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Increased mitochondrial DNA copy-number in CEM cells resistant to delayed toxicity of 2′,3′-dideoxycytidine

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ABSTRACT

The nucleoside analog 2′,3′-dideoxycytidine (ddC) has been used for treatment of human immunodeficiency virus (HIV) infections. ddC causes delayed toxicity when cells are exposed to the drug at low concentration for prolonged periods of time. The delayed toxicity is due to inhibition of mitochondrial DNA (mtDNA) replication, which results in mtDNA depletion and mitochondrial dysfunction. In the present study we have cultured CEM T-lymphoblast cells in the presence of low concentrations of ddC to generate two cell lines resistant to the delayed toxicity of the drug. Both cell lines were resistant to mtDNA depletion by ddC. The mechanism of ddC resistance was investigated and we showed that the resistant cells had decreased mRNA expression of the nucleoside kinases deoxycytidine kinase and thymidine kinase 2. We also studied the mitochondrial DNA in the cells and showed that the ddC-resistant cells had structurally intact mtDNA but 1.5–2-fold increased mtDNA copy-number as well as increased levels of the mitochondrial transcription factor A (Tfam). Our study suggests that cells may increase their level of mtDNA to counteract mtDNA depletion induced by ddC, while keeping pronounced antiviral activity of the drug.

1. Introduction

2′,3′-Dideoxycytidine (ddC) efficiently inhibit replication of human immunodeficiency virus (HIV) and it has been used in combination with other drugs for treatment of HIV infections [1]. ddC exerts its anti-retroviral effects through its phosphorylated derivative ddC-triphosphate (ddCTP). ddCTP is an efficient substrate for HIV reverse transcriptase and causes DNA chain-termination when incorporated into DNA during reverse transcription of the viral genome. The current model of the metabolic pathway for converting ddC to its triphosphate form consists of several steps that include: (i) cellular import of ddC into cells by plasma membrane nucleoside carrier proteins [2]; (ii) phosphorylation of ddC to ddCMP by deoxycytidine kinase (dCK) [3]; (iii) phosphorylation of ddCMP to ddCDP by UMP-CMP kinase [4]; (iv) phosphorylation of ddCDP to ddCTP by nucleoside diphosphate kinases (NDPK) [5]. The first phosphorylation step catalysed by dCK is rate limiting and this enzyme

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Abbreviations: ddC, 2′,3′-dideoxycytidine; ddCTP, 2′,3′-dideoxycytidine 5′-triphosphate; dCK, dexoycytidine kinase [EC 2.7.1.74]; TK1, thymidine kinase 1 [EC 2.7.1.21]; TK2, thymidine kinase 2 [EC 2.7.1.21]; HIV, human immunodeficiency virus; mtDNA, mitochondrial DNA; RT-PCR, reverse transcriptase polymerase chain reaction; NDPK, nucleoside diphosphate kinase [EC 2.7.4.6]; Tfam, mitochondrial transcription factor A; EtBr, ethidium bromide.

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has shown to be essential for the anti-HIV activity of ddC as cells deficient in dCK activity are resistant to the drug [6].

The clinical utility of ddC in anti-HIV therapy is mainly limited by the development of viral resistance to the drug through mutations in the HIV reverse transcriptase but resistance can also occur by cellular mechanisms such as decreased expression of nucleoside kinases [7,8].

Another clinical problem is toxic adverse side effects caused by ddC therapy. The toxic effects of ddC in cell model systems are either acute and occur at high drug concentrations, or delayed and occur after prolonged exposure to lower drug concentrations. The acute toxicity of ddC is caused by inhibition of nuclear DNA replication whereas the delayed toxicity has been associated with mitochondrial dysfunctions and depletion of mitochondrial DNA (mtDNA) [9]. Although ddCTP is a poor substrate for nuclear DNA polymerases compared to the HIV reverse transcriptase, ddCTP will inhibit cellular polymerases at higher concentrations [10]. The delayed toxicity is caused by incorporation of ddCTP into mtDNA by mitochondrial DNA polymerase γ and subsequent inhibition of mtDNA replication. In contrast to the nuclear polymerases, mitochondrial DNA polymerase γ can efficiently use ddCTP as a substrate. Inhibition of mtDNA replication results in depletion of mtDNA and causes mitochondrial dysfunction and ultimately cell death.

MtDNA depletion and adverse effects from mitochondrial dysfunction is a problem that occurs for several anti-viral nucleoside analogs including 3′-azido-2′,3′-dideoxythymidine [9]. Strategies to avoid mitochondrial toxicity, without affecting the anti-HIV activity of nucleoside analogs, should be an important therapeutic improvement. In order to identify critical events involved in mitochondrial toxicity, we have generated and characterized CEM T-lymphoblast cell lines resistant to the delayed toxicity of ddC. We show in the present study that the resistant cells had altered expression of the nucleoside kinases, increased levels of mtDNA and exhibited resistance to mtDNA depletion.

2. Materials and methods

2.1. Cell cultivation and selection of ddC-resistant cells

The T-lymphoblast CCRF-CEM cell lines were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (Gibco, Life Technologies, Paisley, UK), 100 U/ml penicillin and 0.1 mg/ml streptomycin in a humidified atmosphere with 5% CO_2 at 37 °C. CEM cells were cultured under dose-escalating conditions at concentrations of ddC (ranging between 0.025 and 0.25 μM) to select cells with decreased sensitivity to mitochondrial toxicity. The CEM/ddC1 cell line was cultured in the presence of 0.25 μM ddC for 50 days. This cell line was subsequently cultured in the presence of 1 μM ddC for 125 days to generate CEM/ddC2. Cell proliferation assays to determine the acute toxicity of nucleoside analogs were performed on ≈2.5×10^5 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 72 h and the cell number was determined by use of a Coulter counter type ZM (Coulter Electronics, Fullerton, CA).

2.2. Quantification of TK1, TK2, dCK, and DNC mRNAs

We used real-time reverse transcriptase PCR (RT-PCR) to quantify the levels of thymidine kinase 1 (TK1), thymidine kinase 2 (TK2), dCK and deoxyribonucleotide carrier protein (DNC) mRNAs. Total RNA was isolated from logarithmically growing cells using RNeasy kit according to the manufacturer's instruction (Qiagen, VWR International AB, Sweden). The cDNA was synthesized in a 20 μl reaction volume containing 2 μg of total RNA, 200 ng random hexamers primers, 0.5 mM of each dNTP, 5 mM, dithiothreitol, 2 units RNaseOUT (Invitrogen, Carlsbad, CA) and SuperScript RT (Invitrogen). The RNA was denaturated for 5 min at 65 °C and the cDNA transcription was performed for 50 min at 37 °C. Oligonucleotide primers for β-actin (Fwd: 5′-TCCTCC-TGAGCGCAAGTACTC, Rev: 5′-GCATTTGCGGTGGACGAT, Probe: 5′-TGTGGATCAGCAAGCAGGAGTAT-GACGAGT), TK1 (Fwd: 5′-GTGGCTGTCATAGGCATCGA, Rev: 5′-CCAAATGGCT-TCCTCTGGAA, Probe: CCAACGCCGGGAAGACCGTAATTG), TK2 (Fwd: 5′-GGAATGGTTTGACTGGATCTTGAG, Rev: 5′-GCGGAATGACCTTCTCCTCTT, Probe: 5′-CCTTCGGCACAA-TCCTGAGACTTGTTACCA), dCK (Fwd: 5′-GGACCCGCATC-AAGAAAATC, Rev: 5′-AGTACTTTGAACATTGCACCATCTG, Probe: AACATCGCT-GCAGGGAAGTCAACATTTGT), and DNC (Fwd: 5′-ACCTGTACAAGTGG-GCCATACC, Rev: 5′-GCTCA-AACCCTCCAACCTGTAG, Probe: 5′-ACCCTGACA-TATCCG-CTGGACCTCCTCA) were designed using Primer Express software (PerkinElmer Applied Biosystems, Foster City, CA, USA). RT-PCR reactions were performed on Applied Biosystems 7300 Real-time PCR System (PerkinElmer Applied Biosystems) using the TaqMan Universal PCR master kit (PerkinElmer Applied Biosystems) for the PCR reactions. The reaction volume was 25 μl and contained 12.5 μl of TaqMan buffer, 2.5 μl of the cDNA and primers for β-actin (0.2 μM Fwd primer, 0.4 μM Rev primer, 0.1 μM probe), TK1 (0.4 μM Fwd primer, 0.4 μM Rev primer, 0.2 μM probe); TK2 (0.4 μM Fwd primer, 0.4 μM Rev primer, 0.1 μM probe); dCK (0.4 μM Fwd primer, 0.4 μM Rev primer, 0.1 μM probe), or DNC (0.2 μM Fwd primer, 0.2 μM Rev primer, 0.2 μM probe). The PCR reactions were incubated for 2 min at 50 °C for AmpErase UNG enzyme activation, 10 min at 95 °C and subsequently 50 cycles of 15 s at 95 °C and 60 s at 60 °C.

2.3. Quantification of dNTP and ATP pools

The levels of dNTPs were determined from 2×10^6 logarithmically growing cells. The extraction and quantification of the dNTPs was performed as described [11]. The ATP level was determined from 1×10^6 cells. Each sample was treated with 200 μl lysis buffer (100 mM KPO_4 pH 7.8, 2 mM EDTA, 1 mM DTT, Triton X-100) The ATP was quantified with the ATP determination kit (FL-AA, Sigma–Aldrich, St. Louis, MO).

2.4. Quantification and characterization of mtDNA

Genomic DNA was extracted from 4×10^6 cells using the Easy DNA extraction kit (Invitrogen). MtDNA levels were quantified by real-time quantitative PCR as described [12]. Each assay included genomic DNA standards (each concentration measured three times), non-template controls and the genomic

DNA from the CEM cell lines tested (each sample measured four times). Southern blot analysis of mtDNA was performed on 10 μg DNA digested with BamH1 separated on a 0.8% agarose gel. The gel was blotted onto a Hybond N^+ nylon membrane (Amersham, Little Chalfont, UK). The filter was hybridized with [^{32}P]dCTP-labelled mtDNA probe (a fragment of 1484 bp) (PerkinElmer Applied Biosystems). Prehybridization, hybridization and washing were performed following the membrane's instructions. The washed membrane was developed and analyzed. Sequencing of mtDNA was performed on a DNA fragment comprising bp 5028–6531 of the mitochondrial genome. Oligonucleotide primers with flanking Kpn1 and BamH1 restriction enzyme sites (5′-GGTACCTAGGATGAATAATAGCAGTTCTACCGTACAAC and 5′-CGGGATCCAGTAGTATAGTGATGCCAGCAGCTAGGA) were used to PCR amplify the fragment with Pfu Ultra High-Fidelity DNA Polymerase (Stratagene, La Jolla, CA). The 1503 bp PCR product was isolated by agarose gel electrophoresis and purified from gel using a QIAquick gel extraction kit. The fragments were restriction digested, purified and cloned into the BamH1-Kpn1 sites of the pBluescript plasmid vector. Twenty individual clones of plasmid DNA were selected from each cell line. The plasmids were propagated in the *Escherichia coli* strain DH5α and purified with QIAgen Mini Prep kit. The DNA sequences of the plasmid were determined using the ABI Prism 310 Genetic Analyzer (PerkinElmer, Applied Biosystems).

2.5. Quantification of Tfam protein expression

The CEM cells were cultured in the presence or absence of 1 μM ddC or 0.25 μg/ml EtBr with addition of fresh medium and ddC or EtBr every other day. Every fourth day, 2×10^6 CEM cells were harvested, washed in PBS and resuspended in gel-loading buffer containing 50 mM Tris–HCl pH 6.8, 2% SDS, 10% glycerol and 100 mM dithiothreitol. Each sample was boiled for 10 min followed by sonication. The lysate was cleared by centrifugation. The supernatants were collected and quantified with TotalPellet Kit (Sigma) using bovine serum albumin as a protein standard. Ten micrograms of protein sample was separated on a 15% Tris–HCl sodium dodecyl sulfate protein gel (BioRad Laboratories, Hercules, CA). The proteins were transferred to a nitrocellulose membrane (Hybond-C Extra, Amersham) where after the membrane was blocked with 5% milk for 1 h in RT. To determine the level of Tfam expression a Western blot analysis was performed using a polyclonal primary antibody and an anti-rabbit secondary antibody conjugated to horseradish peroxidase [13]. The antibody signal was detected by chemiluminescence (ECL Detection System, Amersham). As a loading control we used a monoclonal mouse antibody against anti-α-tubulin (Calbiochem, VWR International AB, Sweden).

2.6. Microarray analysis

Total RNA was extracted and purified as described above. The quality of the RNA samples was checked by determination of the OD 260/280 values and by use of Agilent Bioanalyzer. The RNA was reverse transcribed, labelled and hybridized to Affymetrix GeneChips® Human Genome U133 Plus 2.0 Arrays (Affymetrix, High Wycombe, UK). The arrays were analyzed at the Bioinformatics and Expression Analysis core facility at Karolinska Institutet (Novum, Sweden). Gene Chip Operating Software Version 1.4 was used to analyze the data with the setting "All probe set scaling to target signal 100". The data were compared between CEM/wt versus CEM/ddC1 and CEM/ddC2, respectively.

3. Results

3.1. Generation of CEM cells resistant to delayed ddC toxicity

We used a CEM T-lymphoblast cell line to study the mechanism of ddC toxicity. When these cells were cultured in the presence of a variety of ddC concentrations for 3 days, the 50% cytostatic concentration (IC_{50}) of ddC under these experimental conditions was 3.2 ± 0.6 μM. In order to generate cells resistant to delayed ddC toxicity, we cultured the cells in the presence of low concentrations of ddC that did not afford acute cytotoxicity (i.e., 10–100-fold lower concentrations than the IC_{50}). CEM cells continuously cultured in the presence of 0.5 μM or higher concentrations of ddC died within 30 days of cultivation. Cells cultured in the presence of 0.25 μM ddC showed initial growth inhibition but recovered and showed a normal growth rate after 50 days of culture. This cell line (CEM/ddC1) was further cultured in the presence of 1 μM ddC for an additional 125 days to generate a cell line with higher levels of ddC resistance (CEM/ddC2). The growth rate and doubling time of both CEM/ddC1 and CEM/ddC2 cell lines was similar to the wild-type CEM cells (data not shown). The acute cytotoxicity of different nucleoside analogs was determined for the different CEM cell lines (Table 1). There was a threefold decrease in sensitivity of the CEM/ddC1 cells to ddC and a fourfold decrease in sensitivity of the CEM/ddC2 cells to ddC compared to the wild-type cells. The sensitivity against other

Table 1 – The acute cytoxicity (IC_{50}; mean ± S.D.) of the CEM cells lines to nucleoside analogs

	IC_{50} (μM)		
	CEM/wt	CEM/ddC1	CEM/ddC2
ddC	3.2 ± 0.6	9.1 ± 0.9	12 ± 7
AraC	0.035 ± 0.006	0.036 ± 0.008	0.042 ± 0.019
AraT	18 ± 12	13 ± 5	36 ± 15
FIAU	3.0 ± 0.7	0.59 ± 0.28	0.49 ± 0.09
dFdC	0.14	0.07 ± 0.07	0.084 ± 0.009
BVDU	302 ± 32	294 ± 33	361 ± 32
dFdG	0.021 ± 0.003	0.029 ± 0.021	0.022 ± 0.007
AraG	1.2 ± 0.1	0.79 ± 0.57	1.3 ± 0.9
CdA	0.19 ± 0.04	0.13 ± 0.08	0.28 ± 0.01
IDU	24 ± 13	24 ± 26	10 ± 1

AraC, 1-β-D-arabinofuranosylcytosine; araT, 1-β-D-arabinofuranosylthymine; FIAU, (E)-1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-iodouracil; dFdC, 2′,2′-difluorodeoxycytidine; BVDU, 5-(2-bromovinyl)-2′-deoxyuridine; dFdG, 2′,2′-difluorodeoxyguanosine; araG, 9-β-D-arabinofuranosylguanine; CdA, 2-chloro-2′-deoxyadenosine; IDU, 5-iododeoxyuridine. The acute cytoxicity of the CEM cells lines to nucleoside analogs.

Table 2 – The anti-HIV activity (EC_{50}; mean ± S.D.) in HIV-1-infected CEM cells to nucleoside analogs

	EC_{50} (μM)		
	CEM/wt	CEM/ddC1	CEM/ddC2
ddC	0.067 ± 0.023	0.12 ± 0.06	0.25 ± 0.07
AZT	0.0055 ± 0.0056	0.0052 ± 0.0017	0.0060 ± 0.0028
D4T	0.43 ± 0.36	0.12 ± 0.11	0.28 ± 0.04
ddG	1.0 ± 0.0	1.35 ± 0.21	1.2 ± 0.0
ddI	12.0 ± 8.0	12.0 ± 0.0	19.0 ± 15.6
(R)PMPA	6.0 ± 2.0	7.0 ± 1.4	6.5 ± 0.7
3TC	0.019 ± 0.018	0.04 ± 0.0	0.10 ± 0.08
Nevirapine	0.0072 ± 0.0011	0.0060 ± 0.0028	0.0060 ± 0.0028

AZT, 3′-azido-2′,3′-dideoxythymidine; D4T, 2′,3′-didehydro-3′-deoxythymidine; ddG, 2′,3′-dideoxyguanosine; ddI, 2′,3′-dideoxyinosine; 3TC, 2′-deoxy-3′-thiacytidine (lamivudine); (R)PMPA, (R)-9-(2-phosphonylmethoxypropyl)adenine (tenofovir); the anti-HIV activity in HIV-1-infected CEM cells to nucleoside analogs.

deoxycytidine analogs including 1-β-D-arabinofuranosyl-cytosine and 2′,2′-difluorodeoxycytidine was unaffected and similar for all the three cell lines. Several other pyrimidine and purine nucleoside analogs neither exhibited any difference in cytotoxicity towards the ddC-resistant cell lines with the exception of (E)-1-(2-deoxy-2-fluoro-£-D-arabinofuranosyl)-5-iodouracil (FIAU) that showed ≈5-fold increased inhibitory activity against the ddC-resistant cells. We also analyzed the anti-HIV-1 effects of ddC and several other nucleoside analogs in the cell lines (Table 2). The CEM/ddC1 and CEM/ddC2 cells showed two to threefold decreased sensitivity to the inhibitory effect of ddC and 3TC against HIV replication and there was poor, if any, statistical significance in the differences of these antiviral values between the wild-type and ddC-resistant cell lines. The anti-HIV activity of several other nucleoside analogs and non-nucleoside analog reverse transcriptase inhibitors was clearly unaffected.

3.2. *Characterization of nucleoside kinases and dNTP pools*

Altered expression of nucleoside kinases is a possible mechanism for resistance to nucleoside analogs. We determined the mRNA expression levels of dCK, TK1 and TK2 in the ddC-resistant cell lines (Fig. 1). We also studied the mRNA expression of DNC, a mitochondrial membrane protein suggested to be involved in transport of deoxyribonucleotides into mitochondria [14]. These experiments showed that both CEM/ddC1 and CEM/ddC2 cells had ≈30% decreased expression of dCK and TK2 compared to the wild-type cells. The mRNA levels of TK1 and DNC was not altered in the ddC-resistant cell lines. We also studied the steady-state dNTP and ATP pools in exponentially growing CEM cells (Fig. 2). The dATP and dTTP pools were decreased approximately 20–40% in the CEM/ddC1 and CEM/ddC2 cells compared to the CEM/wt cells (Fig. 2A). There was also a small decrease in the dCTP pool. The dGTP pool was slightly increased in the CEM/ddC1 cells. The level of ATP showed a ≈1.5-fold increase in the ddC-resistant cell lines compared to the CEM/wt cells (Fig. 2B).

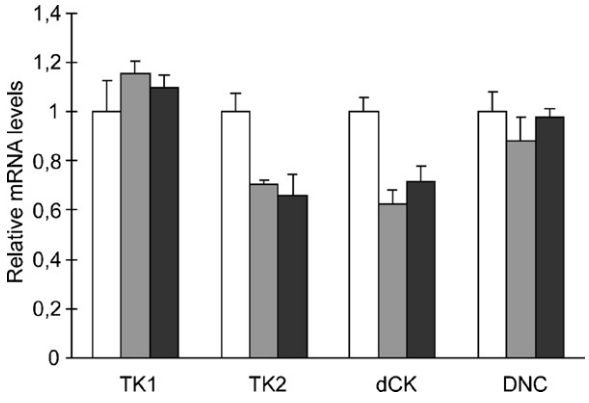

Fig. 1 – RT-PCR quantification of relative TK1, TK2, dCK and DNC mRNA levels (mean ± S.D.) in CEM/wt (white bars), CEM/ddC1 (grey bars) and CEM/ddC2 (black bars) cell lines.

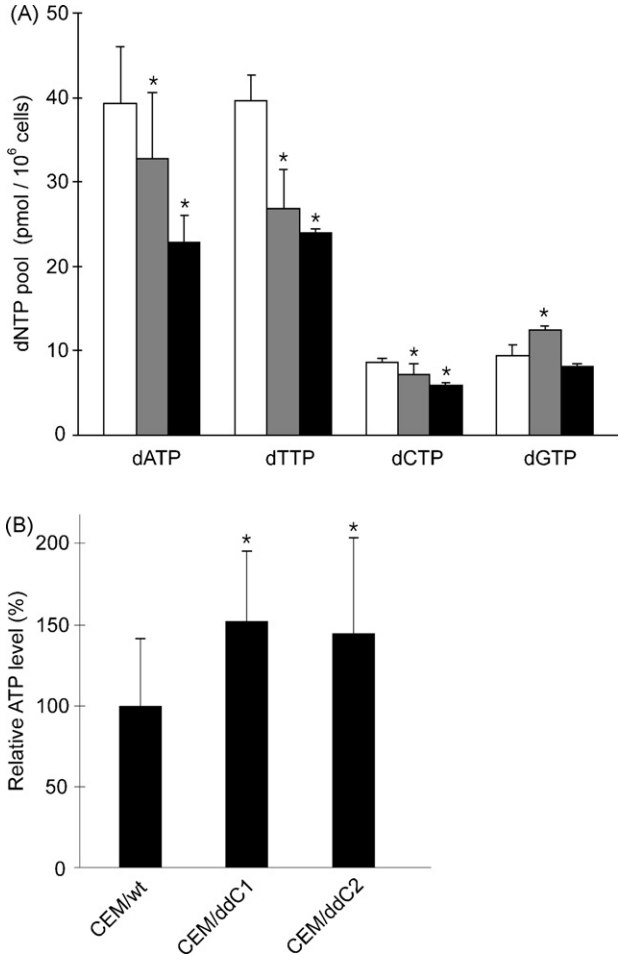

Fig. 2 – Steady-state dNTP (A) and ATP (B) pools (mean ± S.D.; *$p < 0.05$) in CEM/wt (white bars), CEM/ddC1 (grey bars) and CEM/ddC2 (black bars) cell lines.

3.3. *Characterization of mtDNA*

The mtDNA levels in the ddC-resistant cell lines were quantified using an RT-PCR assay (Fig. 3A). These experiments showed that both ddC-resistant cell lines had increased mtDNA levels compared to the wild-type cell line. The increase was ≈2-fold for CEM/ddC1 and ≈1.5-fold for CEM/ddC2. Southern blot

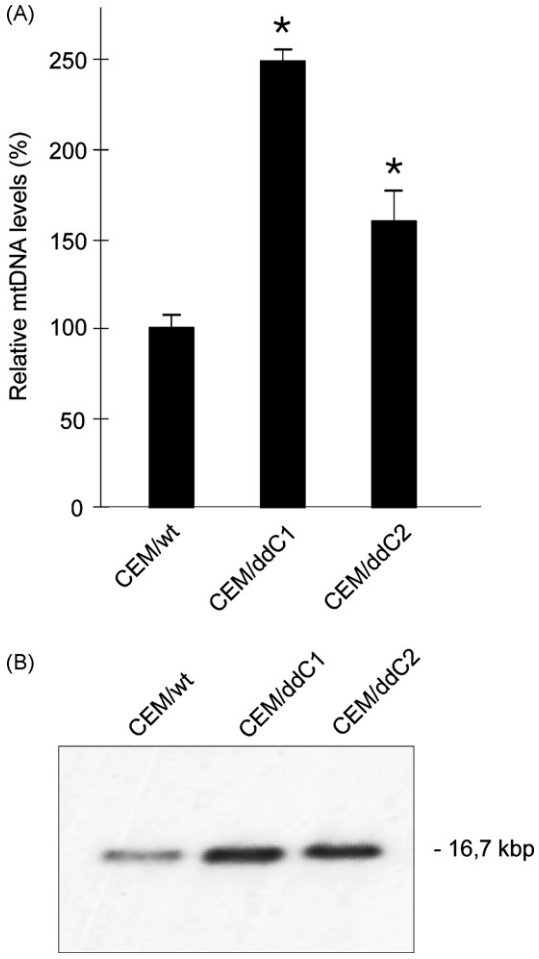

Fig. 3 – mtDNA in the ddC-resistant CEM cells. (A) RT-PCR quantification of mtDNA levels relative to nuclear DNA levels (mean ± S.D.; $^{*}p < 0.05$) in the cell lines. (B) Southern blot analysis of BamH1 digested mtDNA from the cell lines.

analysis of BamH1 restriction digested mtDNA confirmed that the level of mitochondrial DNA was increased in the CEM/ddC1 and CEM/ddC2 cell lines (Fig. 3B). The analysis also showed that the mtDNA had intact size and no mtDNA with major deletions was detected. The increased level of structurally intact mtDNA shows that the ddC-resistant cells had an increased mtDNA copy-number. A fragment of mitochondrial DNA (bp 5028–6511) of the wild-type cell line and the two ddC-resistant cell lines was cloned and the DNA sequenced to determine whether any mutations in mtDNA had occurred. Sequencing of 20 independent clones of mtDNA from each cell line showed no mutations in CEM/ddC1 but three mutations in CEM/ddC2. In two of the clones the mutations were located on bp 5220 (T → C). In two other clones the mutations were located on bp 5530 (A → G) and on bp 6511 (T → G) respectively. Sequence analysis showed that these three regions encode NaDH dehydrogenase subunit 2, tRNA tryptophan and cytochrome c oxidase subunit I, respectively. The occurrence of a few mutations may be within the normal variation of the mitochondrial DNA after selection through many cell divisions, considering the fidelity of the mitochondrial DNA polymerase [15]. A similar rate of two to three mutations per 10^3 base pairs has been reported to accumulate in wild-type mice at the age of 25 weeks [16]. Further studies will be performed to address the question if the observed mitochondrial mutations contribute to the resistance phenotype.

3.4. *mtDNA depletion in the ddC-resistant cells*

ddC-induced mtDNA depletion was studied when the cells were grown in the presence of 1 μM ddC (Fig. 4A). The mtDNA levels of wild-type cells decreased >90% after 12 days. The CEM/ddC1 cell showed a relative resistance to mtDNA depletion but exhibited a ≈50–60% decrease after 8–12 days. The mtDNA levels in CEM/ddC2 were not affected by the ddC treatment. Ethidium bromide is a potent inhibitor of mtDNA replication [17]. Cells cultured in the presence of 0.25 μg/ml EtBr showed rapid depletion of mtDNA without any difference between the wild-type or ddC-resistant cells (Fig. 4B). In summary, our findings show that both the ddC-resistant cell lines had decreased sensitivity of mtDNA depletion induced by ddC but retained their sensitivity for mtDNA depletion by other drugs that inhibit mtDNA replication. Mitochondrial

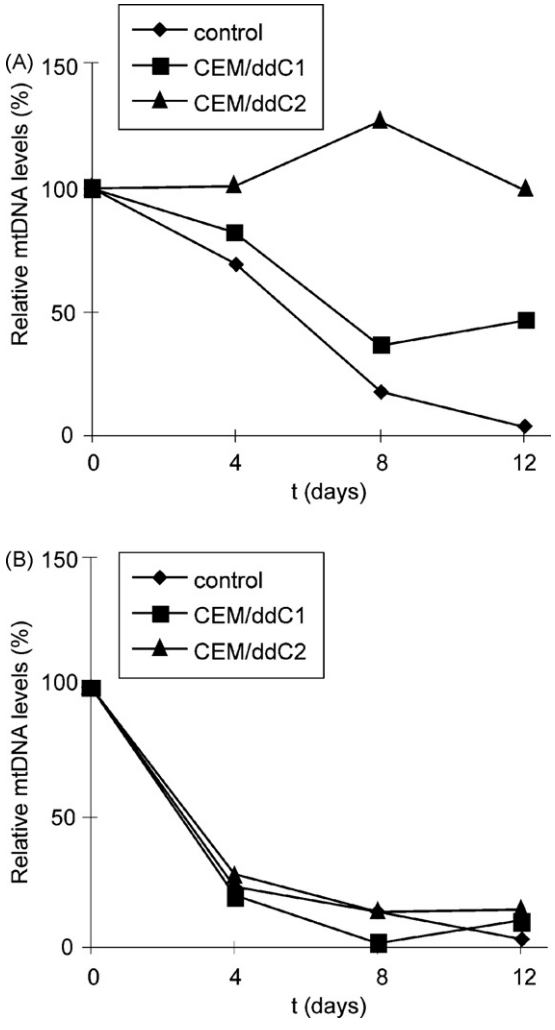

Fig. 4 – Relative mtDNA levels in CEM cell lines treated with 1 μM ddC (A) or 0.25 μg/ml EtBr (B).

transcription factor A (Tfam) is required for replication and the initiation of transcription of mtDNA [18]. The levels of Tfam has been correlated to the level of functional mtDNA and suggested to be used as a marker for mtDNA levels. We quantified the levels of Tfam in the CEM cell lines by Western blot analysis (Fig. 5A). Both CEM/ddC1 and CEM/ddC2 had increased expression of Tfam with ≈1.5-fold increased levels in CEM/ddC2. Tfam levels decreased in the wild-type CEM cells when the cells were cultured in the presence of 1 μM ddC (Fig. 5B). Both CEM/ddC1 and CEM/ddC2 showed a relative resistance to this decrease of Tfam expression. EtBr incubation of the cells also decreased the Tfam expression (Fig. 5C). The

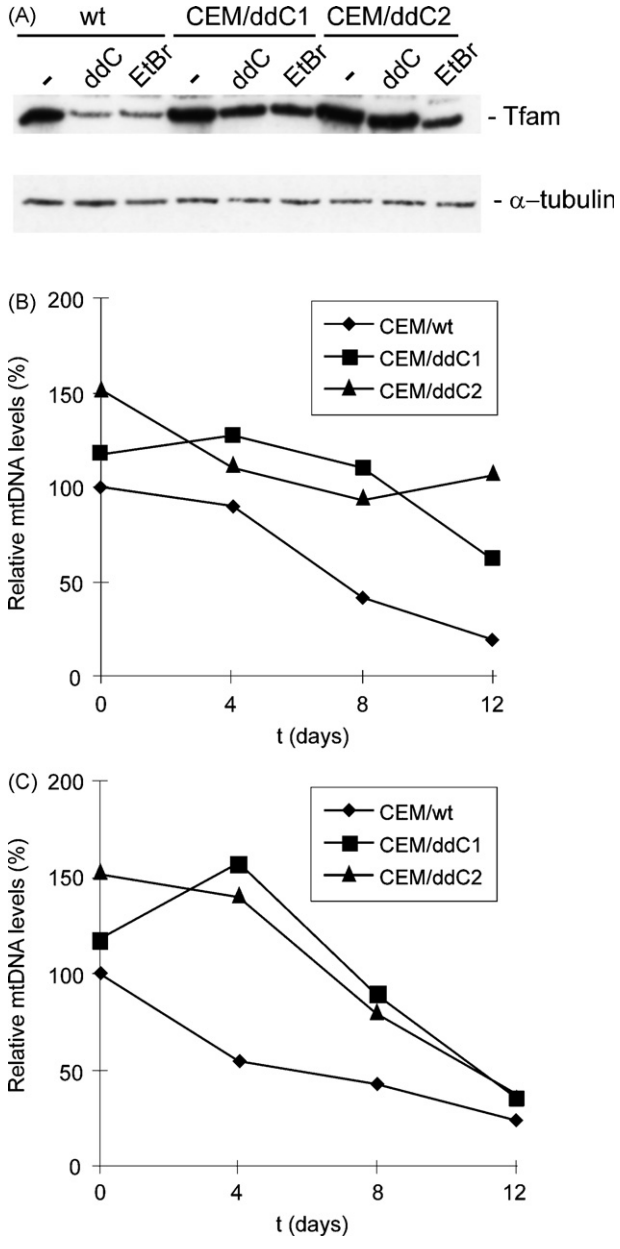

Fig. 5 – Tfam protein expression in the CEM cells quantified by Western blot analysis. (A) Western blot analysis of Tfam expression after 8 days of culture in the absence (–) or presence of 1 μM ddC and 0.2 5 μg/ml EtBr. (B) Tfam expression in CEM cells treated with 1 μM ddC (B) or 0.25 μg/ml EtB (C) for 0–12 days.

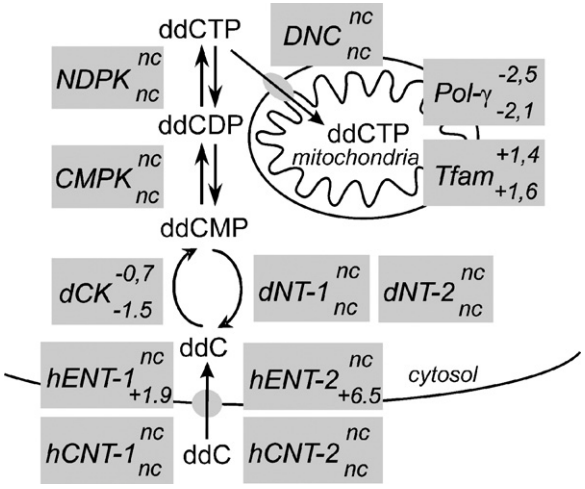

Fig. 6 – The pathway of ddC import, phosphorylation and mtDNA incorporation and analysis of relative changes in mRNA expression of participating genes by microarray analysis. Fold increase (+), decrease (–) or not changed (nc) expression of the indicated genes in CEM/ddC1 (superscript) or CEM/ddC2 (subscript) compared to the wild-type CEM/wt cells. CMPK, UMP-CMP kinase.

CEM/ddC1 and CEM/ddC2 cells showed a slower rate of decreased Tfam compared to CEM/wt but the Tfam levels were similar for all cells after 12 days culture.

3.5. *Microarray analysis of gene expression in ddC-resistant cells*

The pathway of ddC metabolism involves several steps including transport across the plasma membrane, phosphorylation by several kinases and mitochondrial import (Fig. 6). We used a microarray analysis to determine the relative expression of several genes involved in this pathway and compared their expression between the ddC-resistant cells and the wild-type CEM cell line. Similar to the RT-PCR quantification of dCK mRNA expression, a decrease in dCK expression was detected in the ddC-resistant cells by the microarray analysis. Expression of dCK was decreased 0.7- and 1.5-fold in the CEM/ddC1 and CEM/ddC2 cell lines compared to CEM/wt, respectively. There was no change in expression of UMP-CMP kinase, NDPK or the deoxyribonucleotidases dNT-1 or dNT-2. Among the plasma membrane nucleoside carrier proteins we detected an increased expression of ENT-1 and ENT-2 in CEM/ddC2 but no change in the expression of these proteins in CEM/ddC1. Similar to the results showing an increase in Tfam protein expression, we also detected an increase in Tfam mRNA levels in the ddC-resistant cells. A decreased expression of the mitochondrial DNA polymerase γ was also observed in both CEM/ddC1 and CEM/ddC2.

4. Discussion

We have generated and characterized two CEM cell lines that were resistant to the delayed toxicity (i.e., mtDNA depletion)

induced by ddC. The ddC-resistant cell lines exhibited 1.5–2-fold increased mtDNA levels. Southern blot analysis indicated that the mitochondrial DNA was structurally intact and our data showed that the mtDNA copy-number was increased in the ddC-resistant cells compared to the wild-type cells. mtDNA has been quantified in peripheral blood leucocytes and monocytes from patients undergoing anti-HIV therapy with nucleoside analogs [19]. MtDNA levels in the blood cells from many of these patients were decreased especially if the patients have developed clinically significant adverse effects of the drug. However, studies indicate that some patients treated with combinations of antiretroviral drugs including ddC have increased mtDNA copy-number in blood cells [20–22]. Analysis of mtDNA copy-number at repeated time-points during long-term antiretroviral therapy has been performed in a few of these patients. These analyses show an initial decrease of mtDNA followed by recovery of mtDNA to pretreatment levels or even higher mtDNA levels than at the time of initiation of drug therapy [22]. The recovery of mtDNA levels occurred without interruption of the previously mtDNA depleting drug combinations. These findings together with the present study suggests that cells may develop resistance to the delayed toxic effects of ddC (i.e., mtDNA depletion induced by nucleoside analogs) and that an increase in mtDNA copy-number may be associated with this mechanism of resistance. Development of drug resistance is frequently a negative event that will decrease the efficacy of the treatment, such as drug resistance that occurs in chemotherapy of cancer and in antimicrobial therapy. Most often resistance develops related to the mode of action of a compound by alterations of the molecular targets, decreased expression of activating enzymes (i.e., nucleoside kinases) or through increased elimination of the drug. Counteraction of mtDNA depletion induced by ddC represents a mechanism of drug resistance that may be beneficial to the patient as it may decrease adverse effects due to mitochondrial dysfunction, while the metabolism of ddC is only partly altered. Under such conditions, the drug retains a large part of its activity as an anti-HIV agent and no cross-resistance to other deoxycytidine nucleoside analogs is observed. In addition to the increase of mtDNA, the ddC-resistant cells exhibited resistance to mtDNA depletion by ddC. The resistant cells had decreased mRNA expression of the nucleoside kinases dCK and TK2 as well as altered nucleotide pools. Loss of dCK activity has previously been reported in cells resistant to ddC [23]. However, the cells generated in the present study had only ≈30% decreased expression of dCK mRNA and these cells retained sensitivity to the cytostatic activity of other deoxycytidine nucleoside analogs, such as araC and dFdC, that are dependent on dCK-mediated phosphorylation for their cytotoxic effects [24,25]. These findings suggest that the decreased dCK expression alone does not explain the mechanism of ddC resistance in the cells. A decrease in TK2 expression has also been shown to occur in ddC-resistant cells [26] and we observed a similar decrease in our study. However, ddC is very poorly phosphorylated by TK2 and it is unclear whether this enzyme contributes to ddC phosphorylation in vivo. We did not observe any decrease in sensitivity to several other nucleoside analogs that are preferred substrates of TK2, which also suggests that the decrease in TK2 activity is not responsible for the resistance to ddC in the cell lines. The expression of UMP-CMP kinase and nucleoside diphosphate kinase that phosphorylated ddC-monophosphate to ddCTP was not altered in the ddC-resistant cells. We observed altered dNTP pool in the ddC-resistant cells where the major changes were decreased dATP, dTTP and dCTP pools. The altered dNTP pools could be caused by the decreased expression of nucleoside kinases, but as the major part of the dNTPs are derived from de novo synthesis via ribonucleotide reductase, it is more likely that other yet unidentified changes in the cells are responsible for the altered pools. An alteration of the dCTP pool versus the ddCTP pool cannot be excluded by our data but the micoarray analysis of the cells does not show any significant change of the expression of the de novo enzymes (data not shown). Since the origin of the dCTP pool is mainly provided by the ribonucleotide reductase pathway it is not likely that the small change in dCK expression would alter the dCTP pool significantly [27]. Tfam is a nuclear encoded protein essential for human mtDNA transcription and it is also an important regulator of mtDNA copy-number [28]. The mtDNA copy-number is proportional to the level of Tfam and increased expression of this protein has been shown to increase mtDNA copy-number [18]. We observe an increased expression of Tfam in the ddC-resistant cells with elevation of both mRNA and protein levels. These findings suggest that altered expression of Tfam may be responsible for the increased mtDNA levels in the resistant cells. However, the mtDNA levels also in turn affect Tfam levels and it is also most likely that the increase in Tfam expression reflects the increase in mtDNA due to another molecular event in the ddC-resistant cells. Another possible mechanism for a ddC-resistant phenotype is deficient transport of ddC into cells across the plasma membrane or reduced transport of ddCTP into the mitochondria. Deficiencies of nucleoside transport across the plasma membrane may be associated with resistance to nucleoside analogs [29]. The mRNA expression of several proteins involved in ddC transport and phosphorylation showed increased expression of the equilibrative nucleoside carrier proteins ENT-1 and ENT-2 in the CEM/ddC2 whereas their expression in CEM/ddC1 was similar to the wild-type cells. ddC is in vitro transported across the plasma membrane by both ENT-1 and ENT-2 [2]. However, the increased expression of the proteins does not explain the ddC resistance unless their functions are impaired. Less is known about the proteins involved in ddCTP import into mitochondria. Several candidate carrier proteins have been identified that mediate mitochondrial dNTP transport in vitro. One such putative carrier protein is the deoxynucleotide carrier protein DNC. However, the role of DNC as a nucleotide carrier protein has recently been questioned as down regulation of DNC expression did not affect nucleoside analog toxicity and DNC knock-out studies showed no change in the mitochondrial dNTP pools in cells [30,31]. We determined the expression of DNC mRNA in the ddC-resistant cell lines but did not find any difference in expression.

In summary, we have identified multiple changes in CEM cells that resulted in resistance to the delayed toxic effects on mitochondrial DNA caused by prolonged treatment with ddC. Our data demonstrate that the resistance phenotype may be caused by a combination of metabolic alterations and suggest

the existence of adaptation mechanisms of cells to handle toxic effects on mitochondria. Our most important finding is that the resistance to ddC toxicity is not necessary linked to a pronounced anti-HIV resistance of ddC or cross-resistance to other dCK-activated nucleoside analogs. Further studies will be initiated to support the hypothesis that drug-induced mitochondrial toxicity can be decreased or abolished without affecting the pharmacological activation of ddC and other related compounds which allow to keep the clinical use and efficacy of such drugs.

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