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Mitochondrial expression of the
Drosophila melanogaster multisubstrate deoxyribonucleoside
kinase

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Abbreviations: *Dm*-dNK , *Drosophila melanogaster* deoxyribonucleoside kinase; OST TK^{-/-}, osteosarcoma TK1 deficient cells; dThd, deoxythymidine; AraA, 9-β-D-arabinofuranosyladenine; AraG, 9-β-D-arabinofuranosylguanine; AraC, 1-β-D-arabinofuranosylcytosine; dFdC, 2',2'-difluoro-2'-deoxycytidine; BVDU, (E)-5-(2-bromovinyl)-2'-deoxyuridine; BVaraU, 1-β-D-arabinofuranosyl-5-(E)-(2-bromovinyl)uracil; 5-BdUrd, 5-bromo-2'-deoxyuridine; 5-FdUrd, 5-fluoro-2'-deoxyuridine

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ABSTRACT

The multisubstrate deoxyribonucleoside kinase from *Drosophila melanogaster* deoxyribonucleoside kinase (*Dm*-dNK) is studied as a candidate suicide gene for applications in combined gene/chemotherapy of cancer. We have created an engineered *Dm*-dNK nucleoside kinase that is targeted to the mitochondrial matrix. The enzyme was expressed in a thymidine kinase 1-deficient osteosarcoma cell line and the sensitivity of the cells to cytotoxic nucleoside analogs was determined when the enzyme was targeted to either the nucleus or the mitochondrial matrix. Although the total dThd phosphorylation activity was similar in cells expressing *Dm*-dNK in the nucleus or in the mitochondria, the cells expressing the enzyme in the mitochondria showed higher sensitivity to the antiproliferative activity of several pyrimidine nucleoside analogs such as (E)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU), 5-bromo-2'-deoxyuridine (5-BdUrd) and 5-fluoro-2'-deoxyuridine (5-FdUrd). Labeling studies using [³H]-dThd showed that the cells expressing the mitochondrial enzyme had an increased incorporation of [³H]-dThd into DNA, shown to be due to a higher [³H]-dTTP specific activity of the total dTTP pool in the cells where *Dm*-dNK was targeted to the mitochondria. The difference in the specific activity of the dTTP pool is a result of different contributions of the *de novo* and the salvage pathways for the dTTP synthesis in transduced cells.

In summary, these findings suggest that mitochondrial targeting of *Dm*-dNK facilitates nucleoside and nucleoside analog phosphorylation and could be used as a strategy to enhance the efficacy of nucleoside analog phosphorylation and concomitantly their cytostatic potential.

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Cytotoxic nucleoside analogs are used as chemotherapeutic agents for treatment of cancer. The nucleoside analogs are phosphorylated in cells by nucleoside and nucleotide kinases to their triphosphates forms. The phosphorylated nucleoside analogs are incorporated into nuclear DNA during DNA replication and repair. Nucleoside kinases are presently being investigated for possible use as suicide genes in combined gene/chemotherapy of cancer (Springer and Niculescu-Duvaz, 2000). This strategy is based on that a nucleoside kinase is expressed in the cancer cells and that the enzyme phosphorylates and thereby activates cytotoxic nucleoside analogs. In addition to killing the cells expressing the suicide nucleoside kinase, adjacent cells are also killed by the transfer of phosphorylated metabolites via gap-junctions, a phenomenon known as the bystander effect (Freeman et al., 1993; Mesnil et al., 1996). The herpes simplex type-1 thymidine kinase used in combination with the nucleoside analog ganciclovir is the most commonly studied combination of a nucleoside kinase and a nucleoside analog in gene therapy (Balzarini et al., 1985; Moolten, 1986; Moolten and Wells, 1990; Springer and Niculescu-Duvaz, 2000). Other nucleoside kinases such as varicella zoster virus thymidine kinase and the human deoxycytidine kinase have also been investigated for possible use as suicide genes (Degreve et al., 1997; Manome et al., 1996). We have cloned a multisubstrate deoxyribonucleoside kinase from the fruit fly *Drosophila melanogaster* (*Dm-dNK*) and evaluated the use of this enzyme as a suicide gene (Johansson et al., 1999; Zheng et al., 2000; Zheng et al., 2001). In contrast to the human thymidine kinase 1 (TK1) that is able to phosphorylate dThd and deoxyuridine (dUrd) (Munch-Petersen et al., 1995), or to human thymidine kinase 2 (TK2) which accepts dThd, dUrd and deoxycytidine (dCyd) as substrates, *Dm-dNK* can phosphorylate all the natural pyrimidine and purine substrates and also a wide range of nucleoside analogs (Johansson et al., 1999).

Dm-dNK localizes to the cell nucleus when the enzyme is expressed in human cells, which is mediated by a nuclear localization signal in its C-terminal region (Zheng et al.,

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2000). We have previously performed mutagenesis of the nuclear localization signal and investigated the effect of expression of a cytosolic *Dm*-dNK in cancer cells (Zheng et al., 2001). We found no difference either in enzyme activity, cellular sensitivity to nucleoside analogs, or bystander cell killing when the enzyme was expressed in the cytosol or in the nucleus. These results are consistent with a model of rapid equilibration of the dNTP pools between the nucleus and the cytosol mediated by the nuclear pore complexes (Johansson et al., 1997; Zhu et al., 2000).

Mitochondrial DNA is replicated separately from the nuclear DNA and the enzymes involved in mitochondrial DNA replication differ from those catalyzing nuclear DNA replication. Mitochondrial DNA replication occurs independently of the cell cycle phase and is less efficiently repaired as compared to the nuclear DNA (Bogenghagen and Clayton, 1977; Bogenghagen, 1999). The inner mitochondrial membrane functionally separates the mitochondrial matrix from the cytosol and several studies suggest that the mitochondrial dNTP pool is separated from the cytosolic pool (Berk and Clayton, 1973; Bestwick et al., 1982; Bogenghagen and Clayton, 1976). However, dNTP transporter proteins have been identified in the inner mitochondrial membrane and a communication between the cytosolic and mitochondrial dNTP pools has been demonstrated (Bridges et al., 1999; Pontarin et al., 2003; Rampazzo et al., 2004; Rosenberg et al., 2002). We decided to study the effects of targeting *Dm*-dNK to the mitochondrial matrix in a human cell line and compare these cells to cells expressing the wild-type nuclear enzyme. In summary, we show that an engineered *Dm*-dNK with a mitochondrial targeting sequence is localized to the mitochondria and that it retains high enzymatic activity. The cells expressing *Dm*-dNK in the mitochondria showed increased sensitivity to several nucleoside analogs compared to the cells expressing the nuclear enzyme.

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MATERIALS AND METHODS

Construction of plasmid vectors and sequencing

The pEGFP-N1 vector (Clontech, Mountain view, CA, USA) was used for the plasmids constructed. The cDNA sequence encoding the 31 amino acid N-terminal mitochondrial import signal of cytochrome C oxidase subunit VIII was cloned upstream of *Dm*-dNK. A single amino acid mutation (R247S) was introduced in the C-terminal (Zheng et al., 2000). The plasmid was purified using the Plasmid Midi kit (Qiagen, Hilden, Germany).

The DNA sequence of the constructed plasmid was verified by DNA sequence determination using an ABI310 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). To recheck the DNA sequence of the constructs, the total DNA was extracted from the transfected cells using GenElute Mammalian Genomic DNA Miniprep kit (Sigma-Aldrich, St. Louis, MO, USA) and then amplified by PCR using as primers the pEGFP-N1 sequencing oligos. The fragments obtained were sequence using ABI310 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Cell culture and transfection

The OST TK⁻ cells were cultured in Dulbecco's Modified Eagle's Medium (D-MEM). The media were supplemented with 10 % (v/v) fetal calf serum (Gibco BRL, Carlsbad, CA, USA), 100 U / ml penicillin, 0.1 mg/ml streptomycin. Cells were grown at 37 °C in a humidified incubator with a gas phase of 5 % CO₂.

The plasmids were transfected into the human cell lines using FuGENE 6 transfection reagent (Roche, Basel, Switzerland). 1 µg plasmid DNA and 3 µl FuGENE 6 were dissolved in D-MEM medium (Gibco BRL, Carlsbad, CA, USA) and transfection was performed as described in the manufacturer's protocol. The cells were cultured three weeks in the presence of 1 mg/ml Geneticin (Life Technologies, Inc., Grand Island, NY) to select for stably transfected cell clones. The cells were sorted twice with FACS sorter (BD FACS Vantage SE

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with DIVA software equipped with a 488 nm laser) to select the cells expressing only GFP. GFP fluorescence was observed in a Nikon Eclipse E600 microscope equipped with a SPOT RT digital camera. The mitochondria were counterstained with MitoTracker (Invitrogen, Carlsbad, CA, USA).

Enzyme assays

10^6 cells were centrifuged and the pellet was treated for 1h on ice with 100 μ l of extraction buffer (50 mM Tris-HCL pH 7.6, 20% glycerol, 0.5% NP-40, 2 mM dithiothreitol, 0.5 mM phenylmethyl sulfonyl fluoride, 5 mM benzamidine). The samples were centrifuged for 30 min and the supernatant was kept and stored at -80 °C. The protein concentration was determined with Bradford Protein Assay (Bio-Rad, Hercules, CA, USA) using bovine serum albumin (BSA) as the concentration standard.

The activity of the extracted enzymes was assayed in a 50 μ l reaction mixture containing: 50 mM Tris-HCl pH 7.6, 0.1 mg / ml BSA, 2.5 mM ATP, 5 mM MgCl₂, 5 mM dithiothreitol, 0.15 μ M [methyl-³H]-thymidine (MT6032, Moravek Biochemicals, Brea, CA, USA), and 5mM thymidine. 10 μ g of protein extract was incubated 30 min at 37°C and every 10 min 10 μ l aliquots were spotted on Whatman DE-81 filter paper disks. The filters were dried 1 h, washed 3 times for 5 min in 5 mM ammonium formate and once in sterile water for 1 min. The filter bound nucleoside monophosphates were eluted with 500 μ l of 0.1 M HCl and 0.1 M KCl. Then 3 ml of Ready safe liquid scintillation reagent (Beckman, Fullerton, CA, USA) was add and the radioactivity quantified by scintillation counting.

dTTP pool measurement

To measure the dTTP pool at least three OST TK⁻ cell dishes for each cell line were prepared. One of them was used to quantify the number of cells by FACS, while the others were used for the dNTP pool extraction. Cells dishes were transferred on an ice bath. The medium was removed and the cells were washed twice with 5 ml ice-cold PBS. Cells for the

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FACS analysis were treated with 0.4 ml of Trypsin/EDTA, resuspended in 4 ml PBS and counted. Cells for the dTTP pool measurement were treated with 1.5 ml of 60% methanol, scraped from the dish, transferred into 2 ml tubes and stored at -20 °C for 8h. Subsequently the tubes were centrifuged at $16000 \times g$, the supernatant was transferred into a new tube and evaporated to dryness in a vacuum centrifuge. Based on the number of cells the dry pellet was resuspended in 100 μ l of 10 mM Tris HCl pH 7.8 per 10^6 cells.

For the dTTP quantification we used the enzymatic assay proposed by Sherman et al. with synthetic oligonucleotides as template primers for the measurement of deoxyribonucleoside triphosphates (Sherman and Fyfe, 1989).

Nucleotide incorporation assay

The cells, at 40%-50% confluence, were treated for 24h with 1 μ M [methyl- 3 H]-thymidine (MT6032, Moravek Biochemicals, Brea, CA, USA), afterwards 5×10^6 cells were centrifuged and the pellet was incubated on ice for 1h with 10% trichloroacetic acid (TCA) to remove unincorporated nucleotides and nucleic acid polymers shorter than ≈ 20 nucleotides. After centrifugation the pellet was resuspended in 500 μ l of 0.5% sodium dodecyl sulfate (SDS) and 0.5 M of NaOH, and afterwards transferred into scintillation tubes with 3 ml of Ready safe liquid scintillation reagent and the radioactivity measured by scintillation counting.

Serum starvation assay

5×10^6 cells were incubated for 48h in D-MEM without human serum, afterwards [methyl- 3 H]-thymidine (MT6032, Moravek Biochemicals, Brea, CA, USA) was added to a final concentration of 1 μ M. After 1h, 2h, 4h and 24h the cells were washed 2 times with 5 ml of PBS and then they were treated as previously stated.

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Inhibition of cell proliferation by nucleoside analogues

To evaluate the cytostatic activity of the nucleoside analogues against OST TK⁻ and the *Dm*-dNK OST TK⁻ cells, 10⁴ cells/well were plated in 96-well microtiter plates and allowed to adhere. Cells were subsequently incubated at 37°C in a humidified CO₂-controlled atmosphere, in the presence of 5-fold dilutions (in normal growth medium) of the compounds. After 3 days, the cells were detached with trypsin solution (Gibco) and counted in a Coulter Counter (Coulter Electronics Ltd., Harpenden, Hertz, UK). The IC₅₀ was defined as the drug concentration required to inhibit cell proliferation by 50%.

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RESULTS

A genetically engineered mutant of *Dm*-dNK was targeted to the mitochondria in cell lines to study the effect of nucleoside analogs phosphorylated in the mitochondrial matrix. We fused the mitochondrial import signal of cytochrome c oxidase subunit VIII to the N-terminus of *Dm*-dNK. To easily visualize the subcellular location of the protein, we also fused the *Dm*-dNK to GFP. Transfection of an OST TK⁻ cell line with the construct resulted however in a predominantly nuclear localization of the protein (data not shown). *Dm*-dNK localizes to the cell nucleus when expressed in human cell lines, mediated by a nuclear localization signal present in the C-terminal region of the protein (Zheng et al., 2000). It is likely that the nuclear localization signal trapped the protein in the nucleus and prevented the mitochondrial import. A mutation of the arginine residue to serine was introduced in position 247 to remove the nuclear localization signal (Zheng et al., 2000). This mutant was fused to the mitochondrial import signal in the N-terminus and to the GFP in the C-terminus (Mito-*Dm*-dNK-GFP) (Figure 1A). Expression of this fusion enzyme in the OST TK⁻ cell line resulted in a dotted fluorescence pattern (Figure 1B). The fluorescence overlapped with the fluorescence of Mitotracker mitochondrial stain, indicating that the expressed protein was located in the mitochondria. Expression of the wild-type *Dm*-dNK fused to GFP (*Dm*-dNK-GFP) resulted as previously reported in a nuclear localization of the enzyme (Figure 1B) (Zheng et al., 2000). At least three stably transduced cell lines for each of the four constructs were generated and in these cell cultures > 90 % of the cells exhibited green fluorescence.

In order to test the enzymatic activity of the expressed *Dm*-dNK proteins, we determined dThd phosphorylation activity in cell protein extracts (Figure 2). The osteosarcoma cells used were deficient in TK1 enzyme activity and showed accordingly only very low levels of dThd phosphorylation. The dThd kinase activity was increased > 200-fold in the cells expressing *Dm*-dNK compared to untransduced cells or cells expressing GFP

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alone. There was no significant difference ($p > 0.05$) in dThd kinase activity in the cells expressing the wild-type *Dm*-dNK-GFP or the mitochondrial targeted Mito-*Dm*-dNK-GFP.

We determined the sensitivity of the cells to the anti-proliferative activity of several pyrimidine nucleoside analogs (Table 1). The cell line expressing *Dm*-dNK-GFP showed > 5- to 10-fold increase in sensitivity to araT, BVDU and 5-FdUrd. The increase in sensitivity was highest for araT that had > 150-fold increased sensitivity followed by 5-FdUrd and BVDU that both exhibited a 70- and 80-fold increased sensitivity, respectively, compared to control cells. Mitochondrial expression of *Dm*-dNK also increased the sensitivity of these three nucleoside analogs. Interestingly, expression of Mito-*Dm*-dNK-GFP increased the sensitivity to 5-BdUrd by > 10-fold. Cells expressing the mitochondrial enzyme also showed an additional 4-fold higher sensitivity to 5-FdUrd and 8-fold higher sensitivity to BVDU compared with the *Dm*-dNK-GFP transduced cells. Accordingly, these data suggest that although the total level of *Dm*-dNK kinase activity is similar in the cell lines, the cells expressing the enzyme in the mitochondrial matrix exhibited a higher sensitivity to the cytostatic activity of several of the investigated pyrimidine nucleoside analogs.

The total dTTP pools in the cells were also determined and found to be higher for the *Dm*-dNK transduced cells than for the untransduced TK1-deficient cells (Figure 3). The *Dm*-dNK expressing cells had increased dTTP levels but there was no significant difference of the dTTP pools between the control cells expressing *Dm*-dNK-GFP and Mito-*Dm*-dNK-GFP ($p > 0.05$). Accordingly, the enzyme activities as well as the dTTP pools were comparable in the cells expressing *Dm*-dNK in the nucleus or in the mitochondrial matrix.

We performed labeling studies with [³H]-dThd to investigate the reason for the differences in nucleoside analog sensitivity between the cell lines. [³H]-dThd incorporation into genomic DNA was determined and the results indicated that the cells expressing Mito-*Dm*-dNK-GFP had \approx 4-fold higher incorporation of [³H]-dThd into DNA (Figure 4A). We

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also determined the [³H]-dThd incorporation into mtDNA by serum starvation to induce cell cycle arrest. The results did not show any differences in [³H]-dThd DNA incorporation in cells expressing *Dm*-dNK in the nucleus or in the mitochondria (Figure 4B). These findings suggest that the difference in [³H]-dThd incorporation was due to a difference in nuclear DNA incorporation rather than the incorporation into mitochondrial DNA.

Since the growth rate of all the cell lines was similar and not dependent on if the cells expressed *Dm*-dNK (data not shown), the increased [³H]-dThd DNA incorporation cannot be explained by a difference in the rate of DNA synthesis. Therefore a possible explanation for the differences in [³H]-dThd incorporation is a different specific activity of the dTTP pool, that is the part of [³H]-dTTP of the total dTTP pool, between the cells expressing *Dm*-dNK-GFP as compared to the cells expressing Mito-*Dm*-dNK-GFP. We determined the specific activities of the dTTP pools and found that the Mito-*Dm*-dNK-GFP expressing cell line had a 5-fold higher dTTP pool specific activity compared to the *Dm*-dNK-GFP expressing cells (Table 2). Calculating the rate of total dTTP incorporation into DNA from these data showed that the rate of DNA synthesis was similar in the cell lines expressing *Dm*-dNK as was observed also by cell counting. Accordingly, the increase in [³H]-dThd DNA incorporation in the Mito-*Dm*-dNK-GFP expressing cells was due to an increase in the specific [³H]-dTTP activity of the total dTTP pool.

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DISCUSSION

We have created a mitochondrial targeted *Dm*-dNK to study the effects of a mitochondrial expression of the enzyme in a cancer cell model. Our main finding is that the mitochondrial expression of *Dm*-dNK resulted in an increased sensitivity to several cytotoxic pyrimidine nucleoside analogs compared with a nuclear expression of the enzyme. In particular the sensitivity to araT and BVDU, that in non-transduced cells are preferred substrate for human TK2 (Arner et al., 1992; Franzolin et al., 2006), and that previously has been shown to dramatically increase by the expression of wt *Dm*-dNK (Zheng et al., 2000; Zheng et al., 2001), showed an even higher toxicity in cells where *Dm*-dNK was targeted to the mitochondria.

The high catalytic rate of *Dm*-dNK is clearly demonstrated by the dThd phosphorylation activity in crude protein extracts of the transduced cells. The result of the *Dm*-dNK expression is enhanced sensitivity to certain nucleoside analogs not only by the osteosarcoma TK⁻ cells, but also by other cell lines such as the human pancreatic adenocarcinoma MIA PaCa-2 cells, H9 cells and CEM cells (Bertoli et al., 2005; Zheng et al., 2000). Another effect of the expression of *Dm*-dNK is a considerable increase of the dTTP pool, which is consistent with a previous report where the presence of *Dm*-dNK in H9 and CEM cells resulted in alteration of the dNTP pools, with the most significant increase being the dTTP pool (Bertoli et al., 2005).

In an attempt to understand the mechanisms for the high sensitivity of the cells expressing Mito-*Dm*-dNK-GFP, we performed labeling studies using [³H]-dThd as substrate to follow the pathway of dThd metabolism. Our study showed that mitochondrial expression of *Dm*-dNK, as compared to nuclear expression, resulted in a higher specific [³H]-dTTP activity of the total dTTP pool and an increased incorporation of [³H]-dTTP into nuclear DNA. A possible explanation is a relative compartmentalization of the dTTP pool that may be the result of the very high catalytic activity of *Dm*-dNK, that exceeds the transport

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capacity, which has been demonstrated to equilibrate the dTTP pool between the cytosol and mitochondria (Pontarin et al., 2003). Thus the differences of the specific activities of the dTTP pools, depending on the site of phosphorylation, could be explained by a dTTP pool compartmentalization caused by insufficient transport mechanisms between the cytosolic and mitochondrial compartments. The lower specific activity of the dTTP pool synthesized in the cytosol may be a result of a dilution of the [³H]-dTTP due to unlabeled dTTP synthesized via the *de novo* pathway and a feedback inhibition of *Dm*-dNK by dTTP. On the contrary, the Mito-*Dm*-dNK-GFP could be less susceptible of the feedback inhibition from the cytosolic pool of dTTP, and possibly the continuous export of dTDP or dTTP from the mitochondrial matrix to the cytosol could create the condition for a non-feedback inhibition of Mito-*Dm*-dNK-GFP. These effects are caused by the expression of an extremely active enzyme, such as *Dm*-dNK, and may not be the case in normal cells where mitochondrial TK2 is considerably less active and therefore contributes at a much lower level to the total dTTP pool. Differences in feedback inhibition of *Dm*-dNK by high concentrations of dTTP are an interesting possibility when the enzyme is expressed in different sub cellular locations. It has been shown by Knecht et al. that 10 μM dTTP inhibits by 50% the dThd phosphorylation activity of purified *Dm*-dNK *in vitro* (Knecht et al., 2000). The estimated dTTP pool concentrations in the present study are ~ 18 μM in the control cells, ~ 35 μM in *Dm*-dNK-GFP cells and ~ 28 μM in the Mito-*Dm*-dNK-GFP cells. These concentrations may thus be at levels with regulatory effects on the *Dm*-dNK activity also in living cells. However more information is needed regarding the dynamics and regulation of the dTTP pool within mammalian mitochondria (Hosseini et al., 2007; Mathews and Song, 2007; Song et al., 2005).

It is likely that also the pyrimidine nucleoside analogs are phosphorylated at a similar rate as dThd by the enzyme in the mitochondria and consequently that the mitochondrial location of the enzyme would facilitate nucleoside analog phosphorylation. Previous studies

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have suggested that nucleoside analogs phosphorylated within the mitochondrial matrix become trapped and cannot be exported to the cytosol or nucleus (Berk and Clayton, 1973; Bestwick et al., 1982). However, although certain nucleosides and nucleoside analogs appear to be preferentially incorporated into mitochondrial DNA, there is strong evidence that there is communication between the mitochondrial and cytosolic/nuclear dNTP pools (Ferraro et al., 2006).

Expression of nucleoside kinases in the mitochondria may be a strategy to improve combined chemotherapy/suicide gene therapy of cancer. Our data show that several nucleoside analogs have a higher inhibitory potential when the *Dm*-dNK suicide gene is expressed in the mitochondria than in the cytosol or nucleus. However, mitochondrial targeting of suicide nucleoside kinases appears not to enhance the cellular sensitivity for all nucleoside analogs. We have previously expressed a mitochondrial-targeted mutant of human dCK and tested the sensitivity of these cells to several nucleoside analogs (Zhu et al., 2000). In this model system we did not find any enhanced sensitivity for nucleoside analogs when dCK was located in the mitochondria compared to a nuclear or cytosolic location of the enzyme. We do not know the reason for this discrepancy but the enhanced phosphorylation of nucleoside analogs within the mitochondria may be dependent both on the nature of the nucleoside kinase, as well as the particular nucleoside analog investigated.

Accordingly, our study provide evidence that mitochondrial targeting of *Dm*-dNK may increase the sensitivity to thymidine nucleoside analogs but further studies are required to determine if this may become a novel strategy for suicide gene therapy.

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REFERENCES

- Arner ES, Spasokoukotskaja T and Eriksson S (1992) Selective assays for thymidine kinase 1 and 2 and deoxycytidine kinase and their activities in extracts from human cells and tissues. *Biochem Biophys Res Commun* **188**(2):712-718.
- Balzarini J, de Clercq E, Ayusawa D and Seno T (1985) Murine mammary FM3A carcinoma cells transformed with the herpes simplex virus type 1 thymidine kinase gene are highly sensitive to the growth-inhibitory properties of (E)-5-(2-bromovinyl)-2'-deoxyuridine and related compounds. *FEBS Lett* **185**(1):95-100.
- Berk AJ and Clayton DA (1973) A genetically distinct thymidine kinase in mammalian mitochondria. Exclusive labeling of mitochondrial deoxyribonucleic acid. *J Biol Chem* **248**(8):2722-2729.
- Bertoli A, Franco M, Balzarini J, Johansson M and Karlsson A (2005) Altered deoxyribonucleotide pools in T-lymphoblastoid cells expressing the multisubstrate nucleoside kinase of *Drosophila melanogaster*. *FEBS J* **272**(15):3918-3928.
- Bestwick RK, Moffett GL and Mathews CK (1982) Selective expansion of mitochondrial nucleoside triphosphate pools in antimetabolite-treated HeLa cells. *J Biol Chem* **257**(16):9300-9304.
- Bogenhagen D and Clayton DA (1976) Thymidylate nucleotide supply for mitochondrial DNA synthesis in mouse L-cells. Effect of 5-fluorodeoxyuridine and methotrexate in thymidine kinase plus and thymidine kinase minus cells. *J Biol Chem* **251**(10):2938-2944.
- Bogenhagen D and Clayton DA (1977) Mouse L cell mitochondrial DNA molecules are selected randomly for replication throughout the cell cycle. *Cell* **11**(4):719-727.
- Bogenhagen DF (1999) Repair of mtDNA in vertebrates. *Am J Hum Genet* **64**(5):1276-1281.

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Bridges EG, Jiang Z and Cheng YC (1999) Characterization of a dCTP transport activity reconstituted from human mitochondria. *J Biol Chem* **274**(8):4620-4625.

Degreve B, Andrei G, Izquierdo M, Piette J, Morin K, Knaus EE, Wiebe LI, Basrah I, Walker RT, De Clercq E and Balzarini J (1997) Varicella-zoster virus thymidine kinase gene and antiherpetic pyrimidine nucleoside analogues in a combined gene/chemotherapy treatment for cancer. *Gene Ther* **4**(10):1107-1114.

Ferraro P, Nicolosi L, Bernardi P, Reichard P and Bianchi V (2006) Mitochondrial deoxynucleotide pool sizes in mouse liver and evidence for a transport mechanism for thymidine monophosphate. *Proc Natl Acad Sci U S A* **103**(49):18586-18591.

Franzolin E, Rampazzo C, Perez-Perez MJ, Hernandez AI, Balzarini J and Bianchi V (2006) Bromovinyl-deoxyuridine: A selective substrate for mitochondrial thymidine kinase in cell extracts. *Biochem Biophys Res Commun* **344**(1):30-36.

Freeman SM, Abboud CN, Whartenby KA, Packman CH, Koeplin DS, Moolten FL and Abraham GN (1993) The "bystander effect": tumor regression when a fraction of the tumor mass is genetically modified. *Cancer Res* **53**(21):5274-5283.

Hosseini SH, Kohler JJ, Haase CP, Tioleco N, Stuart T, Keebaugh E, Ludaway T, Russ R, Green E, Long R, Wang L, Eriksson S and Lewis W (2007) Targeted transgenic overexpression of mitochondrial thymidine kinase (TK2) alters mitochondrial DNA (mtDNA) and mitochondrial polypeptide abundance: transgenic TK2, mtDNA, and antiretrovirals. *Am J Pathol* **170**(3):865-874.

Johansson M, Brismar S and Karlsson A (1997) Human deoxycytidine kinase is located in the cell nucleus. *Proc Natl Acad Sci U S A* **94**(22):11941-11945.

Johansson M, van Rompay AR, Degreve B, Balzarini J and Karlsson A (1999) Cloning and characterization of the multisubstrate deoxyribonucleoside kinase of *Drosophila melanogaster*. *J Biol Chem* **274**(34):23814-23819.

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- Knecht W, Munch-Petersen B and Piskur J (2000) Identification of residues involved in the specificity and regulation of the highly efficient multisubstrate deoxyribonucleoside kinase from *Drosophila melanogaster*. *J Mol Biol* **301**(4):827-837.
- Manome Y, Wen PY, Dong Y, Tanaka T, Mitchell BS, Kufe DW and Fine HA (1996) Viral vector transduction of the human deoxycytidine kinase cDNA sensitizes glioma cells to the cytotoxic effects of cytosine arabinoside in vitro and in vivo. *Nat Med* **2**(5):567-573.
- Mathews CK and Song S (2007) Maintaining precursor pools for mitochondrial DNA replication. *Faseb J*, *21*, 2294-2303.
- Mesnil M, Piccoli C, Tiraby G, Willecke K and Yamasaki H (1996) Bystander killing of cancer cells by herpes simplex virus thymidine kinase gene is mediated by connexins. *Proc Natl Acad Sci U S A* **93**(5):1831-1835.
- Moolten FL (1986) Tumor chemosensitivity conferred by inserted herpes thymidine kinase genes: paradigm for a prospective cancer control strategy. *Cancer Res* **46**(10):5276-5281.
- Moolten FL and Wells JM (1990) Curability of tumors bearing herpes thymidine kinase genes transferred by retroviral vectors. *J Natl Cancer Inst* **82**(4):297-300.
- Munch-Petersen B, Cloos L, Jensen HK and Tyrsted G (1995) Human thymidine kinase 1. Regulation in normal and malignant cells. *Adv Enzyme Regul* **35**:69-89.
- Pontarin G, Gallinaro L, Ferraro P, Reichard P and Bianchi V (2003) Origins of mitochondrial thymidine triphosphate: dynamic relations to cytosolic pools. *Proc Natl Acad Sci U S A* **100**(21):12159-12164.
- Rampazzo C, Ferraro P, Pontarin G, Fabris S, Reichard P and Bianchi V (2004) Mitochondrial deoxyribonucleotides, pool sizes, synthesis, and regulation. *J Biol Chem* **279**(17):17019-17026.

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Rosenberg MJ, Agarwala R, Bouffard G, Davis J, Fiermonte G, Hilliard MS, Koch T, Kalikin LM, Makalowska I, Morton DH, Petty EM, Weber JL, Palmieri F, Kelley RI, Schaffer AA and Biesecker LG (2002) Mutant deoxynucleotide carrier is associated with congenital microcephaly. *Nat Genet* **32**(1):175-179.

Sherman PA and Fyfe JA (1989) Enzymatic assay for deoxyribonucleoside triphosphates using synthetic oligonucleotides as template primers. *Anal Biochem* **180**(2):222-226.

Song S, Pursell ZF, Copeland WC, Longley MJ, Kunkel TA and Mathews CK (2005) DNA precursor asymmetries in mammalian tissue mitochondria and possible contribution to mutagenesis through reduced replication fidelity. *Proc Natl Acad Sci U S A* **102**(14):4990-4995.

Springer CJ and Niculescu-Duvaz I (2000) Prodrug-activating systems in suicide gene therapy. *J Clin Invest* **105**(9):1161-1167.

Zheng X, Johansson M and Karlsson A (2000) Retroviral transduction of cancer cell lines with the gene encoding *Drosophila melanogaster* multisubstrate deoxyribonucleoside kinase. *J Biol Chem* **275**(50):39125-39129.

Zheng X, Johansson M and Karlsson A (2001) Bystander effects of cancer cell lines transduced with the multisubstrate deoxyribonucleoside kinase of *Drosophila melanogaster* and synergistic enhancement by hydroxyurea. *Mol Pharmacol* **60**(2):262-266.

Zheng X, Johansson M and Karlsson A (2001) Nucleoside analog cytotoxicity and bystander cell killing of cancer cells expressing *Drosophila melanogaster* deoxyribonucleoside kinase in the nucleus or cytosol. *Biochem Biophys Res Commun* **289**(1):229-233.

Zhu C, Johansson M and Karlsson A (2000) Incorporation of nucleoside analogs into nuclear or mitochondrial DNA is determined by the intracellular phosphorylation site. *J Biol Chem* **275**(35):26727-26731.

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. Expression of *Dm*-dNK targeted to the mitochondria. A. Plasmid vector constructs containing a CMV promoter used to express GFP, wild-type *Dm*-dNK fused to GFP (*Dm*-dNK-GFP), mitochondrial targeted GFP (Mito-GFP), and mitochondrial targeted *Dm*-dNK fused to GFP (Mito-*Dm*-dNK-GFP). The black box indicates the single amino acid mutation in the nuclear targeting sequence of *Dm*-dNK. COX8, cytochrome C oxidase subunit VIII mitochondrial targeting sequence. B. Fluorescent microscopy of OST TK⁻ cells expressing the GFP proteins. The mitochondria were contra-stained with Mitotracker.

Figure 2. *Dm*-dNK activity, determined as [³H]-dThd phosphorylation in crude extracts of untransduced and transduced OST TK⁻ cells (Control cells include OST TK⁻ wt, OST TK⁻ GFP and OST TK⁻ Mito-GFP).

Figure 3. dTTP pools of OST TK⁻ and OST TK⁻ expressing *Drosophila melanogaster* nucleoside kinase (*Dm*-dNK) into nucleus or mitochondria. Each data point represents the mean value ± SD of at least two separate experiments carried out in duplicate (Control cells include OST TK⁻ wt, GFP and Mito-GFP).

Figure 4. A. [³H]-dThd incorporation into DNA in untransduced and transduced OST TK⁻ cells. B. [³H]-dThd incorporation into DNA. Untransduced and transduced OST TK⁻ cells were subjected to serum starvation for 48 h. (Control cells include OST TK⁻ wt, GFP and Mito-GFP)

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	IC ₅₀ (μM)			
	GFP	<i>Dm</i> -dNK-GFP	Mito-GFP	Mito- <i>Dm</i> -dNK-GFP
araC	0.079	0.094	0.078	0.080
araG	145 ± 12	90 ± 29	175 ± 19	34 ± 14
araT	339 ± 228	2.0 ± 0.8	> 500	1.5 ± 0.1
5-BdUrd	> 500	240 ± 51	371 ± 137	22 ± 15
BVDU	216 ± 53	2.8 ± 0.8	241	0.33 ± 0.02
BVaraU	314 ± 73	299	427	294
dFdC	0.011	0.0080 ± 0.0003	0.0096 ± 0.0021	0.0052 ± 0.0008
5-FdUrd	7.4 ± 2.0	0.11 ± 0.06	6.0 ± 1.8	0.027 ± 0.003

Table 1. Nucleoside analog sensitivity of the OST TK⁻ cell line expressing GFP, wild type *Dm*-dNK fused to GFP (*Dm*-dNK-GFP), mitochondrial targeted GFP (Mito-GFP) or mitochondrial targeted *Dm*-dNK fused to GFP (Mito-*Dm*-dNK-GFP).

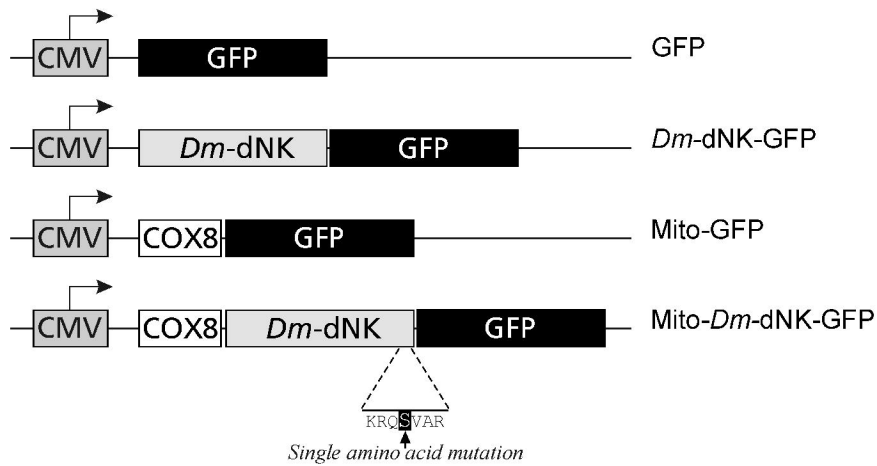
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	³ H]-dTTP DNA incorporation (CPM/min/10 ⁶ cells)	Specific [³ H]-dTTP activity of the dTTP pool (CPM / pmol)	Total dTTP DNA incorporation (pmol/min/10 ⁶ cells)
OST TK ⁻ cells	3	11	0,27
<i>Dm</i> -dNK-GFP	60	22	2,72
Mito- <i>Dm</i> -dNK-GFP	300	121	2,48

Table 2. [³H]-dThd labeling studies on dTTP and DNA metabolism in the controls or *Dm*-dNK expressing cell lines.

Figure 1

A



B

