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Biochemical Pharmacology 68 (2004) 601–609
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Detection of an alternatively spliced form of deoxycytidine kinase mRNA in the 2′-2′-difluorodeoxycytidine (gemcitabine)-resistant human ovarian cancer cell line AG6000

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Received 22 March 2004; accepted 6 May 2004

Abstract

Gemcitabine (2′-2′-difluorodeoxycytidine (dFdC)) is a deoxycytidine analogue that is effective against solid tumors, including lung cancer and ovarian cancer. dFdC requires the phosphorylation by deoxycytidine kinase (dCK) as a primary step in its activation. Deficiency of dCK is associated with resistance against this compound both in vitro in cancer cell lines and in clinical practice in acute myeloid leukemia and solid tumors. The human ovarian cancer cell line AG6000 is 100,000-fold resistant against dFdC compared to its parent cell line A2780. This cell line proved to be dCK deficient in enzyme activity assays and by Western blot analysis, but by RT-PCR, a normal and a truncated dCK mRNA was found. Sequencing revealed that exon 3 was deleted from the dCK cDNA, resulting in a 74-aa-long open-reading frame due to the generation of a premature stop codon. No gross genomic alteration was observed at the dCK locus, suggesting the involvement of post-transcription mechanisms. Transient transfection experiments indicated that the truncated dCK transcripts are not translated to protein. To study the functional role of the truncated dCK transcripts, both A2780 cells and AG6000 cells were stably transfected with human and rat dCK. The results indicated that over-expression of full-length dCK genes in AG6000 failed to completely reverse the sensitivity to dFdC or other drugs.

Keywords: Deoxycytidine kinase; Gemcitabine; Chlorodeoxyadenosine; Alternative splicing; Deoxynucleoside analogues; Resistance

1. Introduction

Deoxycytidine kinase (dCK, EC 2.7.1.74) is a salvage pathway enzyme that catalyses the phosphorylation of deoxycytidine (dC) into dCMP, using ATP or UTP as phosphate donors [1–3]. The enzyme has broad substrate specificity, including deoxyadenosine (dA) and deoxyguanosine (dG), and plays a role in the maintenance of normal deoxyribonucleotide pools in the cell. In addition, dCK phosphorylates several important anti-cancer and anti-viral drugs, such as dFdC, araC (arabinofuranosyl-cytosine), aza-CdR (aza-2′-deoxycytidine), CdA, and fludarabine [4,5]. Deficiency of dCK activity is associated with a disturbance of the deoxynucleotide pools and resistance to therapeutic drugs [6–9]. The kinetic regulatory mechanisms of this enzyme have been established [10–13], and recently, the crystal structure has been determined [14]. At gene level, dCK is predominantly expressed in lymphoid cells, indicating cell-specific regulation [12]. Although dCK expression is upregulated in cancer cells, the enzyme is not cell cycle regulated [1]. The human dCK gene is located on chromosome 4 (4ql3.3–4q21.1] and promoter activity has been demonstrated to be mediated via protein–protein interactions between USF and SP1 transcription factors [15,16]. However, the molecular mechanism that

Abbreviations: dFdC, 2′-2′-difluorodeoxycytidine; CdA, 2-chloro-2-deoxyadenosine; dCK, deoxycytidine kinase; RT-PCR, reverse transcription-polymerase chain reaction

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0006-2952/$ – see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2004.05.007

leads to tissue-specific and proliferation-dependent transcription of dCK has not yet been fully clarified.

Gemcitabine (2′,2′-difluorodeoxycytidine (dFdC)) is a dC analogue with a substitution of the hydrogen atoms at the 2′-position in the pentose moiety by two fluoro atoms. Gemcitabine is transported to the cells by active nucleoside transport systems [17,18]. In the cells, dFdC is initially phosphorylated by deoxycytidine kinase (dCK) to dFdCMP and subsequently to dFdCTP, which can be incorporated both in DNA and RNA [19,20]. Gemcitabine showed anti-tumor activity against several experimental and clinical solid tumors, including non-small cell lung, ovarian and pancreatic cancers [21–26].

Deoxynucleoside analogue-based chemotherapy currently faces three major obstacles, variable efficacy between individual patients, toxic side effects, and development of drug resistance. All these phenomena are believed to be related to the variations in the levels and activities of drug-metabolising enzymes between the different normal and tumor cell types and individuals. Drug resistance is the main reason for the eventual treatment failure in cancer therapy. Earlier, the lack of dCK activity was found to be one of the causes for the acquired resistance in clinical trials [28], and in a model system of xenografts dCK activity was found to be related to antitumor effects [27]. The generation of alternative spliced transcripts of dCK was also observed in patients with acute myeloid leukemia classified as resistant to araC, suggesting a possible mechanism for dCK downregulation [28,29].

In this study, the focus was to understand the mechanism(s) involved in the development of acquired resistance to gemcitabine. Earlier, Ruiz van Haperen et al. [30] described the generation of gemcitabine-resistant cell lines from the human ovarian carcinoma cell line A2780. The cell lines were developed following a continuous exposure to increasing concentrations of gemcitabine, resulting in the 100,000-fold resistant AG6000 cells associated with a total lack of dCK activity. Western blot analysis did not reveal any dCK protein, and RT-PCR-amplified mRNA demonstrated that AG6000 expressed a normal length amplicon of 701 bp and an aberrant amplicon of about 500 bp [30,31]. In this paper, we investigated the potential role of this aberrant transcript in dCK expression and whether the expression of transfected dCK protein can compensate for the deficient dCK.

2. Materials and methods

2.1. Chemicals

Gemcitabine was from Eli Lilly, Inc. (Indianapolis, IN, USA). 2-Chloro-2′-deoxyadenosine was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of analytical grade and commercially available.

2.2. Cell culture

A2780 human ovarian carcinoma cells and its gemcitabine-resistant variant were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, UK), supplemented with 5% heat-inactivated fetal calf serum (Gibco, UK) at 37 °C and 5% CO_2. AG6000 was cultured without gemcitabine, since tests had proven that the resistance did not alter.

2.3. Protein extracts and immunoblot assays

Whole cell extracts were prepared from A2780, AG6000 cells and their transfected clones as described in [30]. Proteins were extracted by an urea extraction buffer (8 M urea, 100 mM $NaPO_4$, pH 8.0, 10 mM Tris-HCl, pH 8.0). For immunoblot analysis, proteins were fractionated on SDS–polyacrylamide gels, immobilized onto nitrocellulose membranes, and probed with the dCK-pep antibody at 1:5000 dilution [32], followed by incubation with horseradish peroxidase-conjugated anti-rabbit IgG (Jackson Laboratories, 1:20,000 dilution). Immunocomplexes were visualized by the enhanced chemiluminescence reaction (ECL, Amersham Pharmacia Biotech). The monoclonal antibody for the myc epitope (9E10 hybridoma) was kindly provided by N.T. Ktistakis, Babraham Institute, and used at 1:1000 dilutions.

2.4. RNA and poly(A) RNA preparation

Total RNAs from cultured cells (10^7 cells) were prepared by the guanidium-isothiocyanate acid phenol extraction method as described in [33]. Poly(A) RNA was isolated by affinity chromatography on an oligo-dT-cellulose column [33].

2.5. Oligonucleotides and polymerase chain reaction

Oligonucleotides primers were designed based on the human dCK-cDNA and genomic sequences [1,15]. Both primers 5′-AGCCCTCTTTGCCGGACGAGCTCTGGG and 5′-CTGGAACCATTTGGCTGCCTGTAGTCT were generated from the 5′-UTR and 3′-UTR of human dCK sequences, respectively. dCK-cDNA was amplified, subcloned into the T-vector following the procedure described by the manufactures (Invitrogen). In order to clone the alternatively spliced dCK-mRNA, 5′-TCGAGCT CCATGG CCACCCGCCCAAGAG and 5′-CATGGATCC GATGC-AAAAATATACC harboring restriction sites for *Sac*I and *Bam*HI, respectively, were synthesized. The exon–intron junctions of exon 3 were determined using oligonucleotides primers selected and synthesized according to [28].

2.6. Southern blot analysis

Southern blot analysis was performed according to [33], with minor modifications. The genomic DNA from A2780

and AG6000 was digested with restriction endonucleases (*Eco*RI, *Hin*dIII or *Eco*RI and *Hin*dIII) and fractionated in 1% agarose gel. After capillary transfer to nitrocellulose membranes, the blot was hybridized with the cDNA probes encompassing the entire human dCK-cDNA or a fragment generated from dCK exon 3. The membranes were exposed to Kodak films overnight at −80 °C.

2.7. RNase protection

2.7.1. Ribo-probe synthesis

A 225-bp band, containing exons 2 and 3 of the human dCK was cloned into the pBs-KS vector (Clontech) at *Pst*I and *Sac*I restriction sites. Following the manufacturer protocol, T7-RNA polymerase (Promega) was used to generate anti-sense ribo-probe under in vitro conditions.

2.7.2. ^{32}P-labeled RNA:RNA hybridization

Poly(A) RNA (15 ng) was hybridized with 10^5 cpm anti-sense riboprobe in a buffer containing 75% formaldehyde, 500 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0, as described in [33].

2.7.3. Digestion with RNase A

The hybridized RNA was digested with RNase A, and the protected RNA species were extracted and precipitated. The RNA pellet was resuspended in formamide-loading buffer. A total of 5000 cpm of the riboprobe were loaded on a 6% polyacrylamide–urea gel.

2.8. Semi-quantitative RT-PCR

In order to determine the gene expression in the parent cell lines and that in the resistant and transfected variants, we used semi-quantitative PCR as described previously [27,34]. The primers span the exon 2 and 3 boundary.

2.9. Cell-free transcription and translation

RNA was transcribed from constructs containing the full length dCK-cDNA and dCKΔ195ex3, and extracted according to the Protocols and Applications Guide (Promega). RNA was denatured and applied to cell-free translation system: Flexi Rabbit Reticulocyte Lysate (Promega) in the presence of 0.4 μCi [^{35}S]methionine. The products were fractionated by SDS–polyacrylamide gel and visualized by autoradiography.

2.10. Transfection of eukaryotic cell lines

Cells were transfected by calcium phosphate-DNA co-precipitation [35]. In order to determine whether transfections with dCK-cDNA would restore or enhance drug sensitivity, a different approach was used with both human [11] and rat dCK-cDNA [36]. The human dCK-cDNA and the aberrant fragment dCKΔ195ex3 were cloned into the mammalian expressing vectors pCMV-myc for transient transfection assay, to detect exogenous protein by the myc epitope. The full-length human dCK-cDNA [11] was cloned into the pCB6, pcDNA3, and pSVZeo vectors. Both pCB6 and pcDNA3 are suitable for eukaryotic expression and neomycin selection. pSVZeo can be used for bacterial and eukaryotic expression because of a selectable Zeomycin resistance. The full-coding region of the rat dCK cloned in the pRc/CMV vector (containing the neomycin resistance gene, Invitrogen) was obtained from M. Veuger (Department of Hematology, Leiden University Medical Center, Leiden, [36]).

By means of a commercial lipofectin agent (Dotap, Gibco) and calcium phosphate–DNA co-precipitation, the expression vectors containing human or rat dCK were transfected into A2780 and AG6000 cells. After selection with the suitable agent, about 30 clones of each transfection were screened.

2.11. Chemo-sensitivity testing

The sensitivity of A2780, AG6000, and the transfected clones was assessed using the sulforhodamine B (SRB) assay, essentially as described previously [37]. Cells were seeded in triplicate wells in 96-well flat-bottom plates (Costar, The Netherlands) at day 0. After 24 h, deoxynucleoside analogues were added, and after 72 h, drug exposure growth inhibition was evaluated.

3. Results

3.1. Characterization of a dCK-deficient cell line

The cell line AG6000 was selected from A2780, a human ovarian carcinoma cell line after treatment with increasing concentrations of dFdC. Earlier studies [30] revealed that AG6000 cell line lacks dCK enzymatic activity, while RT-PCR analysis demonstrated the presence of two species of the dCK transcripts. Western blot analysis using specific polyclonal antibodies directed against the very C-terminal 15 amino acids of the full-length dCK protein [32], indicated that the AG6000 cell line lacks full-length dCK protein. In contrast to the parental A2780 cell line, a clear band of 30 kDa was observed (Fig. 1). We cannot exclude that C-terminally truncated forms of dCK are still expressed in AG6000 cells, but would not be detected by our antibodies. Such forms however should lack dCK activity.

The dCK mRNA transcripts were analyzed by RT-PCR in both the A2780 and AG6000 cell lines. As described earlier by [30], only a wild-type dCK amplicon was detected in A2780 cells; however, AG6000 cells expressed a normal length amplicon, besides an aberrant mRNA transcript of 628 bp. The RT-PCR products from triplicate experiments were cloned and characterized. Sequence

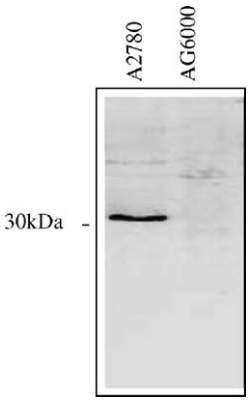

Fig. 1. Detection of dCK protein by Western blot analysis. Whole cell extracts from the parental A2780 cell line, and the dFdC-resistant AG6000 cell line were fractionated by 12% SDS–polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and probed with the dCK-pep antibodies.

analysis revealed the existence of a full-length dCK transcript of 823 bp identical to the wild-type dCK mRNA in AG6000 cells, while in the other shorter form exon 3 was completely missing, with no other point mutations. Alternative splicing joins exon 2 directly to exon 4, and this event generates a frame shift with a stop codon at amino acid position 75 (Fig. 2). The shorter form of dCK transcripts was tentatively named dCKΔ195ex3.

3.2. Analysis of the genomic structure of the dCK gene

To investigate a possible genomic rearrangement of the dCK locus in AG6000 cell line, Southern blot analysis was performed using genomic DNA from both the parental A2780 and the drug-resistant AG6000 cell lines. The genomic DNAs from both cell lines were digested with the restriction enzymes *Eco*RI, *Hin*dIII and an *Eco*RI/*Hin*dIII combination (Fig. 3). Hybridization with

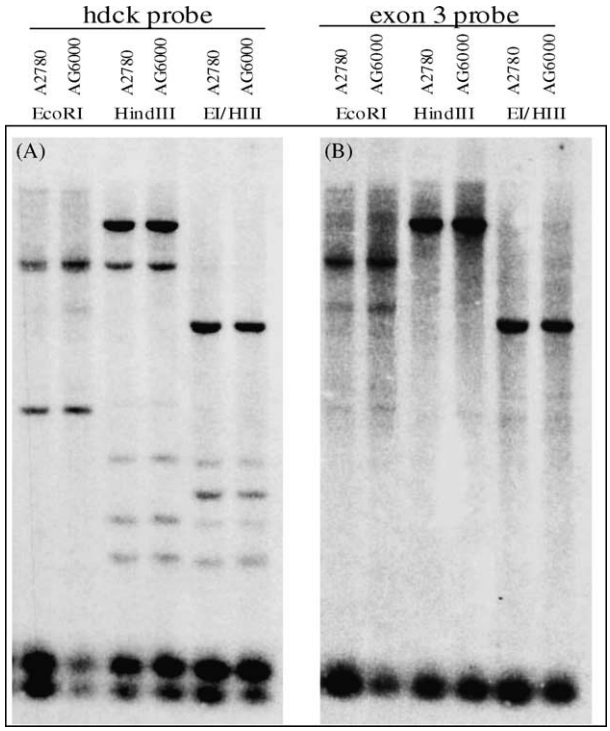

Fig. 3. Southern blot analysis of the dCK genomic region in both A2780 and AG6000 cell lines. The genomic DNA was digested by restriction enzymes *Eco*RI, *Hin*dIII and *Eco*RI plus *Hin*dIII, separated on 1% agarose gels, transferred to nitro-cellulose membrane and probed with in (A) the full-length dCK-cDNA and in (B) the exon 3 fragment.

human dCK full-length cDNA and with exon 3 containing probes did not show any gross genomic rearrangement of the dCK locus in the drug-resistant cell line. To verify this finding, possible point mutations within the splicing sites were investigated. PCR analysis, using primers generated against the splicing sites flanking exon 3, did not reveal any point mutations in these regions (data not shown).

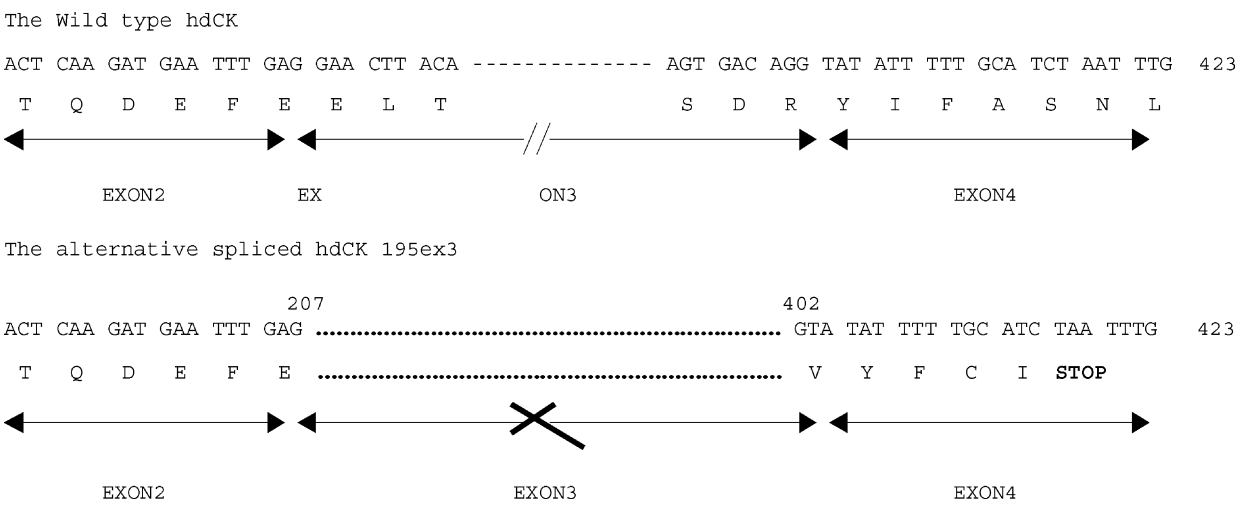

Fig. 2. A scheme showing the wild type and the alternatively spliced dCK detected in the drug-resistant AG6000 cell line. The wild-type exons 2–4 are shown. In the alternative-spliced dCK form, exon 3 is missing, thus exons 2 and 4 are directly joined (dotted lines). This event generates a frame shift with a new stop codon.

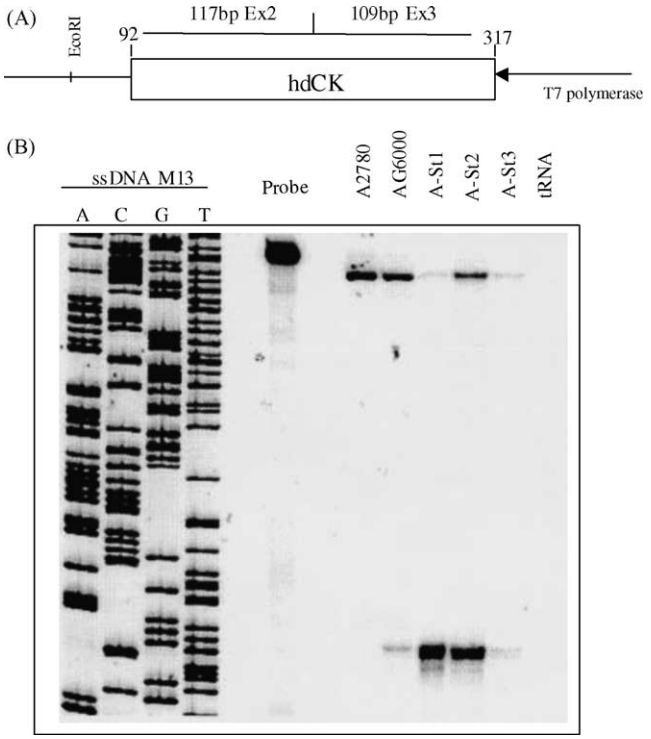

Fig. 4. Determination of the relative abundance of the aberrant dCKΔ195ex3 in the AG6000 cell line and in stably transfected A2780 cells. An RNase protection experiment was performed using the 236-nt-long riboprobe synthesized from p-Blue Script containing 226 bp of the second and the third exons of dCK-cDNA (A). The probe containing extra sequences from the vector was hybridized with Poly(A) RNA, which was prepared from both A2780 and AG6000 cell lines and yeast tRNA as a control (B). The intensities of the 226- and 117-bp protected fragments that correspond to the full-length dCK mRNA and to the dCKΔ195ex3 were quantified by phospho-image analysis. The values obtained for AG6000 cells were normalized according to the total deoxyuridine content of each fragment and expressed as a percentage (28.4% for the 117-bp fragment and 71.6% for the 226-bp fragment) to the total dCK-mRNA population.

3.3. RNase protection analysis

To determine the expression levels of the aberrant dCKΔ195ex3 relative to the full length dCK transcripts, an RNase protection experiment was performed using poly(A) RNA prepared from both A2780 and AG6000 cells. A 236-nt-long anti-sense riboprobe, containing 226 base pairs from the second and the third exons of the dCK-cDNA, was synthesized in a cell-free system. This probe should give rise to a 226-bp (exons 2 and 3, Fig. 4A) protected fragment when hybridized to the wild-type dCK mRNA and to a 117-bp (only exon 2) fragment when hybridized to the dCKΔ195ex3 mRNA. The result of this assay demonstrated expression of dCKΔ195ex3 only in AG6000 at the level of about 28.4% of the total dCK mRNA population (Fig. 4B, lanes: A2780 and AG6000).

3.4. Detection of dCKΔ195ex3

The alternative splicing in dCKΔ195ex3 fragment generates a frame shift with a stop codon at amino acid position 75. Thus, a truncated polypeptide of about 10 kDa is expected to be generated. In order to test whether this truncated dCK polypeptide is translated into a protein, both the full-length dCK and the dCKΔ195ex3 have been expressed as myc fusion proteins. The myc epitope allows the detection of the exogenous full-length dCK as well as the truncated form using the monoclonal anti-myc antiserum. Western blot analysis of transiently transfected A2780 cells revealed an expression of wild-type myc-dCK whereas no expression was detected for the myc-dCKΔ195ex3 (Fig. 5).

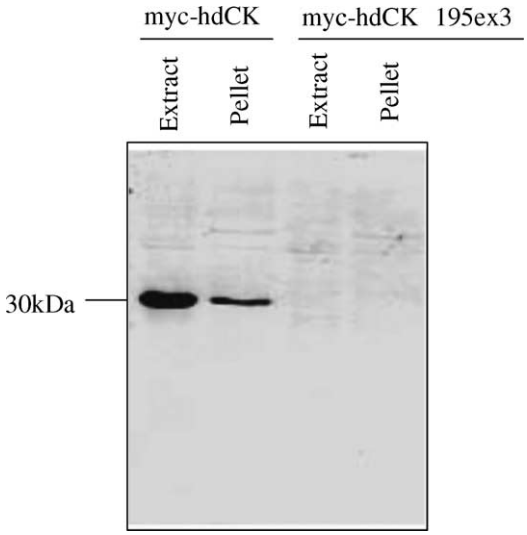

Fig. 5. Western blot analysis of cell extracts from the A2780 cell line transiently transfected with pCMV-myc-dCK and pCMV-myc-dCKΔ195ex3 constructs. Whole cell extracts and the insoluble material (pellet) were separated by 12% SDS–polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and probed with the myc-tag antibody.

The transient transfection results raised the hypothesis that myc-dCKΔ195ex3 was not translated to a protein. To test this, two plasmids were constructed to express the wild type and the truncated dCK, with no epitopes. These constructs were used for cell-free transcription and translation assays. The cell-free transcripts from the dCKΔ195ex3 demonstrated that the truncated dCK form is not translated to a detectable protein.

3.5. Generation of stable cell lines expressing the dCKΔ195ex3 in A2780 cell line

In order to determine whether the truncated form of dCK mRNA is a cause or a consequence of the drug resistance phenotype observed in AG6000 cell line, and since the intracellular transcription translation system did not give a clear-cut answer, the dCKΔ195ex3 expression cassette was stably transfected into the genome of A2780 cells under the control of cytomegalo virus (CMV) promoter. This system was also expected to mimic the cellular situation in AG6000 cells. The over-expressed transcripts of dCKΔ195ex3 have been detected by RNase protection assay (using the riboprobe that has been described in

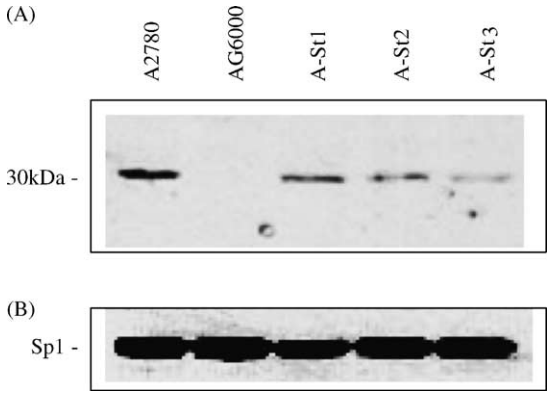

Fig. 6. A western blot of whole cell extracts prepared from A2780, AG6000 and the stably transfected A2780 cell lines A-Stl, A-St2 and A-St3. (A) Protein extracts were separated by 12% SDS–polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes and probed with dCK-pep antibody. (B) The Western blot was stripped and re-probed with antibodies recognizing the SP1 protein, as a control.

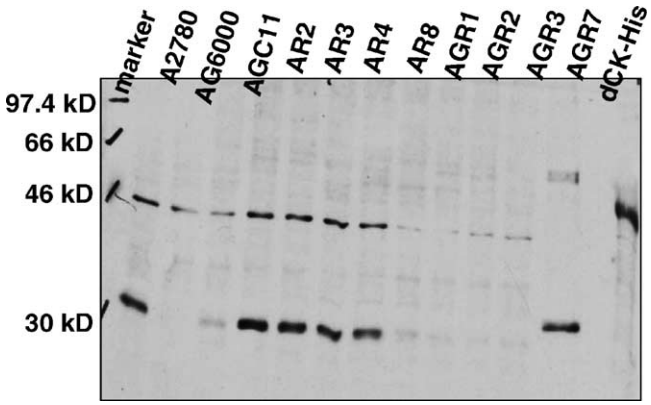

Fig. 7. Western blots of whole cell extracts prepared from A2780, AG6000 and all clones transfected with human or rat dCK cDNA. Whole cell extracts were separated by 12% SDS–polyacrylamide gel electrophoresis and blotted as described in Section 2.

Fig. 4, lanes A-ST1, A-ST2 and A-ST3). Results from integrated transfections have shown a redundancy in dCK mRNA expression independent of dCKΔ195ex3 copy number introduced to the cells (Fig. 4). However, Western blot analysis (Fig. 6) did not show any severe reduction in the wild-type dCK proteins levels, indicating that the expression of dCKΔ195ex3 transcripts could not account for the loss of dCK protein observed in the AG6000 cell line.

3.6. Generation of stable cell lines expressing the human and rat dCK in AG6000 cell line

In order to determine whether the truncated form of dCK mRNA would affect translation of not only native dCK mRNA but also artificially introduced dCK mRNA, we transfected AG6000 cells with either human or rat cDNA. Western blot analysis indeed revealed an increased expression of dCK protein in cells transfected with human cDNA, but the rat dCK was not recognized (Fig. 7). In order to determine whether these cells would also express dCK mRNA to a larger extent, we used quantitative RT-PCR using competitive templates as an internal standard for the assay [27,34]. However, it was not possible to determine the expression quantitatively since additional bands were found in both the parent AG6000 and the clones transfected with human dCK (Fig. 8). Since the primers were designed to span exon 3, the additional band of 231 base pairs fits with exon 3 skipping. The increased expression of a band at 425 fits with an increased expression of human dCK in the transfected lines. Quantitation of dCK mRNA is based on the ratio between native dCK cDNA and the competitive template, which are both recognized by the same primer set. The competitive template gives the shorter product. However, due to presence of additional bands also competing for the same primers, the exact extent of competition could not be quantified, precluding exact calculation of dCK mRNA expression. However, it was evident that in the cells transfected with the human dCK, an increase in the expression of the 425-base-pair band was observed in concurrence with the increased expression of human dCK. Despite the homology (88%) between the rat and the human dCK, the primers used in this study did not recognize rat dCK. We also tried to use a recently developed real-time RT-PCR method [38]to quantify the extent of gene expression of the transfected gene, but this method

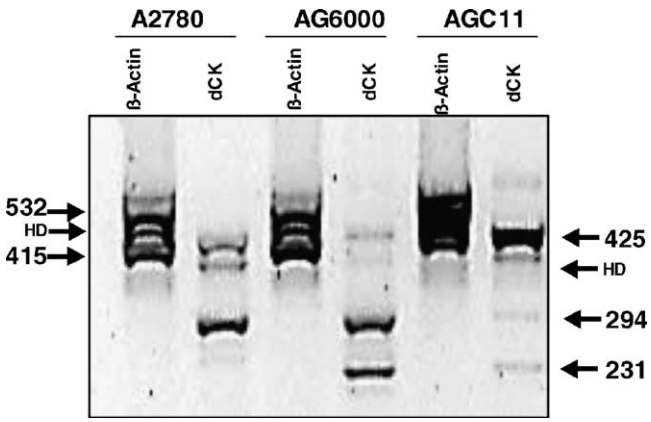

Fig. 8. UV visible bands of β-actin and dCK gene and competitive template (CT) products of a CT–RT-PCR with dCK cDNA from A2780, AG6000 and AGC11 cells separated on agarose gels. The blots shows three bands for both dCK and β-actin: 532 and 425 are the number of the base pairs (bp) encoded by the forward and reverse primers for the native cDNA of β-actin and dCK, respectively; 415 and 294 are the number of bp encoded by the CT for β-actin and dCK, respectively. The intermediate band is a heterodimer (HD), which can be formed between the native cDNA and the CT. In the AG6000 and AGC11, an additional band was found at 231 bp, due to fact that the primers were designed to span exon 3, leading to the appearance of this shorter transcript. The bands were scanned and the optical density (OD) was used to calculate a ratio between the native cDNA and CT of each target; thereafter, these ratios were used to calculate the ratio for dCK and β-actin [27,34]. However, only in the A2780 cells, an expression could be calculated (6.06×10^{-3} dCK/β-actin), but not in the AG6000 and AGC11 due to the presence of the additional band. In the AGC11, it is evident that the native 415 band is increased as a results of the transfection. Due to the competition in the assay for the same primer set, the CT of 294 bp and the additional transcript at 231 are less abundant.

Table 1
IC50 values in the ovarian cancer cell lines A2780, AG6000, and stably transfected cell lines

Cell line	Drug sensitivity IC_{50} values (μM)			
	dFdC	CdA	Ara-C	Aza-CdR
A2780	0.004 ± 0.001	0.20 ± 0.15	0.038 ± 0.025	0.65 ± 0.21
AR2 (rdCK)	0.003 ± 0.001	0.10 ± 0.08	0.020 ± 0.015	0.35 ± 0.07
AR3 (rdCK)	0.004 ± 0.002	0.14 ± 0.10	0.019 ± 0.016	0.70 ± 0.42
AR4 (rdCK)	0.004 ± 0.001	0.14 ± 0.10	0.008 ± 0.002	0.28 ± 0.11
AR8 (rdCK)	0.005 ± 0.001	0.14 ± 0.10	0.024 ± 0.023	0.65 ± 0.21
AG6000	69 ± 28	>100	42 ± 10	>500
AGN (Neo-vector)	65 ± 49	>100	68 ± 46	>500
AGZ (Zeo-vector)	40 ± 28	>100	45 ± 21	>500
AGN-hdCKl	1.0 ± 0.4	80 ± 34	14 ± 11	>500
AGZ-hdCK5	0.03 ± 0.01	38 ± 5.3	0.27 ± 0.08	>500
AGR1 (rdCK)	0.37 ± 0.55	4.9 ± 3.5	0.40 ± 0.17	22 ± 24
AGR2 (rdCK)	0.11 ± 0.08	7.6 ± 2.9	0.57 ± 0.08	5.3 ± 2.52
AGR3 (rdCK)	0.05 ± 0.01	2.0 ± 1.8	0.30 ± 0.01	3 ± 1.41
AGR7 (rdCK)	0.48 ± 0.13	7.4 ± 2.0	2.67 ± 0.5	400 ± 141
AGC11 (hdCK)	0.09 ± 0.015	20 ± 10	1.4	>500

The IC50 value (μM) is defined as the concentration of the compound that inhibited cells growth by 50%. Cell growth inhibition data are expressed as mean ± S.D. of three experiments performed in triplicate. Cells were incubated with the tested compounds for 72 h as previously described in [30].

failed to distinguish different transcripts (see Fig. 8), because they all intercalate CYBR Green. A signal in AG6000 cells of about 25% of that in the parent A2780 cells was detected, which was also observed in rat dCK-transfected AG6000 cells even when other primers were used. However, efficiency varied, possibly due to differences in sequences between rat and human dCK. Therefore, we considered these signals as not relevant and not informative. The success of transfection was therefore evaluated by measuring sensitivity to dCK-dependent drugs.

Cells selected in Geneticin or Zeomycin were then tested for their sensitivity to dFdC and CdA. The latter analogue was used to assure that an increased sensitivity was due to an selective increase of dCK expression (Table 1). Different levels of dFdC sensitivity could be achieved, AG6000 cells transfected with human or rat dCK were 60- to 2000-fold more sensitive to this drug. A comparable sensitivity pattern of the transfected cells was seen for CdA. However, it seemed that most cells transfected with the hdCK were less sensitive to both aza-CdR and ara-C. Actually human dCK did not change sensitivity to aza-CdR at all. In order to test whether we could increase the sensitivity of the parental A2780 cells, they were transfected in a similar way, but transfection did not change their drug sensitivity. The mock transfectants of AG6000 (AGN and AGZ, Table 1) were still resistant to gemcitabine.

4. Discussion

Deficiency in dCK activity has been considered as one of the main mechanisms responsible for the development of resistance to deoxynucleoside analogues. Gemcitabine (dFdC) is a dC analogue that must be phosphorylated by dCK in order to be active. The resistant cell line AG6000 has a stable resistance phenotype, which was established following the in vitro exposure of the human ovarian carcinoma A2780 cell line to increasing concentrations of dFdC [30]. No dCK activity or full-length protein could be detected in extracts from AG6000 cells. These results indicated that the observed phenotype is mainly due to dCK deficiency. Analysis of various drug-resistant cell lines at the molecular level indicated the existence of several mutations or genomic rearrangements within the dCK gene. Owens et al. [39] described a deletion of 155 bp in the dCK-coding region, corresponding to the fifth exon, in a human T-lymphoblast cell line that is resistant to AraC. These authors also reported a miss-sense point mutation within the functional domain, ATP-binding site, of dCK protein. The expression of the two mutant cDNAs in *Escherichia coli* demonstrated a complete loss of in vitro dCK activity [39]. Later, Northern blot analysis revealed a remarkable reduction in dCK mRNA expression in an AraC-resistant human leukemic cell line, indicating that the expression of dCK could be affected at the transcriptional level or at the level of mRNA stability [40]. Recently, Veuger et al. [28] reported the presence of inactive, alternatively spliced dCK mRNA transcripts in lymphoblasts obtained from patients with acute myeloid leukemia who displayed resistance to AraC. dCK-cDNAs, generated from these spliced mRNA species, yielded non-functional truncated proteins [29].

In the dFdC-resistant AG6000, these molecular abnormalities could not be identified, but the data indicate a novel alteration in this cell. RT-PCR experiments revealed that the dCK deficiency is not due to a lack of transcription of the dCK gene, but probably to a post-transcriptional mechanism that impairs the translation of dCK mRNA. RT-PCR analysis revealed the existence of two dCK transcripts in the drug-resistant cell line AG6000, but not in the parental A2780 cell line. These transcripts corresponded to the full-length dCK mRNA and an additional alternatively

spliced dCK mRNA-lacking exon 3. Our studies showed that the alternatively spliced dCKΔ195ex3 is not translated into a stable protein. In contrast, the full-length dCK transcripts isolated from AG6000 cell line could be translated into full-length protein, but not in AG6000 cells.

No gross genomic rearrangements at the dCK locus were detected by Southern blot analysis, as determined by the use of specific primers complementary to the 5′-flanking region of the dCK transcripts, revealing no point mutations or deletions in this region. Also, PCR analysis of the exon–intron boundary surrounding exon 3 did not show any point mutation in this region. Furthermore, the RT-PCR products corresponding to the full-length dCK from AG6000 cells was cloned and could produce a full-length protein when expressed from a mammalian expression vector in A2780 cells. In addition, the RT-PCR product was sequenced and was not found to contain any point mutations or deletions.

RNase protection analysis demonstrated that the spliced form of the dCK mRNA accounts for 28.4% of the total dCK transcripts in AG6000 cell line, indicating that it is generated by alternative splicing at the post-transcriptional level. In other words, if there was any mutation within the splice sites of dCK gene, in either allele, we should have detected equal amounts of the wild type and the mutated transcripts. However, instability of the altered mRNA species cannot be ruled out as an explanation for the observed difference. Also the RT-PCR using competitive template revealed a shorter product of 231 base pairs in AG6000 cells, in line with exon 3 skipping.

From these experiments, one can suggest that AG6000 cells may contain an inhibitor that specifically blocks dCK mRNA translation. Since the only difference we could reveal so far between A2780 and AG6000 was the presence of the truncated mRNA species, we examined whether its expression may cause the inhibition of dCK translation. Our results suggest that the generation of the alternatively spliced form of dCK mRNA is not a cause but rather a consequence or a byproduct of the drug-resistant phenotype observed in the AG6000 cell line. Veuger et al. [28] reported similar observation in acute myeloid cells made resistant to AraC, a dC analogue also dependent on dCK for activation. It seems that the existence of alternatively spliced forms of dCK is associated with a general mechanism of drug resistance. A possible post-transcriptional mechanism that may interfere with the production of dCK protein in AG6000 cells could be the generation of an anti-sense RNA species due to the drug treatment. In order to test this, we performed RNase protection analysis using the full-length dCK riboprobe, which failed to reveal any anti-sense RNA complementary to dCK in AG6000 cell line. In this assay, low molecular weight RNA species has also been taken into consideration [41], arguing against a potential mechanism that involves RNA interference.

Another mechanism that could be involved is a rapid degradation of the translated dCK protein in AG6000 cells, which could be mediated by the action of other induced proteins in these cells due to drug treatment. Beausejour et al. [42] demonstrated that dCK mRNA could be a substrate for ribozymes, thus the possibility of the expression of ribozymes that can cleave specifically dCK mRNA sequences cannot be excluded. This hypothesis was further supported when AG6000 cell were transfected with the full-length human and rat dCK regulated by the CMV promoter. Surprisingly, none of the generated cell lines was able to fully reverse the resistance, indicating the existence of unknown mechanism that prevent the translation of dCK and/or rapid degradation of the enzyme in these cell lines. However, the sensitivity to CdA and aza-CdR of the clones transfected with the rat dCK was closer to the wild-type A2780 than those transfected with human dCK. This may indicate that the regulation is specific for human dCK. These differences between human and rat dCK were most pronounced when sensitivity for aza-CdR was evaluated. These data are in line with the observation that aza-CdR is a good substrate for rat dCK [8]. Therefore, in order to restore sensitivity to deoxynucleoside analogues, it is recommended not to use human dCK, but e.g. rat dCK or a broad-substrate deoxynucleoside kinase, such as that from *Drosophila* [43,44].

In conclusion, this study demonstrated that the formation of alternatively spliced form of dCK-mRNA in the dFdC-resistant cell line (AG6000) is a consequence of the drug resistance. The phenomenon could be mediated by the action of unknown mechanism in which specific ribozymes could be involved.

Acknowledgments

This study was supported by grant BMH4-CT96-0479 of the European Community and the Swedish Research Council. We also thank A. Adema for performing additional real-time RT-PCR experiments.

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