

# Pretreatment Deoxycytidine Kinase Levels Predict *in Vivo* Gemcitabine Sensitivity<sup>1</sup>

Judith R. Kroep, Willem J. P. Loves, Clasina L. van der Wilt, Enrique Alvarez, Iannis Talianidis, Epie Boven, Boudewijn J. M. Braakhuis, Cornelis J. van Groeningen, Herbert M. Pinedo, and Godefridus J. Peters<sup>2</sup>

Departments of Medical Oncology [J. R. K., W. J. P. L., C. L. v. d. W., E. B., C. J. v. G., H. M. P., G. J. P.] and Head and Neck Surgery [B. J. M. B.], VU University Medical Center, Amsterdam, 1007 MB The Netherlands; Eli Lilly Research Laboratories, Indianapolis, Indiana 46285 [E. A.]; and Institute for Molecular Biology, 71110 Heraklion, Crete, Greece [I. T.]

## Abstract

Deoxycytidine kinase (dCK) is essential for the phosphorylation of gemcitabine (2',2'-difluorodeoxycytidine), a deoxycytidine analogue active against various solid tumors. Cytidine deaminase (CDA) catalyzes the degradation of gemcitabine. We determined whether dCK and/or CDA levels would predict response to gemcitabine.

Activities of dCK and CDA were measured in a panel of eight gemcitabine-sensitive and -resistant tumors of a different origin (pancreas, lung, colon, ovary, and head and neck) grown as s.c. tumors in mice.

Sensitivity to gemcitabine was expressed as treated *versus* control (tumor volume treated mice/control mice). Gemcitabine was given on days 0, 3, 6, and 9 (q3dx4) at its maximum tolerated dose. In addition, we measured the mRNA expression and protein levels of dCK in seven human tumor xenografts.

dCK activity (mean  $\pm$  SE) ranged from  $3.3 \pm 0.3$  to  $18.4 \pm 1.2$  nmol/h/mg protein. Sensitivity to gemcitabine, expressed as treated *versus* control, ranged from 0.98 to 0.02, and the activity of CDA varied from  $2 \pm 2$  to  $411 \pm 4$  nmol/h/mg protein. In contrast to CDA, dCK activity was clearly related to gemcitabine sensitivity ( $\rho = -0.93$ ;  $P < 0.001$ ). This indicates that dCK might be an important prognostic marker for gemcitabine sensitivity.

Protein levels were significantly related to both dCK activity ( $r = 0.96$ ;  $P < 0.001$ ) and gemcitabine sensitivity ( $\rho = -0.96$ ;  $P < 0.001$ ). dCK expression as determined by competitive template reverse transcriptase PCR was significantly related with the dCK activity ( $r = 0.88$ ;  $P = 0.025$ ) and protein levels ( $\rho = 0.80$ ;  $P = 0.052$ ) but not with gemcitabine

sensitivity, suggesting a post-translational regulation of dCK.

In conclusion, the clear correlation between dCK levels and gemcitabine sensitivity in various murine tumors and human tumor xenografts may be a prognostic parameter when considering gemcitabine therapy.

## Introduction

dCK<sup>3</sup> (EC 2.7.1.74) is the rate-limiting enzyme in the salvage of deoxyribonucleosides, providing dNTPs for replicative and repair DNA synthesis (1, 2). In addition to the naturally occurring 2'-deoxyribonucleosides, dCK phosphorylates several clinically important antiviral and cytotoxic deoxyribonucleoside analogues, such as ara-C, 2-chlorodeoxyadenosine (CdA) and dFdC (gemcitabine; Refs. 3–5). Cell lines selected for resistance to these analogues showed frequently a deficiency in dCK activity (6–11) caused by mutational inactivation of dCK. Transfection and induction of dCK resulted in resensitization of cells to ara-C and gemcitabine (12–14).

CDA (EC 3.5.4.5) catalyzes the deamination of cytidine, deoxycytidine, and their analogues (5, 15). The enzyme has been associated with sensitivity and resistance to ara-C (16, 17). Transfection of the gene resulted in resistance to nucleoside analogues (18).

Gemcitabine, which is active against various solid tumors (19, 20), has multiple mechanisms of action. Gemcitabine is phosphorylated to its mononucleotide by dCK and subsequently by nucleotide kinases to its active metabolites, gemcitabine diphosphate (dFdCDP) and dFdCTP. dFdCTP is mainly incorporated into DNA leading to masked chain termination (5, 21). In addition, the active metabolites can inhibit ribonucleotide reductase, dCMP deaminase, and CTP synthetase (22, 23), enhancing the gemcitabine activation, leading to cell death.

Prediction of gemcitabine sensitivity might be achieved by determination of its active metabolite dFdCTP, which is related to gemcitabine sensitivity in cell lines (24, 25). However, dFdCTP accumulation cannot be determined routinely in tumors from patients to be treated with gemcitabine. Because dCK activity is vital in gemcitabine sensitivity, we therefore investigated in our *in vivo* panel whether gemcitabine sensitivity was related to dCK activity. Because dCK is known to be regulated at transcriptional or post-transcrip-

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<sup>2</sup> To whom requests for reprints should be addressed, at Department of Medical Oncology, VU University Medical Center, 1007 MB Amsterdam, The Netherlands. Phone: 31-20-444-2633; Fax: 31-20-444-3844; E-mail: gj.peters@vumc.nl.

<sup>3</sup> The abbreviations used are: dCK, deoxycytidine kinase; dFdC, 2',2'-difluorodeoxycytidine; CDA, cytidine deaminase; CT-RT-PCR, competitive template reverse transcriptase-PCR; ara-C, 1- $\beta$ -D-arabinofuranosylcytosine; CdA, 2-chlorodeoxyadenosine; dFdCTP, gemcitabine triphosphate; [<sup>3</sup>H]CdA, [8-<sup>3</sup>H]-2-chlorodeoxyadenosine; dNTP, deoxyribonucleotide; TK2, thymidine kinase 2; ECL, enhanced chemiluminescence; RT-PCR, reverse transcription-PCR.

tional levels, we also determined the dCK protein expression using Western blotting and its gene expression with CT-RT-PCR. The latter assay would enable usage of small tumor fragments.

## Materials and Methods

**Chemicals and Reagents.** [<sup>3</sup>H]CdA (24.2 Ci/mmol) was purchased from Moravek, Brea, CA, RNAzol from Campro Scientific (Veenendaal, The Netherlands), dNTPs, random hexamers, and Taq polymerase (5 IU/ $\mu$ l) from Pharmacia Biotech (Roosendaal, The Netherlands), and research agarose from Biozym (Landgraaf, The Netherlands). The antibody to human dCK was described previously (26). All of the other chemicals were of analytical grade.

**Tumor Models.** Tumors of five different histological types were studied: the murine tumor PANC-02 (pancreas) and the human tumor xenografts BxPC3, PANC-01 (pancreas), H460, CALU-6 (NSCLC), WiDr (colon), HNX-22B (head & neck), and A2780 (ovary). Murine tumors were implanted s.c. in C57/Bl6 mice (27), whereas human tumors were implanted in female nude mice (28, 29). Gemcitabine was given i.p. as a q3dx4 schedule at its maximum tolerated dose, and anti-tumor activity was evaluated using the ratio between the average tumor volume of treated mice (T) divided by that of control mice (C): T/C. For each tumor type the median T/C of six tumors was used. For an evaluation of the antitumor activity in relation to dCK activity we used the maximum T/C, *i.e.*, the lowest T/C value which could be calculated during treatment. For evaluation of the enzyme activities, Western blotting, and gene expression, tumors were excised and immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ .

**Enzyme Activity Assays.** dCK was determined essentially as described previously (30). To measure dCK selectively and bypass TK2 mediated phosphorylation of deoxycytidine, we used [<sup>3</sup>H]CdA as the substrate (31), which is not activated by TK2. Frozen tissues were pulverized using a microdismembrator as described previously (32), suspended (0.33 g/ml) in extraction buffer [50 mM Tris-HCl (pH 7.6) and 2 mM DTT], and sonicated. Twenty-five  $\mu$ l of appropriately diluted supernatant (protein range: 5.9–20.3  $\mu$ g/ $\mu$ l) was added to a reaction mixture (50  $\mu$ l total volume) containing (final concentrations) 50  $\mu$ M [<sup>3</sup>H]CdA (0.16  $\mu$ Ci/nmol), 50 mM Tris-HCl (pH 7.6), 2 mM DTT, 5 mM MgCl<sub>2</sub>, 5 mM ATP, and 10 mM sodium fluoride. After incubation (15 and 30 min) the reaction was terminated by heating (3 min at 95 $^{\circ}$ ). The substrate and the product, CdA monophosphate, were separated by TLC on polyethyleneimine cellulose using H<sub>2</sub>O after which radioactivity was measured. CDA activity assays (10) were performed with 500  $\mu$ M gemcitabine as the substrate. Protein concentrations were measured using the standard Bio-Rad Bradford assay (33). Enzyme activities were expressed as nmol product formed/h/mg protein (nmol/h/mg protein).

**Western Blots.** Immunoblotting of human dCK was performed essentially as described (26). Briefly, protein extracts (30  $\mu$ g) were electrophoresed on 12.5% SDS polyacrylamide gels and by electroblotting transferred to nitrocellulose membranes (Hybond ECL membranes, Amersham, Buckinghamshire, United Kingdom). Blots were blocked overnight in

blocking buffer (5% BSA in Tris-buffered saline with 0.1% Tween 20). Rabbit antihuman dCK polyclonal antibody (1:5,000; 1 h at room temperature) and goat antirabbit secondary antibody conjugated to horseradish peroxidase (1:40,000; 1 h at room temperature) were used for Western blot. Blots were developed using the ECL Western blot detection kit (Amersham). To identify the identity of the dCK band, purified recombinant dCK (10 ng), AG6000 cells lacking dCK activity (9), and parental A2780 ovarian cancer cells were also tested on every blot. Relative levels of expression were determined by densitometric scanning (Imaging Densitometer, model GS-690; Bio-Rad) of the X-ray films (Hyperfilm ECL). dCK protein levels were expressed as ng dCK/mg total protein.

**Quantitative CT-RT-PCR.** For evaluation of dCK mRNA expression, frozen tissues were pulverized as described under "enzyme activity assays," whereafter the pulverized tissues were suspended in RNAzol to isolate RNA, which was processed to prepare cDNA as described previously for cell lines (34). CT-RT-PCR assay was performed based on principles as described previously (34, 35). Competitive templates are identical to  $\sim$ 75% of the coding region (34, 35), starting with the forward primers for the  $\beta$ -actin and dCK genes, and are added in a known amount to the reaction mixture serving as an internal standard in the assay by competing with native cDNA. Construction of the competitive template was initiated by synthesizing a 425-bp oligonucleotide covering exons 2, 3, and partly 4 of the dCK gene using native cDNA, and the forward (5'-GAAGGGAACATCGCTGCAGG) and reverse (5'-TGGCCAAATTGGTTATTCATCC) primers. This oligonucleotide was mixed with the forward primer for the dCK gene and a specific primer to construct the competitive template (5'-TGGCCAAATTGGTTATTCATCCCTTGAGCTTGCCATTCAGAGAGG), consisting of the reverse primer and a second part, which encodes for a sequence upstream of the reverse primer for the dCK. This resulted in a short product of  $\sim$ 294 bp. This competitive template was additionally purified and quantified as described (34, 35). The competitive template and the native cDNA were balanced to assure linear amplification for both. Because the concentrations of the competitive templates are known, the ratio of the cDNA compared with the competitive template gives the concentration of the mRNA of the target gene. The sequences for the  $\beta$ -actin primers were published previously (34, 35). In these papers various housekeeping genes were compared, but for all seven of the target genes the use of each housekeeping gene resulted in a similar pattern. More details will be published elsewhere.<sup>4</sup>

For the quantitative PCR the volume of the reaction mixtures was 50  $\mu$ l, containing 0.2  $\mu$ g of the forward and reverse primers, 1.0 unit Taq polymerase, 5.0  $\mu$ l PCR buffer, 0.2 mM dNTPs, and a mixture of competitive templates. The reaction mixtures were cycled in a thermocycler (PTC-200 Peltier

<sup>4</sup> C. L. Van der Wilt, J. R. Kroep, W. J. P. Loves, G. J. L. Kaspers, M. G. Rots, C. J. Van Groeningen, and G. J. Peters. Deoxycytidine kinase mRNA expression measured by a competitive template based RT-PCR method related to enzyme activity and protein expression, submitted for publication.

**Table 1** Gemcitabine sensitivity, dCK activity, and CDA activity in a murine tumor and various human tumor xenografts

Values are mean  $\pm$  SE of three separate tumors and experiments.

Xenograft	Sensitivity <sup>a</sup>	dCK activity <sup>b</sup>	CDA activity <sup>c</sup>
HNX-22B	0.98	3.3 $\pm$ 0.3	191 $\pm$ 106
BxPC3	0.91	6.2 $\pm$ 0.5	83 $\pm$ 66
PANC-02 <sup>d</sup>	0.59	6.5 $\pm$ 1.7	158 $\pm$ 44
H460	0.55	9.7 $\pm$ 1.5	411 $\pm$ 4
WiDr	0.28	9.6 $\pm$ 1.8	299 $\pm$ 31
PANC-01	0.30	14.6 $\pm$ 0.3	190 $\pm$ 9
Calu-6	0.25	17.5 $\pm$ 3.1	2 $\pm$ 2
A2780	0.02	18.4 $\pm$ 1.2	76 $\pm$ 49

<sup>a</sup> Sensitivity to gemcitabine is expressed as maximal T/C (tumor volume treated mice/tumor volume control mice).

<sup>b</sup> dCK activity is expressed as nmol/h/mg protein.

<sup>c</sup> CDA activity is expressed as nmol/h/mg protein.

<sup>d</sup> A murine tumor.

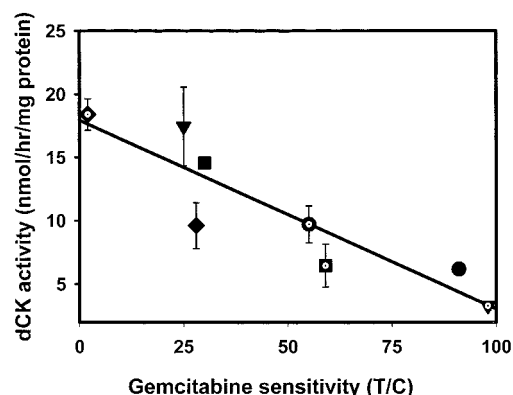
Thermal Cycler; MJ Research, Watertown, MA), whereafter the product was electrophoresed on a agarose gel and quantified by scanning on a GS-690 Bio-Rad scanner (Hercules, CA). Levels of expression were reported as units dCK-mRNA/10<sup>6</sup>  $\beta$ -actin mRNA molecules.

**Statistics.** The Pearson/Spearman correlation test and regression analysis were used to quantitate the relationship between the dCK activity and dCK expression, as well as between the dCK activity/expression and antitumor activity. The computer program SPSS (version 9.0; SPSS, Inc., Chicago, IL) was used for statistical analysis.

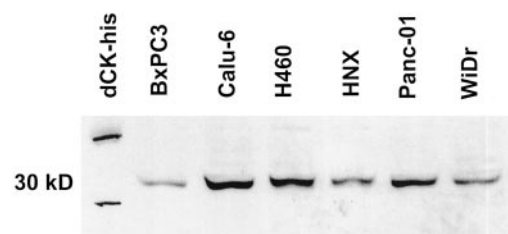
## Results

**Gemcitabine Sensitivity in Relation with dCK and CDA Activities.** Activities (mean  $\pm$  SE) of the main gemcitabine activating and inactivating enzymes, dCK and CDA, respectively, are summarized in Table 1. The same table shows the sensitivity of the various tumors tested *in vivo* for gemcitabine given at its maximum tolerated dose in the q3dx4 schedule (28, 29). dCK activity in control tumors ranged from 3.3  $\pm$  0.3 to 18.4  $\pm$  1.2 nmol/h/mg protein, whereas CDA activities showed a larger variation, ranging from 2  $\pm$  2 to 411  $\pm$  4 nmol/h/protein. Sensitivity of the tumors to gemcitabine varied from resistant (0.98) to extremely sensitive (0.02). A clear relation ( $\rho = -0.93$ ;  $P < 0.001$ ) was observed between the dCK activity and gemcitabine sensitivity (Fig. 1) in this panel. Both CDA enzyme levels and the dCK:CDA ratio did not correlate with the gemcitabine sensitivity. These results indicate that dCK activity levels in solid tumors might be important as a prognostic marker for response to gemcitabine therapy.

**Relation between dCK Expression and dCK Enzyme Activity.** The anti-dCK antibody recognized a 30,000  $M_r$  protein band in crude cellular extracts (26). Purified dCK-his, representing the subunit of the dCK polypeptide dimer with a histidine tag, and the dCK-deficient variant of A2780 cells, AG6000, were included as positive and negative controls, respectively. In the latter no band was observed at 30,000  $M_r$ . The anti-dCK antibody did not recognize the 30,000  $M_r$  polypeptide in the murine tumor. Fig. 2 shows an example of



**Fig. 1.** Clear relationship between dCK activity (nmol/h/mg protein) and T/C (tumor volume of treated mice/tumor volume of control mice) for the murine tumor PANC-02 (dotted square) and various human tumor xenografts BxPC3 (●), PANC-01 (■), H460 (dotted circle), CALU-6 (▲), WiDr (◆), HNX-22B (dotted triangle), and A2780 (dotted diamond);  $\rho = -0.93$ ;  $P < 0.001$ .



**Fig. 2.** Western analysis of dCK expression at the 30,000  $M_r$  position in some of the xenografts with the dCK-his as reference. Lanes represent, as indicated in the figure, dCK-his, BxPC3, Calu-6, H460, HNX-22B, PANC-01, and WiDr, respectively. Because of the his-tag, dCK-his has a higher molecular weight than natural dCK.

the Western analysis, whereas Fig. 3 depicts the relationship between immunoreactive and catalytically active dCK in seven different human tumor xenografts. The levels of dCK protein varied from 0.12 ng/mg protein for the head and neck squamous cell carcinoma, HNX-22B, to 3.9 ng/mg protein for the ovarian tumor, A2780. dCK protein levels and activity were significantly related ( $r = 0.945$ ;  $P < 0.001$ ) as determined by exponential regression. Furthermore, dCK protein levels were also related to the gemcitabine sensitivity of the tumor panel ( $\rho = -0.96$ ;  $P < 0.001$ ).

dCK mRNA expression was measured with a RT-PCR in the human tumor xenografts using competitive templates as internal standard for both dCK and the housekeeping gene  $\beta$ -actin (Fig. 4). dCK mRNA was measured in all of the human tumor xenografts except for H460 and HNX-22B from which mRNA could not be isolated at good quality in sufficient amounts. Per tumor, the intra-assay variation had a median relative SD of 11% and was always  $<27\%$ . The mRNA levels (mean  $\pm$  SE) showed a 2.2-fold variation among the various xenografts, ranging from 4.9  $\pm$  0.6 to 11.0  $\pm$  1.7 (ratio  $10^{-3} \times$  dCK/ $\beta$ -actin). dCK mRNA was significantly related to dCK activity ( $r = 0.88$ ;  $P = 0.025$ ; Fig. 5) and protein levels ( $\rho = 0.80$ ;  $P = 0.052$ ); however the relation was not significant

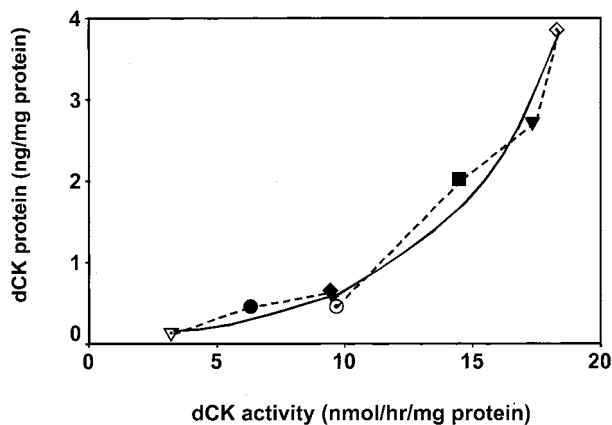


Fig. 3. Relation between dCK protein levels (ng/mg total protein) and dCK activity (nmol/h/mg total protein) in various human tumor xenografts (exponential regression curve fit:  $r = 0.964$ ;  $P < 0.001$ ). Values are the mean of three separate tumors and experiments; bars,  $\pm$  SE.

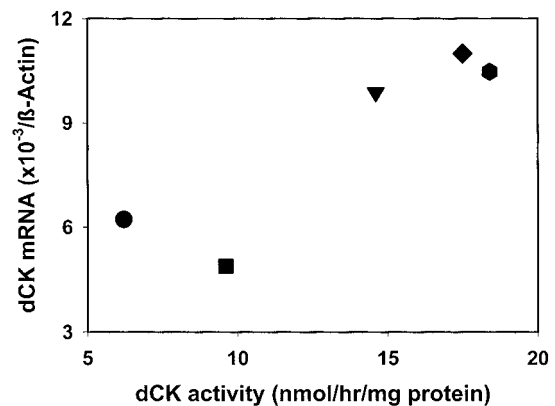


Fig. 5. Relationship between dCK activity (nmol/h/mg protein) and dCK mRNA level ( $\times 10^{-3} \times \text{dCK}/\beta\text{-actin}$ ;  $r = 0.88$ ;  $P = 0.025$ ) in the following human tumor xenografts: BxPC3, (●); WiDr (■); Panc-01 (▲); Calu-6 (◆), A2780 (●). Values are mean  $\pm$  SE of three different tumors, whereas the dCK mRNA levels were determined minimally three times on the same cDNA.

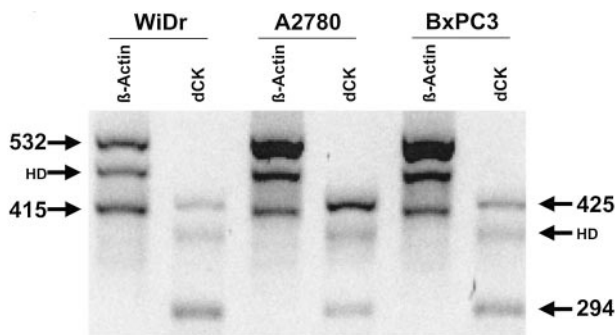


Fig. 4. Expression of dCK mRNA in three human xenografts. The blot shows three bands for both dCK and  $\beta$ -actin; 425 and 532 are the number of bp encoded by the forward and reverse primers for the native cDNA of dCK and  $\beta$ -actin, respectively; 294 and 415 are the number of bp encoded by the competitive templates for dCK and  $\beta$ -actin, respectively. HD represent a heterodimer, which can be formed between the native cDNA and the competitive template. The contribution of the HD was calculated as described previously (34, 35). The bands were scanned and the optical density was used to calculate a ratio between the native cDNA and competitive template of each target; thereafter these ratios were used to calculate the ratio for dCK: $\beta$ -actin.

with the sensitivity of the xenograft panel to gemcitabine ( $\rho = -0.60$ ;  $P = 0.142$ ).

## Discussion

Pretreatment dCK activity in various solid tumors grown in mice was clearly related to the gemcitabine sensitivity, indicating that dCK levels might predict response to gemcitabine. Our study is the first describing this direct relationship between dCK levels and gemcitabine sensitivity in nonselected tumors. Previously, we did not observe this relation (28, 30), most likely because dCK was measured with deoxycytidine as a substrate. It has now been recognized that deoxycytidine can also be activated by TK2. Several of the studied tumors contained high and variable amounts of TK2. One of the advantages of the use of CdA as a substrate is that it bypasses the TK2 effect, whereas it cannot be deami-

nated in contrast to deoxycytidine (36, 37). CdA can also be phosphorylated by deoxyguanosine kinase. However, this effect is believed to be  $<10\%$  (37). The latter can be supported by our observed relation between dCK protein expression and activity.

Various leukemias are treated with deoxynucleoside analogues, which are activated by dCK, such as ara-C and CdA. For these deoxynucleosides dCK activity has been associated with their antitumor activity (38–40). In addition, cells resistant to deoxynucleoside analogues frequently showed a dCK deficiency (6–11). Furthermore, transfection and induction of the dCK gene resulted in sensitization of both leukemic (12, 13) and solid tumor cells (14, 41) to deoxynucleoside analogues. Interestingly, the solid tumor cells, which could be sensitized *in vitro* by transfection of the dCK gene (41), also showed increased sensitivity to gemcitabine *in vivo* (42). These data underline the importance of dCK activity in the sensitivity of gemcitabine. The relation between endogenous dCK levels and gemcitabine activity in solid tumors has not been described previously and is in agreement with the findings with other deoxycytidine analogues in leukemia, dCK-deficient cells, and dCK-transfected cells.

Several other cellular mechanisms potentially contribute to gemcitabine sensitivity. dFdCTP accumulation has been related to *in vitro* sensitivity to gemcitabine (24). The efficiency of dCK phosphorylation most likely represents an indication of dFdCTP accumulation and, consequently, sensitivity to gemcitabine. However, dFdCTP cannot be measured as a pretreatment factor. CDA levels were not of prognostic value, corresponding to previous studies using deoxycytidine as a substrate (28, 30). In addition, the ratio of dCK:CDA activity did not correlate with gemcitabine sensitivity. The relative large variation of CDA both between and within different tumor types, as noted before (28), might be an explanation why CDA levels are not prognostic.

Measurement of dCK gene expression or protein levels seems attractive to use small tissue samples and no radioactive substrates as compared with the dCK activity assay.



A highly significant positive relationship was observed between dCK protein and activity levels. This is in accordance with results obtained in leukemic cells (43), and a combination of leukemic and solid tumor cells (31). Furthermore, dCK protein levels correlated with the gemcitabine sensitivity, and might, therefore, be used as a prognostic marker for sensitivity to gemcitabine. We are currently evaluating dCK levels in biopsy specimens from human tumors with a different histological origin. These types of tumors (e.g., lung, colon, glioma, and pancreas) have been reported to be sensitive or resistant to gemcitabine treatment; preliminary data show that a major part of the tumors have dCK levels in the range of the sensitive tumors. The CT-RT-PCR is a sensitive method to assess dCK gene expression (34, 35) even in small tumor samples and only requires the use of any routinely available PCR machine. Previously, a relationship between dCK activity and dCK mRNA has been shown in leukemic cells (44) as well as in a panel of solid tumor, leukemia, and lymphoma cell lines (14). In the various solid tumors dCK mRNA was also related to dCK activity and to dCK protein levels, and tended to be related the gemcitabine sensitivity. The latter might be explained by a post-translational processing of dCK. Furthermore, the number of xenografts that were evaluable in the current study might be too small to detect a significant relation at the mRNA level.

In conclusion, dCK activity and protein levels were clearly related to gemcitabine sensitivity in a panel of one murine tumor and human tumor xenografts of different origin, whereas mRNA levels were related to dCK activity and protein levels. Whether patients with increased dCK levels in the tumor have a better response to gemcitabine is currently under investigation.

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