *SD;* $p < 0.05$).

TABLE 2 Sensitivity (IC_{50}; mean ± SD) of $TK2^+$ and $TK2^-$ fibroblast cell lines to nucleoside analogs. araA, 9-β-D-arabinofuranosyladenine; araT, 1-β-D-arabinofuranosylthymine; AZT, 3′-azido-2′,3′-dideoxythymidine; Bv-araU, 1-β-D-arabinofuranosyl-5-(*E*-2-bromovinyl)uracil; BVDU, 5-(2-bromovinyl)-2′-deoxyuridine; dFdC, 2′,2′-difluorocytidine; ddC, 2′,3′-dideoxycytidine; FdUrd, 5-fluoro-2′-deoxyuridine

	IC_{50} (μM)		
	$TK2^+$-1	$TK2^+$-2	$TK2^-$
araA	105 ± 22	50 ± 18	54 ± 32
araT	98 ± 67	56 ± 18	81 ± 26
AZT	≥ 500	240 ± 97	287 ± 213
Bv-araU	> 500	> 500	> 500
BVDU	310 ± 269	154 ± 0	289 ± 64
dCyd	> 500	≥ 500	468 ± 46
dThd	473 ± 38	258 ± 121	236 ± 200
dFdC	0.0062 ± 0.0017	0.0030 ± 0.0002	0.0036 ± 0.0008
ddC	186 ± 58	38 ± 30	43 ± 12
FdUrd	0.28 ± 0.09	0.48 ± 0.44	0.42 ± 0.33

enzyme for FdUrd toxicity. The two $TK2^+$ control cell lines differed for nucleoside analogs sensitivity where the $TK2^+$-2 cells showed an up to 4-fold higher sensitivity to several of the investigated compounds than the $TK2^+$-1 cell line. The sensitivity of $TK2^-$ cells to the toxicity of the nucleoside analogs was similar to the $TK2^+$-2 cells and the TK2-deficient cells did not show decreased sensitivity to any of the investigated nucleoside analogs. Accordingly, there was no indication that loss of TK2 activity would alter the cell sensitivity towards the compounds investigated.

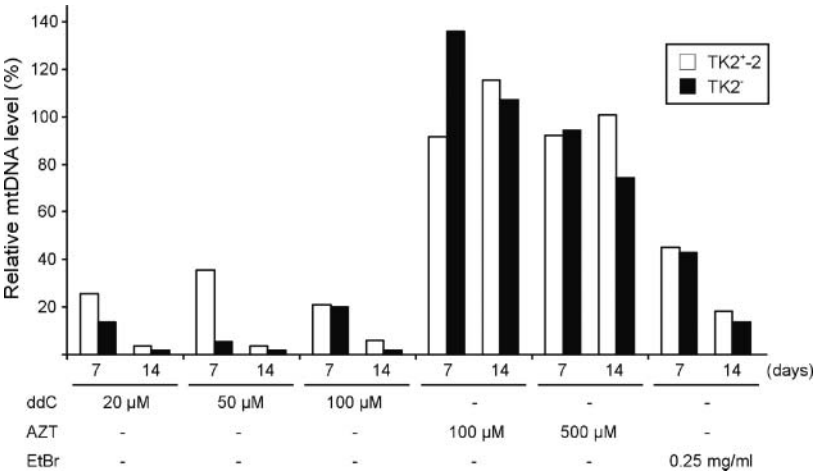

FIGURE 6 RT-PCR quantification of mitochondrial DNA levels relative to nuclear DNA levels in wildtype $TK2^+$-2 and TK2 deficient fibroblast cell line cultured in the presence of different concentrations of AZT, ddC and EtBr, respectively.

4. DISCUSSION AND CONCLUSION

Mitochondrial TK2 investigated in vitro phosphorylates several pyrimidine nucleoside analogs used in anti-cancer and anti-viral therapy.[15] The nucleoside analogs are in addition to recognizing TK2 also substrates of either TK1 or dCK. The importance of the in vivo TK2-mediated nucleoside analog phosphorylation in mitochondria has been unclear. A fibroblast cell line with approximately 8-fold decreased TK2 activity from a patient with TK2 deficiency was investigated. The TK2 deficiency was confirmed by measuring the BVDU phosphorylation in cell extracts as well as by determining the kinetic properties for both thymidine and BVDU using purified recombinant R130W mutated TK2. BVDU is often used as a selective marker for TK2 activity in living cells due to its high recognition by TK2.[16] The incorporation of ^{3}H-dThd in resting cells were also measured to confirm a reduced TK2 activity in the mutant cells. The activity of the nucleoside kinase TK1 is dominating in proliferating cells and TK2 represents only a fraction of the total cellular TK levels. However, when the cells are resting TK1 is almost undetectable and TK2 is the only thymidine phosphorylating kinase.[3]

It was shown that the sensitivity of the TK2 deficient cells towards the nucleoside analogs tested was at least as high as the sensitivity of two wildtype control fibroblast cell lines. The control cells used in this study were fibroblast cell lines from two different age-matched patients. Although the wildtype cells differed in growth rate, the sensitivity to nucleoside analogs was comparable between the three cell lines. These findings suggest that TK1 and dCK are more important than TK2 for nucleoside analog phosphorylation and induction of acute cytotoxicity in our cell models. However, the nucleoside analog cytotoxicity was studied in exponentially growing dividing cells that express high levels of TK1 during the S-phase of the cell cycle. Since TK1 is not present in resting cells it is possible that TK2 may be more important for the delayed nucleoside analog toxicity observed in certain cells. Previous studies have suggested that nucleoside analogs phosphorylated in the mitochondrial matrix become trapped in this subcellular compartment and that the intra-mitochondrial phosphorylation of nucleoside analogs could be an important determinant for the eventual mitochondrial toxicity of some nucleoside analogs.[17] However, subsequent studies showed that deoxyribonucleotides and phosphorylated nucleoside analogs can be transported across the mitochondrial membrane.[18] Accordingly, nucleoside analogs phosphorylated in the mitochondrial matrix by TK2 may also be exported to the cytosolic/nuclear dNTP compartment to exert their pharmacological effects. This is supported by the finding in the present study that the effect of ddC on mtDNA levels was similar in all cell lines investigated indicating that ddC is phosphorylated by dCK and subsequently transported into the mitochondria.

Patients with inherited TK2 deficiency show a tissue specific mtDNA depletion with decreased levels in muscle cells whereas other investigated organs such as blood and skin, show normal mtDNA levels.[19] Dividing fibroblast also import dNTPs from the cytosol and this may explain why any mtDNA depletion was not observed in these cell lines. This is consistent with studies on fibroblasts derived from a patient with dGK deficiency that also exhibited normal mtDNA levels.[19]

Subsequent questions to be addressed involve effects of cell cycle phases on mtDNA synthesis in cells deficient of TK2. The present TK2 deficient cells have a low but maybe significant residual TK2 activity that may result in phenotypic changes only when TK1, and other dTTP or dCTP contributing enzymes, are expressed at very low levels. Considering the severe phenotypic alterations in patients with reported TK2 deficiency it is clear that there are large tissue and organ specific differences of the importance of TK2 for cellular functions. The fibroblast cultures used in the present study demonstrates an example of cells where the contribution of TK2 for normal cell growth seems very low. It is likely that compensatory enzymes must be present in cultured fibroblasts for the synthesis of deoxyribonucleotides for mitochondrial DNA. The challenge will be to understand and use these compensatory mechanisms to treat mitochondrial DNA deficiency in cells that are dependent on TK2 for their survival.

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