Cancer Chemother Pharmacol (2006) 58: 547–554
DOI 10.1007/s00280-006-0195-8

ORIGINAL ARTICLE

Lars Petter Jordheim · Carlos M. Galmarini
Charles Dumontet

Gemcitabine resistance due to deoxycytidine kinase deficiency can be reverted by fruitfly deoxynucleoside kinase, DmdNK, in human uterine sarcoma cells

Received: 24 November 2005 / Accepted: 17 January 2006 / Published online: 4 February 2006

Abstract *Purpose*: Cytotoxic nucleoside analogues are widely used in the treatment of cancers. Resistance to these compounds is frequent and often multifactorial. Deficiency in deoxycytidine kinase (dCK), the rate-limiting activating enzyme, has been reported in a number of in vitro models as well as in various clinical situations. Some strategies to overcome this mechanism of resistance have been proposed there by gene transfer based therapy. *Methods*: We have developed and characterized a gemcitabine-resistant cell line (Messa 10 K) from the human uterine sarcoma Messa strain, and transfected this cell line with the multisubstrate deoxynucleoside kinase from *Drosophila melanogaster* (DmdNK) in order to revert the resistance in Messa 10 K cells which was due to dCK-deficiency. *Results*: Messa 10 K is highly resistant to gemcitabine (122-fold), troxacitabine (>15-fold) and araC (13,556-fold). Quantitative real-time PCR and western blot analysis showed that dCK was not detectable in Messa 10 K cells, presumably because of a genetic modification. The transfection of Messa 10 K cells with DmdNK significantly increased the sensitivity to gemcitabine. *Conclusions*: These results show that genetic modifications in non-hematological malignant cells may be associated with resistance to gemcitabine, and that the gene transfer of non-human genes can be used for the reversion of nucleoside analogue resistance due to dCK deficiency.

Keywords Gemcitabine · Resistance · dCK · DmdNK · Nucleoside analogues

L. P. Jordheim (✉) · C. M. Galmarini · C. Dumontet
INSERM U590, Laboratoire de Cytologie Analytique,
Faculté de Médecine Rockefeller, 8, Avenue Rockefeller,
69008 Lyon, France
E-mail: jordheim@yahoo.com
Tel.: +33-4-78777236
Fax: +33-4-78777088

Introduction

Gemcitabine (2′, 2′-Difluorodeoxycytidine, dFdC) is a deoxyribonucleoside analogue that shows activity against hematological malignancies and solid tumors, both alone [9, 11, 21, 39, 52] and in combination with other chemotherapeutic agents [6, 18, 22]. The cellular metabolism of gemcitabine is similar to that of physiological deoxyribonucleosides [25]. Once inside the cell, it is phosphorylated by deoxycytidine kinase (dCK) to its monophosphorylated form dFdCMP, and further by other intracellular kinases to the metabolically active forms, dFdCDP and dFdCTP [5, 31, 34]. Diphosphorylated gemcitabine is an inhibitor of ribonucleotide reductase [20], thereby causing a decrease in dCTP pools and a decreased feedback inhibition of dCK leading to an enhanced phosphorylation of gemcitabine [7], a process designated as "self-potentiation". The triphosphorylated form is cytotoxic by incorporation into DNA, causing masked chain termination after the incorporation of an additional nucleotide after gemcitabine. Cytotoxicity of gemcitabine in non-dividing cells has been attributed to the fact that dFdCTP can disturb RNA metabolism by inhibiting CTP synthetase (CTPs) or by direct incorporation into newly synthesized RNA.

Resistance to gemcitabine may involve a variety of mechanisms. In humans, cellular influx of gemcitabine involves different membrane equilibrative (ENT) or concentrative (CNT) nucleoside transporters, such as hENT1, hENT2, hCNT1 and hCNT3 [35]. It has been shown that nucleoside transporter-deficient cells display resistance to gemcitabine in vitro [35], and that the expression of hENT1 is correlated with the survival of gemcitabine-treated patients with pancreatic adenocarcinoma [48]. Various authors which includes us have shown that dCK-deficient lines display a gemcitabine-resistant phenotype [13, 14, 26, 37, 42, 43, 45, 50]. Furthermore, gemcitabine and its monophosphorylated form can be inactivated by intracellular enzymes such as

cytidine deaminase and deoxycytidine monophosphate deaminase (dCMPd), producing dFdU from dFdC and dFdUMP from dFdCMP, respectively [5, 19]. Intracellular 5′-nucleotidases are also likely to be involved in the cytotoxic activity of gemcitabine by dephosphorylating dFdCMP or by modifying pools of endogenous deoxyribonucleotides which compete with dFdCDP or dFdCTP. Finally, modifications in intracellular targets of gemcitabine could be involved in resistance to this molecule, as has been shown for the large subunit of ribonucleotide reductase, R1 [3, 8, 27, 44]. The increased use of gemcitabine in the treatment of solid tumors justify the research on resistance in new models.

The high efficiency multisubstrate deoxyribonucleoside kinase from the fruitfly *Drosophila melanogaster* (DmdNK) has been identified as the only deoxyribonucleoside kinase in this insect [41]. This kinase efficiently phosphorylates all four natural deoxyribonucleosides as well as several clinically used nucleoside analogues [23, 40]. Its transduction into human pancreatic adenocarcinoma cells or thymidine kinase 1-deficient human osteosarcoma cells increased the sensitivity to cytotoxic nucleoside analogues such as gemcitabine, araC and cladribine [55]. Therefore, and in addition to the bystander effect observed for this enzyme, DmdNK has been proposed for the use in gene/chemotherapy combinations [53, 54]. Site-directed mutagenesis studies have identified amino acids involved in the kinase activity of and the substrate recognition by DmdNK, and improved kinases have been developed [46, 47]. No data have yet been published regarding the circumvention of dCK deficiency by DmdNK and the eventual reversion of nucleoside analogue resistance observed in cells lacking dCK expression.

In order to develop a model of a human solid tumor resistant to gemcitabine suited for in vitro strategies of reversion of drug resistance, we have developed and characterized the dCK-deficient Messa 10 K model from the human uterine sarcoma cell line Messa, and shown that DmdNK successfully reverses gemcitabine resistance in this cell line.

Materials and methods

Reagents

Compounds used for in vitro experiments were ara-C (Pharmacia, Saint-Quentin-en-Yvelines, France), cisplatin (Merck, Lyon, France), gemcitabine (Lilly, IN, USA), Fara-AMP (Schering Laboratories, Lys-les-Lannoy, France), troxacitabine (Shire BioChem, Ottawa, Canada), etoposide (Laboratoire Sandoz, Rueil-Malmaison, France) and doxorubicin (Pharmacia & Upjohn, Saint-Quentin-en-Yvelines, France). Hydroxyurea, methylthiazoletetrazolium (MTT), isopropanol and NaCl were purchased from Sigma Aldrich (Saint-Quentin, France), HCl from Merck (Strasbourg, France). TRIZol RNA extraction kit, Murine Moloney Leukemia Virus reverse transcriptase (MMLV), Taq DNA Polymerase and RPMI 1640 cell culture media were purchased from Invitrogen (Cergy Pontoise, France), L-glutamine and penicillin-streptomycin from Gibco (Cergy Pontoise, France), and fetal bovine sera from PAN Biotech GmbH (Aidenbach, Germany).

Development of gemcitabine resistant Messa cells

Human uterine sarcoma Messa cells were grown in RPMI 1640 media containing L-glutamine, penicillin (200 UI/ml), streptomycin (200 µg/ml) and fetal bovine serum (10%) at 37°C in the presence of 5% CO_2. Gemcitabine resistant Messa cells were developed by continuous exposure to increasing concentrations of gemcitabine over a period of 6 months. The cells obtained (Messa 10 K) were viable in a media containing 10 µM of gemcitabine and showed no apparent morphological differences or differences in growth rate with the parental Messa wt cells.

Cytotoxicity assays

Messa cells were plated at 20,000 cells per well in 24 well plates (Becton Dickinson, San Jose, CA, USA) in a volume of 900 µl and incubated for 24 h at 37 °C before 100 µl of RPMI containing different drug concentrations was added. After incubation at 37°C for 72 h, MTT (500 µg) was added and, after a 2 h incubation at 37°C, the supernatant was replaced with 300 µl isopropanol/H_2O/HCl (v/v/v 90/9/1) to dissolve the formazan crystals. Spectrophotometric determination of optical density was performed using a microplate reader (Labsystem Multiskanner RC). Inhibitory concentration 50 (IC50) was defined as the concentration inhibiting proliferation to a level equal to 50% that of unexposed controls and the resistance ratio (RR) was calculated as the ratio between the IC50 of the gemcitabine-resistant Messa 10 K cell line and the IC50 of the sensitive parental cell line Messa wt. IC50 values were determined from concentration-effect curves generated using Microsoft® Excel.

Quantitative real-time RT-PCR

Total mRNA was extracted using TRIZol as previously described [15], and quantitative RT-PCR was performed in a Lightcycler® as previously described [26] using primers, probes and PCR conditions as described earlier [27]. Results were analysed with RelQuant software (Roche, Mannheim, Germany) as indicated in the user's manual using ribosomal 18S RNA (Applied Biosystems) as housekeeping gene. Results are given as mean values of three experiments in which gene expressions in Messa

10 K cells are expressed relative to gene expression in parental Messa wt cells.

Western blot

For Western blot analysis, proteins were extracted and treated for electrophoretic separation as previously described [27]. The membrane was incubated with primary antibody (dCK, 1/5,000 (a generous gift from Dr I Talianidis, Greece); hCNT-3, 1/50 (generous gift from Dr J.R. Mackey, Canada); actin, 1/5,000) followed by incubation with peroxidase-conjugated secondary antibodies. Protein signals were detected by chemoluminescence (Amersham) and exposure to Kodak film (Eastman Kodak Company, New Haven, CT, USA).

PCR

Genomic DNA was prepared from Messa wt and Messa 10 K cells with a phenol/chloroform extraction method. dCK (GenBank ID: 1633) exons were amplified by PCR from 250 ng of genomic DNA using Taq DNA Polymerase (Invitrogen) in a final volume of 25 µl containing forward and reverse primers (1.5 nM each), dNTPs (200 mM) and $MgCl_2$ (1.5 mM). PCR conditions included an initial denaturation of 15 min at 94°C followed by 50 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 30 s. Primers were (numbers are positions on chromosome 4): exon 2 forward (5′-GGCAGGGAGC CTTTTCATTT-3′, 72228644), exon 2 reverse (5′-CTC CTCTAAACCAAGAGGCT-3′, 72228963), exon 3 forward (5′-CCAGACCTCAGACAATCTGA-3′, 72253025), exon 3 reverse (5′-CCCAGCTCAGCCAT TCATTA-3′, 72253495), exon 4 forward (5′- GTGCC ACTGGATTTAGGAGA-3′, 72254207), exon 4 reverse (5′-GTTGGGAGGCTGGAGTTAAA-3′, 72254545), exon 5 forward (5′-AGCGTGAGTAGAGAGACA CA-3′, 72256469), exon 5 reverse (5′-CGCTATCAATA CCACCAAGG-3′, 72256888), exon 6 forward (5′-CTGC CAGATGAAGTACTGCA-3′, 72257271), exon 6 reverse (5′-TGTCAGATTCTGAGATTCCG-3′, 72257709), exon 7 forward (5′-AGTTACTTATACAGCTGGGG-3′, 72261649), exon 7 reverse (5′-TATCTGGAACCATT TGGCTGC-3′, 72260149), 5UT forward (5′-AGTCAC CGTTTTCCCCTTCT-3′, 72222833), 5UT reverse (5′-CTTTGTTCAACACGCCAAAA-3′, 72223019).

Transfection with DmdNK

The pGEX-2T vector containing the DmdNK cDNA was a generous gift from J. Piskur and B. Munch-Petersen [29, 40]. DmdNK was cloned into pcDNA3 (Invitrogen) with BamHI and EcoRI. For transfection studies, Messa 10 K cells were seeded in 6-well plates (50,000 cells per well) and transfected with empty or DmdNK containing pcDNA3 vector (1.5 µg per well) and Lipofectin® (Invitrogen, 5 µl per well). After a 10 min incubation of plasmid and Lipofectin® at 25°C, OptiMEM media (Gibco, 500 µl per well) was added. The mixture was added to rinsed cells after a 10 min incubation at 25°C. Cells were then incubated for 4 h at 37°C and 2.5 ml of serum-complemented RPMI media containing different concentrations of gemcitabine were added. Cells were further incubated at 37°C for 72 h. Messa wt cells were treated the same way as Messa 10 K cells but in the absence of plasmids or Lipofectin®. Using this transfection protocol, 50% of Messa 10 K cells were transfected as shown by co-transfection experiments with green fluorescent protein. To quantify the sensitivity to gemcitabine, MTT (1,000 µg) was added, and after 2 h incubation at 37°C, the supernatant was removed and replaced with 1,500 µl isopropanol/H_2O/HCl (v/v/v 90/9/1) to dissolve the formazan crystals. IC50 values were determined as described above.

Statistical analysis

All statistical analyses were performed using Student's *t* test with STATISTICA® version 6 (StatSoft, Inc., Tulsa, OK, USA).

Results

Characterization of gemcitabine resistant Messa cells

IC50 values and resistance ratios for the gemcitabine resistant Messa 10 K cell line and the sensitive parental cell line Messa wt are listed in Table 1. Messa 10 K cells were 122-fold less sensitive to gemcitabine than the parental cell line Messa wt. Significant cross-resistance was observed for araC (RR = 13,556) and troxacitabine (RR > 15), as well as a less pronounced and non-significant resistance to fludarabine (RR = 3.50). Further, Messa 10 K showed no decreased sensitivity to hydroxyurea, cisplatin, doxorubicin or etoposide, suggesting that the resistance mechanism was specific to nucleoside analogues.

To identify cellular mechanisms responsible for the resistance observed in Messa 10 K cells, we quantified the expression of genes known to be involved in the transport, metabolism and the cytotoxicity of nucleoside analogues by quantitative RT-PCR (Fig. 1). The most important difference between the two cell lines concerned dCK. In fact, the expression of dCK was completely abolished in the gemcitabine resistant cell line. As for the membrane nucleoside transporters studied (hENT-1, hENT-2 and hCNT-1), in all cases a slight (but statistically insignificant) decrease in mRNA was detected in Messa 10 K cells, indicating that the overall nucleoside transport might be altered in this cell line. The expression of MRP5, a potential

Table 1 Sensitivity of gemcitabine resistant and parental Messa cells to clinically used anticancer drugs. IC50 values were determined by the MTT assay and resistance ratios were calculated for Messa wt and Messa 10 K cells as described in Materials and methods

	IC50 (μM) Messa wt	IC50 (μM) Messa 10 K	RR	*P t*
Gemcitabine	0.767 ± 1.069	26.7 ± 5.77	122 ± 95.7	0.0015*
Troxacitabine	8.00 ± 3.46	> 100	> 15.0 ± 8.66	0.0000*
AraC	0.700 ± 0.265	9,000 ± 1732	13,556 ± 3,355	0.0008*
Fludarabine	0.233 ± 0.153	0.700 ± 0.265	3.50 ± 1.32	0.057
HU	833 ± 289	1,000 ± 0.00	1.33 ± 0.577	0.37
CisPt	30.0 ± 20.0	36.7 ± 15.3	1.44 ± 0.509	0.67
Doxorubicine	0.229 ± 0.139	0.207 ± 0.124	1.17 ± 0.289	0.92
Etoposide	16.6 ± 5.77	10.0 ± 0.00	0.667 ± 0.289	0.12

All experiments were performed in triplicate on three separate occasions, and results are expressed as mean values ± SE. * were statistically significant. *P t*: *P* value obtained with Student's *t* test

gemcitabine efflux pump, was not modified, nor were the deoxyribonucleoside and nucleotide kinases deoxyguanosine kinase (dGK), thymidine kinase 2 (TK2), uridinemonophosphate-cytidinemonophosphate-kinase (UMPCMPK) and nucleoside diphosphate kinase (NDPK). Regarding the cytosolic thymidine kinase, TK1, we observed a non-significant decrease of the expression in the gemcitabine-resistant cells. Finally, no difference was found between Messa wt cells and Messa 10 K cells in the expression levels of the 5′ nucleotidases cN-II, cN-III, cdN and mdN, for the intracellular gemcitabine targets CTPs, ribonucleotide reductase (subunits R1 and R2) and DNA polymerase α2 or the dFdCMP inactivating protein dCMPd. Thus, the RT-PCR study suggests that the nucleoside analogue resistance observed in Messa 10 K cells is essentially due to dCK deficiency.

The low expression of dCK in Messa 10 K cells was confirmed at the protein level by Western blot (Fig. 2) and by measurement of dCK activity (data not shown). In addition, we studied the protein level of the fourth nucleoside transporter transporting gemcitabine across the cell membrane, hCNT-3. However, no difference was observed in the hCNT-3 protein level between the two cell lines studied.

dCK deficiency is due to genetic modification in gemcitabine resistant Messa cells

To understand the mechanism by which dCK mRNA expression is absent in Messa 10 K cells, we tried to amplify the 7 exons of the human *dCK* gene coding for the mRNA. As shown in Fig. 3, exons 2–7 as well as a 5′ untranscribed region (-1,748 to -1,542) were not detectable by PCR using genomic DNA from Messa 10 K cells, whereas they were all amplified in the Messa wt cells using the same experimental conditions. We were unable to obtain PCR products for exon 1, either in the Messa wt or in the Messa 10 K cells. The quality of DNA from Messa 10 K cells was verified by PCR using primers for a gene situated on another chromosome and no differences were observed between the parental and the gemcitabine-resistant cell lines in this experiment (data not shown). These results suggest a genetic modification on chromosome 4 in the region of the dCK gene in Messa 10 K cells.

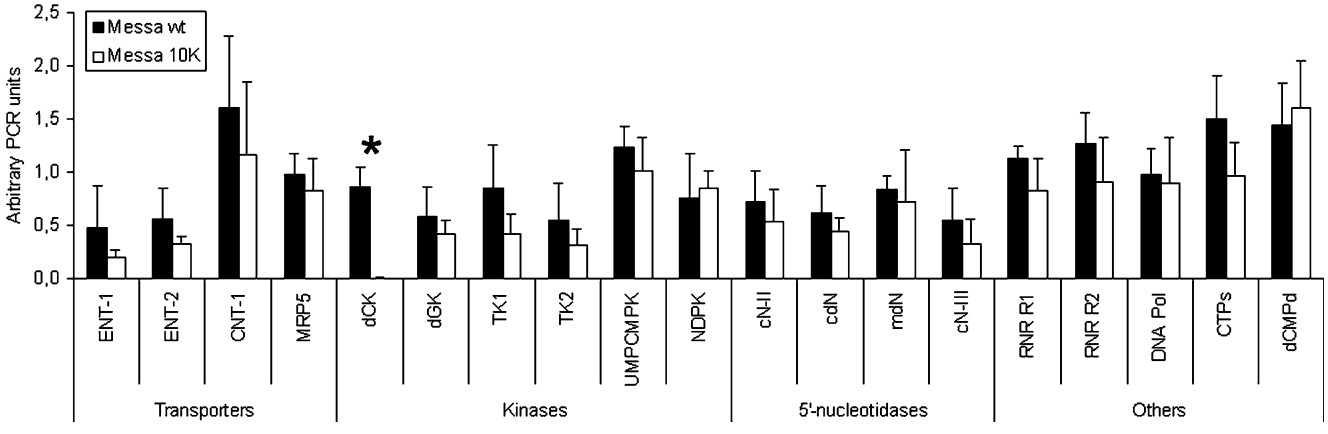

Fig. 1 Expression of genes involved in nucleoside analogue metabolism in gelcitabine-resistant Messa cells. mRNA levels are expressed as arbitrary PCR units as described in Materials and methods. All experiments were done four times. * $P < 0.05$ with Student's *t* test

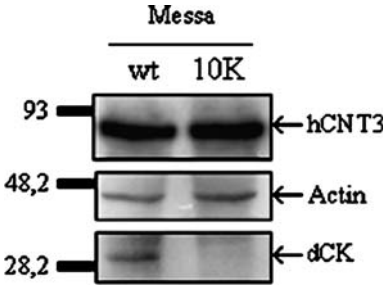

Fig. 2 Expression of dCK and hCNT-3 in gemcitabine resistant Messa cells. Western blot analysis in Messa wt and Messa 10 K cells using anti-dCK, anti-hCNT-3 and anti-actin antibodies

DmdNK reverts gemcitabine resistance in Messa 10 K cells

To study the reversion of gemcitabine resistance due to dCK deficiency by introduction of the deoxynucleoside kinase from fruitfly, Messa 10 K cells were transfected with empty or DmdNK containing pcDNA3 vector as described in Materials and methods. Initial transfection experiments showed that DmdNK mRNA was detected specifically in DmdNK transfected cells, and cotransfection with a GFP-coding plasmid indicated a transfection efficiency of about 50% in our conditions (data not shown). Exposure to gemcitabine was continuous after transfection, to ensure the presence of drug at the time when the expression of DmdNK protein would be the highest. Messa wt cells were treated the same way, however without transfection, to verify whether the transfection of DmdNK would totally restore gemcitabine sensitivity. Mean IC50 values for Messa wt cells, Messa 10 K cells transfected with empty vector and Messa 10 K transfected with DmdNK are presented in Table 2. DmdNK transfected Messa 10 K cells were only 3.83-fold less sensitive than Messa wt cells and showed a 6.16-fold decrease in IC50 value as compared to Messa 10 K cells. The difference in the resistance ratios for control Messa 10 K cells and DmdNK transfected Messa 10 K cells, calculated as the ratio between their IC50 and the IC50 of Messa wt cells, were statistically significant. This result shows that the transient transfection of DmdNK in gemcitabine-resistant dCK-deficient human uterine sarcoma cells partially restored the sensitivity of these cells to the cytotoxic activity of gemcitabine.

Discussion

Gemcitabine is currently indicated in the treatment of bladder, lung and pancreatic cancers and has shown activity in patients with breast cancer. Complete responses are rarely observed with gemcitabine, suggesting that mechanisms of resistance to this compound exist in vivo, or that suboptimal dose-schedules are used. To increase knowledge about nucleoside analogue resistance, and in particular, resistance of solid tumors to gemcitabine, we have developed a gemcitabine-resistant human uterine sarcoma cell line from parental Messa cells. Characterization of these cells showed that the resistant cell line, Messa 10 K, is 122-fold less sensitive to the cytotoxic action of gemcitabine than the parental cell line, Messa wt, and shows cross-resistance to other pyrimidine analogues. These results are similar to those obtained in other studies on nucleoside analogue resistance, and indicate that the resistance mechanism is specific to nucleoside analogues [13, 14, 16, 26, 37, 38, 42, 43, 45, 50].

The cellular mechanism responsible for the resistant phenotype was studied by comparison of results obtained by real time quantitative RT-PCR in the two cell lines. Gemcitabine interacts with a large number of membrane and intracellular proteins (transporters, kinases, deaminases, 5′-nucleotidases, DNA and RNA polymerases) [4], and the quantification of 19 genes in Messa wt and Messa 10 K cells showed that the major difference in the expression pattern was the complete absence of dCK mRNA and protein in Messa 10 K cells (Fig. 1). Such a down-regulation might be responsible for the resistance, as dCK catalyzes the first rate-limiting step in the intracellular phosphorylation of dFdC. dCK deficiency is frequently found in nucleoside analogue resistant cell lines, and has been shown to be clinically relevant [12–14, 16, 26, 28, 42, 43, 45, 50]. In addition to the abolished dCK level, we observed only small non-significant differences for the other enzymes studied. Although we did not perform functional transport studies or determinations of the intracellular concentrations of phosphorylated derivatives of gemcitabine, these observations suggest that the deficiency in dCK strongly contributes to the gemcitabine resistant phenotype of Messa 10 K cells. The interesting fact that Messa 10 K cells are more resistant to araC than to gemcitabine (RR of 13,556 and 122, respectively) could be explained by the modifications observed in these other genes. As these two nucleoside analogues do not have the same mechanism of action

Table 2 Cytotoxicity of gemcitabine in parental Messa cells and gemcitabine resistant Messa cells transfected or not with DmdNK. IC50 values were determined and resistance ratios were calculated for Messa wt and Messa 10 K cells as described in Materials and methods

		Messa wt	Messa 10 K pcDNA.3	Messa 10 K DmdNK	*P t*
Gemcitabine	IC50 (μM)	0.433 ± 0.153	11.7 ± 7.64	1.90 ± 1.65	
	RR		26.4 ± 12.0	3.83 ± 2.77	0.03*

All experiments were performed in triplicate on 3 separate occasions, and results are expressed as mean values ± SE. * were statistically significant. *P t*: *P* value obtained with Student's *t* test

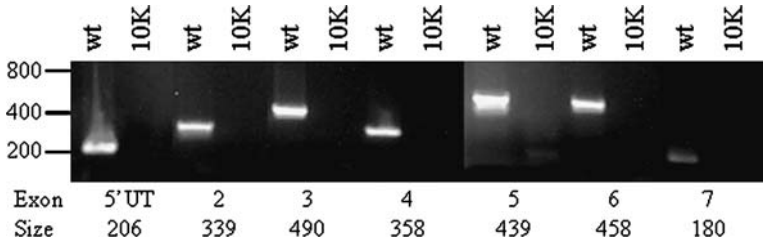

Fig. 3 Study of *dCK* gene in gemcitabine resistant Messa cells. PCR products for human *dCK* gene in Messa wt and 10 K cells were prepared as described in Materials and methods and separated on agarose gel. 5UT corresponds to a 5 untranscribed region (-1,748 to -1,542 bp) of the *dCK* gene

and metabolism, it is possible that changes induced by gemcitabine can influence specifically on araC activity.

Several mechanisms have been proposed for down-regulation of dCK in nucleoside analogue resistant cells [1, 10, 30, 33]. To search for mutations within the *dCK* gene, we tried to amplify each exon of the *dCK* gene in genomic DNA from Messa wt and Messa 10 K cells. As presented above, we identified a deletion on chromosome 4 in the region of the *dCK* gene in the gemcitabine resistant cells, ranging from the 5′UT region to the last exon (Fig. 3). We have also found similar alterations in two other human cell lines and one murine cell line resistant to gemcitabine, indicating that genetic alterations within the *dCK* gene might be a common mechanism to gemcitabine resistance [13, 24, 26]. These chromosomal modifications might be induced secondarily as a result of dNTP pool modifications during gemcitabine treatment [32], and the fact that they seem to be homozygotes might be explained by a loss of heterogenicity during the selection of resistant cells. We also showed that gemcitabine could induce genomic amplification of the *RNR R1* gene in MCF-7 cells [27], and similar results were reported for a gemcitabine resistant murin colon cancer cell line developed in vivo [51]. It seems, therefore, that long-time exposure of cancer cells to gemcitabine can induce variable genetic modifications to allow cell survival.

Different strategies have been proposed to circumvent resistance to nucleoside analogues. For example, we have developed a pronucleotide strategy based on the administration of monophosphorylated derivatives protected by biolabile entities allowing the intracellular delivery of the monophosphorylated analogue [14, 26]. This approach is particularly suited in cases of resistance due to kinase deficiency, and might be applicable in the case of transporter deficiency. Another way to circumvent kinase deficiency could be the introduction of a deoxyribonucleoside kinase into resistant cells. Transfection of dCK or dGK in normal or cancer cells sensitizes the cells to the cytotoxic activity of nucleoside analogues activated by these kinases [2, 17, 36, 49, 56]. The unique deoxynucleoside kinase from *D. melanogaster* has also been studied in this gene therapy approach. In fact, cells expressing this kinase are more sensitive to nucleoside analogues than their parental cells [55]. This has been observed in deoxyribonucleoside kinase proficient cells, as well as in thymidine kinase deficient cancer cells. However, no results have been reported as to the reversion of nucleoside analogue resistance due to dCK deficiency. Therefore, we studied the possibility of reverting this kind of resistance in our gemcitabine resistant human uterine sarcoma cell line Messa 10 K. As reported, the transfection of *DmdNK* gene into Messa 10 K cells induced a significant sensitization to gemcitabine (Table 2). Our transfection study did not allow us to revert totally the gemcitabine resistance in these cells. This can be explained by the fact that only 50% of the cells were efficiently transfected in our experiments, as documented by green fluorescent protein cotransfection. Another explanation could be the presence of other cellular modifications in Messa 10 K cells altering the cellular response of gemcitabine. Our results clearly show that this kinase could be used in the sensitization of dCK-deficient cells to the cytotoxic effects of gemcitabine.

In conclusion, we have developed and characterized a gemcitabine resistant variant of the human uterine sarcoma cell line Messa in which dCK deficiency appears to be a significant resistance mechanism. We also showed that gemcitabine resistance due to dCK deficiency can be reverted by transfection with the deoxynucleoside kinase from *D. melanogaster*. DmdNK thus appears to be an interesting candidate for gene therapy approaches. Future studies will be required to determine whether transfection of normal cells harboring the cancer cells, in particular hematopoietic cells, with this gene enhances their sensitivity to nucleoside analogues.

Acknowledgements LPJ received grants from the Association pour la Recherche contre le Cancer (ARC) and Stiftelsen Thomas Fearnley, Heddy og Nils Astrup. This work was supported by Mjålands Stiftelse for Kreftforskning.

References

1. Antonsson BE, Avramis VI, Nyce J, Holcenberg JS (1987) Effect of 5-azacytidine and congeners on DNA methylation and expression of deoxycytidine kinase in the human lymphoid cell lines CCRF/CEM/0 and CCRF/CEM/dCk-1. Cancer Res 47:3672–3678

2. Beausejour CM, Gagnon- J, Primeau M, Momparler RL (2002) Cytotoxic activity of 2′, 2′-difluorodeoxycytidine, 5-aza-2′-deoxycytidine and cytosine arabinoside in cells transduced with deoxycytidine kinase gene. Biochem Biophys Res Commun 293:1478–1484
3. Bergman AM, Eijk PP, Ruiz van Haperen VW, Smid K, Veerman G, Hubeek I, van den Ijssel P, Ylstra B, Peters GJ (2005) In vivo Induction of resistance to gemcitabine results in increased expression of ribonucleotide reductase subunit M1 as the major determinant. Cancer Res 65:9510–9516
4. Bergman AM, Pinedo HM, Peters GJ (2002) Determinants of resistance to 2′, 2′-difluorodeoxycytidine (gemcitabine). Drug Resist Updat 5:19–33
5. Bouffard DY, Laliberte J, Momparler RL (1993) Kinetic studies on 2′, 2′-difluorodeoxycytidine (Gemcitabine) with purified human deoxycytidine kinase and cytidine deaminase. Biochem Pharmacol 45:1857–1861
6. Dabrow MB, Francesco MR, Gilman PB, Cantor R, Rose L, Meyer TJ (2003) Combined therapy with topotecan and gemcitabine in patients with inoperable or metastatic non-small cell lung cancer. Cancer Invest 21:517–525
7. Datta NS, Shewach DS, Mitchell BS, Fox IH (1989) Kinetic properties and inhibition of human T lymphoblast deoxycytidine kinase. J Biol Chem 264:9359–9364
8. Davidson JD, Ma L, Flagella M, Geeganage S, Gelbert LM, Slapak CA (2004) An increase in the expression of ribonucleotide reductase large subunit 1 is associated with gemcitabine resistance in non-small cell lung cancer cell lines. Cancer Res 64:3761–3766
9. Dumontet C, Fabianowska-Majewska K, Mantincic D, Callet Bauchu E, Tigaud I, Gandhi V, Lepoivre M, Peters GJ, Rolland MO, Wyczechowska D, Fang X, Gazzo S, Voorn DA, Vanier-Viornery A, Mackey J (1999) Common resistance mechanisms to deoxynucleoside analogues in variants of the human erythroleukaemic line K562. Br J Haematol 106:78–85
10. Flasshove M, Strumberg D, Ayscue L, Mitchell BS, Tirier C, Heit W, Seeber S, Schutte J (1994) Structural analysis of the *deoxycytidine kinase* gene in patients with acute myeloid leukemia and resistance to cytosine arabinoside. Leukemia 8:780–785
11. Fowler WC Jr, Van Le L (2003) Gemcitabine as a single-agent treatment for ovarian cancer. Gynecol Oncol 90:S21–23
12. Funato T, Satou J, Nishiyama Y, Fujimaki S, Miura T, Kaku M, Sasaki T (2000) In vitro leukemia cell models of Ara-C resistance. Leuk Res 24:535–541
13. Galmarini CM, Thomas X, Calvo F, Rousselot P, Jafaari AE, Cros E, Dumontet C (2002) Potential mechanisms of resistance to cytarabine in AML patients. Leuk Res 26:621–629
14. Galmarini CM, Clarke ML, Santos CL, Jordheim L, Perigaud C, Gosselin G, Cros E, Mackey JR, Dumontet C (2003) Sensitization of ara-C-resistant lymphoma cells by a pronucleotide analogue. Int J Cancer 107:149–154
15. Galmarini CM, Thomas X, Graham K, El Jafaari A, Cros E, Jordheim L, Mackey JR, Dumontet C (2003) Deoxycytidine kinase and cN-II nucleotidase expression in blast cells predict survival in acute myeloid leukaemia patients treated with cytarabine. Br J Haematol 122:53–60
16. Galmarini CM, Clarke ML, Jordheim L, Santos CL, Cros E, Mackey JR, Dumontet C (2004) Resistance to gemcitabine in a human follicular lymphoma cell line is due to partial deletion of the *deoxycytidine kinase* gene. BMC Pharmacol 4:8
17. Hapke DM, Stegmann AP, Mitchell BS (1996) Retroviral transfer of deoxycytidine kinase into tumor cell lines enhances nucleoside toxicity. Cancer Res 56:2343–2347
18. Heinemann V, Xu YZ, Chubb S, Sen A, Hertel LW, Grindey GB, Plunkett W (1990) Inhibition of ribonucleotide reduction in CCRF–CEM cells by 2′, 2′-difluorodeoxycytidine. Mol Pharmacol 38:567–572
19. Heinemann V, Xu YZ, Chubb S, Sen A, Hertel LW, Grindey GB, Plunkett W (1992) Cellular elimination of 2′, 2′-difluorodeoxycytidine 5′-triphosphate: a mechanism of self-potentiation. Cancer Res 52:533–539
20. Heinemann V (2003) Role of gemcitabine in the treatment of advanced and metastatic breast cancer. Oncology 64:191–206
21. Hochster HS (2003) Newer approaches to gemcitabine-based therapy of pancreatic cancer: fixed-dose-rate infusion and novel agents. Int J Radiat Oncol Biol Phys 56:24–30
22. Hussain SA, James ND (2003) The systemic treatment of advanced and metastatic bladder cancer. Lancet Oncol 4:489–497
23. Johansson M, van Rompay AR, Degreve B, Balzarini J, Karlsson A (1999) Cloning and characterization of the multisubstrate deoxyribonucleoside kinase of *Drosophila melanogaster*. J Biol Chem 274:23814–23819
24. Jordheim L, Galmarini CM, Dumontet C (2003) Drug resistance to cytotoxic nucleoside analogues. Curr Drug Targets 4:443–460
25. Jordheim L, Cros E, Galmarini CM, van Bree C, Dumontet C (2004) *Deoxycytidine kinase* gene alterations in gemcitabine-resistant cell lines. Proc Am Assoc Cancer Res 45:618a
26. Jordheim LP, Cros E, Gouy MH, Galmarini CM, Peyrottes S, Mackey J, Perigaud C, Dumontet C (2004) Characterization of a gemcitabine-resistant murine leukemic cell line: reversion of in vitro resistance by a mononucleotide prodrug. Clin Cancer Res 10:5614–5621
27. Jordheim LP, Guittet O, Lepoivre M, Galmarini CM, Dumontet C (2005) Increased expression of the large subunit of ribonucleotide reductase is involved in resistance to gemcitabine in human mammary adenocarcinoma cells. Mol Cancer Ther 4:1268–1276
28. Kakihara T, Fukuda T, Tanaka A, Emura I, Kishi K, Asami K, Uchiyama M (1998) Expression of *deoxycytidine kinase* (*dCK*) gene in leukemic cells in childhood: decreased expression of *dCK* gene in relapsed leukemia. Leuk Lymphoma 31:405–409
29. Knecht W, Munch-Petersen B, 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 301:827–837
30. Kobayashi T, Kakihara T, Uchiyama M, Fukuda T, Kishi K, Shibata A (1994) Low expression of the *deoxycytidine kinase* (*dCK*) gene in a 1-beta-D-arabinofuranosylcytosine-resistant human leukemic cell line KY-Ra. Leuk Lymphoma 15:503–505
31. Krishnan P, Fu Q, Lam W, Liou JY, Dutschman G, Cheng YC (2002) Phosphorylation of pyrimidine deoxynucleoside analog diphosphates: selective phosphorylation of L-nucleoside analog diphosphates by 3-phosphoglycerate kinase. J Biol Chem 277:5453–5459
32. Kunz BA, Kohalmi SE, Kunkel TA, Mathews CK, McIntosh EM, Reidy JA (1994) International Commission for Protection Against Environmental Mutagens and Carcinogens. Deoxyribonucleoside triphosphate levels: a critical factor in the maintenance of genetic stability. Mutat Res 318:1–64
33. Leegwater PA, De Abreu RA, Albertioni F (1998) Analysis of DNA methylation of the 5′ region of the *deoxycytidine kinase* gene in CCRF-CEM-sensitive and cladribine (CdA)- and 2-chloro-2′-arabino-fluoro-2′-deoxyadenosine (CAFdA)-resistant cells. Cancer Lett 130:169–173
34. Liou JY, Dutschman GE, Lam W, Jiang Z, Cheng YC (2002) Characterization of human UMP/CMP kinase and its phosphorylation of D- and L-form deoxycytidine analogue monophosphates. Cancer Res 62:1624–1631
35. Mackey JR, Mani RS, Selner M, Mowles D, Young JD, Belt JA, Crawford CR, Cass CE (1998) Functional nucleoside transporters are required for gemcitabine influx and manifestation of toxicity in cancer cell lines. Cancer Res 58:4349–4357
36. Manome Y, Wen PY, Dong Y, Tanaka T, Mitchell BS, Kufe DW, 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 2:567–573
37. Mansson E, Spasokoukotskaja T, Sallstrom J, Eriksson S, Albertioni F (1999) Molecular and biochemical mechanisms of fludarabine and cladribine resistance in a human promyelocytic cell line. Cancer Res 59:5956–5963

38. Mansson E, Flordal E, Liliemark J, Spasokoukotskaja T, Elford H, Lagercrantz S, Eriksson S, Albertioni F (2003) Down-regulation of deoxycytidine kinase in human leukemic cell lines resistant to cladribine and clofarabine and increased ribonucleotide reductase activity contributes to fludarabine resistance. Biochem Pharmacol 65:237–247
39. Markman M, Webster K, Zanotti K, Kulp B, Peterson G, Belinson J (2003) Phase 2 trial of single-agent gemcitabine in platinum-paclitaxel refractory ovarian cancer. Gynecol Oncol 90:593–596
40. Munch-Petersen B, Piskur J, Sondergaard L (1998) Four deoxynucleoside kinase activities from *Drosophila melanogaster* are contained within a single monomeric enzyme, a new multifunctional deoxynucleoside kinase. J Biol Chem 273:3926–931
41. Munch-Petersen B, Knecht W, Lenz C, Sondergaard L, Piskur J (2000) Functional expression of a multisubstrate deoxyribonucleoside kinase from *Drosophila melanogaster* and its C-terminal deletion mutants. J Biol Chem 275:6673–6679
42. Obata T, Endo Y, Tanaka M, Matsuda A, Sasaki T (1998) Development and biochemical characterization of a 2′-C-cyano-2′-deoxy-1-beta-D-arabino-pentofuranosylcytosine (CNDAC)-resistant variant of the human fibrosarcoma cell line HT-1080. Cancer Lett 123:53–61
43. Orr RM, Talbot DC, Aherne WG, Fisher TC, Serafinowski P, Harrap KR (1995) 2′-Deoxycytidine kinase deficiency is a major determinant of 2-chloro-2′-deoxyadenosine resistance in lymphoid cell lines. Clin Cancer Res 1:391–398
44. Rosell R, Danenberg KD, Alberola V, Bepler G, Sanchez JJ, Camps C, Provencio M, Isla D, Taron M, Diz P, Artal A (2004) Ribonucleotide reductase messenger RNA expression and survival in gemcitabine/cisplatin-treated advanced non-small cell lung cancer patients. Clin Cancer Res 10:1318–1325
45. Ruiz van Haperen VW, Veerman G, Eriksson S, Boven E, Stegmann AP, Hermsen M, Vermorken JB, Pinedo HM, Peters GJ (1994) Development and molecular characterization of a 2′, 2′-difluorodeoxycytidine-resistant variant of the human ovarian carcinoma cell line A2780. Cancer Res 54:4138–4143
46. Solaroli N, Bjerke M, Amiri MH, Johansson M, Karlsson A (2003) Active site mutants of *Drosophila melanogaster* multisubstrate deoxyribonucleoside kinase. Eur J Biochem 270:2879–2884
47. Solaroli N, Bjerke M, Johansson M, Karlsson A (2004) Investigation of the substrate recognition of *Drosophila melanogaster* nucleoside kinase by site directed mutagenesis. Nucleosides Nucleotides Nucleic Acids 23:1527–1529
48. Spratlin J, Sangha R, Glubrecht D, Dabbagh L, Young JD, Dumontet C, Cass C, Lai R, Mackey JR (2004) The absence of human equilibrative nucleoside transporter 1 is associated with reduced survival in patients with gemcitabine-treated pancreas adenocarcinoma. Clin Cancer Res 10:6956–6961
49. Stegmann AP, Honders WH, Willemze R, Ruiz van Haperen VW, Landegent JE (1995) Transfection of wild-type deoxycytidine kinase (dck) cDNA into an AraC- and DAC-resistant rat leukemic cell line of clonal origin fully restores drug sensitivity. Blood 85:1188–1194
50. van Bree C, Castro Kreder N, Loves WJ, Franken NA, Peters GJ, Haveman J (2002) Sensitivity to ionizing radiation and chemotherapeutic agents in gemcitabine-resistant human tumor cell lines. Int J Radiat Oncol Biol Phys 54:237–244
51. van de Wiel MA, Costa JL, Smid K, Oudejans CB, Bergman AM, Meijer GA, Peters GJ, Ylstra B (2005) Expression Microarray Analysis and Oligo Array Comparative Genomic Hybridization of Acquired Gemcitabine Resistance in Mouse Colon Reveals Selection for Chromosomal Aberrations. Cancer Res 65:10208–10213
52. Vansteenkiste JF, Vandebroek JE, Nackaerts KL, Weynants P, Valcke YJ, Verresen DA, Devogelaere RC, Marien SA, Humblet YP, Dams NL (2001) Clinical-benefit response in advanced non-small-cell lung cancer: A multicentre prospective randomised phase III study of single agent gemcitabine versus cisplatin-vindesine. Ann Oncol 12:1221–1230
53. Zheng X, Johansson M, Karlsson A (2000) Retroviral transduction of cancer cell lines with the gene encoding *Drosophila melanogaster* multisubstrate deoxyribonucleoside kinase. J Biol Chem 275:39125–39129
54. Zheng X, Johansson M, 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 60:262–266
55. Zheng X, Johansson M, 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 289:229–233
56. Zhu C, Johansson M, Permert J, Karlsson A (1998) Enhanced cytotoxicity of nucleoside analogs by overexpression of mitochondrial deoxyguanosine kinase in cancer cell lines. J Biol Chem 273:14707–14711