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Intracellular cytidine deaminase regulates gemcitabine metabolism in pancreatic cancer cell lines

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Cytidine deaminase and gemcitabine metabolism

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Abstract

Cytidine deaminase (CDA) is a determinant of *in vivo* gemcitabine elimination kinetics and cellular toxicity. The impact of CDA activity in pancreatic ductal adenocarcinoma (PDAC) cell lines has not been elucidated. We hypothesized that CDA regulates gemcitabine flux through its inactivation and activation pathways in PDAC cell lines.

Three PDAC cell lines (BxPC-3, MIA PaCa-2 and PANC-1) were incubated with 10 or 100 µM gemcitabine for 60 minutes or 24 hours, with or without tetrahydrouridine (THU), a CDA inhibitor. Extracellular inactive gemcitabine metabolite (dFdU) and intracellular active metabolite (dFdCTP) were quantified with liquid chromatography tandem mass spectrometry. Cellular expression of CDA was assessed with real-time PCR and Western blot.

Gemcitabine conversion to dFdU was extensive in BxPC-3 and low in MIA PaCa-2 and PANC-1, in accordance with their respective CDA expression levels. CDA inhibition was associated with low or undetectable dFdU in all three cell lines. After 24 hours gemcitabine incubation, dFdCTP was highest in MIA PaCa-2 and lowest in BxPC-3. CDA inhibition resulted in a profound dFdCTP increase in BxPC-3, but not in MIA PaCa-2 or PANC-1. dFdCTP concentrations were not higher after exposure to 100 vs. 10 µM gemcitabine when CDA-activities were low (MIA PaCa-2 and PANC-1) or inhibited (BxPC-3).

The results suggest a regulatory role of CDA for gemcitabine activation in PDAC cells, but within limits related to the capacity in the activation pathway in the cell lines.

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Significance statement

The importance of cytidine deaminase (CDA) for cellular gemcitabine toxicity, linking a lower activity to higher toxicity, is well described. An underlying assumption is that CDA, by inactivating gemcitabine, limits the amount available for the intracellular activation pathway. Our study is the first to illustrate this regulatory role of CDA in pancreatic ductal adenocarcinoma cell lines by quantifying intracellular and extracellular gemcitabine metabolite concentrations.

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Introduction

Gemcitabine (2',2'-difluoro-2'-deoxycytidine, dFdC) is a nucleoside analogue used either alone or in combination with other cytostatic agents for treatment of inoperable pancreatic ductal adenocarcinoma (PDAC), and several other human cancers (Norwegian Medicines Agency, 2018). Following intracellular uptake, mainly by transmembrane equilibrative (hENT) and concentrative nucleoside transporter proteins (Wong et al., 2009), gemcitabine undergoes a stepwise phosphorylation process. Deoxycytidine kinase (dCK) catalyses the initial phosphorylation to gemcitabine monophosphate (dFdCMP), and is considered to be the rate limiting step in the activation pathway (Wong et al., 2009). The main active metabolite is gemcitabine triphosphate (dFdCTP), which inhibits DNA-replication. In tumor specimens from PDAC patients, high expression of hENT1 and dCK have been shown to favour the outcome of gemcitabine treatment (Marechal et al., 2012).

Cytidine deaminase (CDA) catalyses the inactivation of gemcitabine to 2',2'-difluoro-2'-deoxyuridine (dFdU) (Gusella et al., 2011; Simon et al., 2015; Cohen et al., 2018). CDA expression and activity in peripheral blood (Bowen et al., 2009) have been attributed both to lack of effect and increased toxicity of gemcitabine (Sugiyama et al., 2007; Ciccolini et al., 2010; Gusella et al., 2011). In PDAC tumor tissue, it has been found that CDA mRNA expression is higher compared to healthy tissues (Mameri et al., 2017). Bacteria and cells such as macrophages in the tumor microenvironment that express CDA might contribute to gemcitabine resistance (Vande Voorde et al., 2014; Weizman et al., 2014; Geller et al., 2017; Hessmann et al., 2018). However, the impact of intracellular CDA on gemcitabine metabolism in cancer cells is less studied (Morita et al., 2003; Vande Voorde et al., 2014).

Mameri and co-workers (Mameri et al., 2017) restored the expression of CDA in two *a priori* CDA-deficient cancer cell lines, and showed that survival of these cells was higher than that of their CDA-deficient counterparts following *in vitro* incubation with gemcitabine. Indeed, similar results have also been achieved by others, indicating a reciprocal relationship between intracellular CDA activity and cellular gemcitabine sensitivity (Morita et al., 2003;

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Giovannetti et al., 2007; Yoshida et al., 2010; Peters et al., 2019). Thus, intracellular conversion of gemcitabine to dFdU is likely to be a mechanism contributing to gemcitabine resistance in this setting (Bardenheuer et al., 2005; Giovannetti et al., 2007; Ohmine et al., 2012; Vande Voorde et al., 2014; Mameri et al., 2017; Tibaldi et al., 2018).

In this study, we hypothesized that CDA plays a regulatory role in intracellular gemcitabine activation in PDAC cells. To test the hypothesis we assessed intracellular and extracellular concentrations of gemcitabine and metabolites after exposure to gemcitabine with and without the use of the CDA inhibitor tetrahydrouridine (THU). We also determined basal mRNA and protein expression profiles of CDA and other main proteins involved in the transport and metabolism of gemcitabine.

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Materials and Methods

Chemicals, Reagents and Consumables

Unless otherwise stated, chemicals and reagents were purchased from Merck KGaA (Darmstadt, Germany) and were of analytical grade. Horse serum and sodium pyruvate were bought from Thermo Fisher Scientific (Oslo, Norway), culture flasks and cryotubes from VWR (Oslo, Norway), centrifuge tubes from Sarstedt (Oslo, Norway), and tetrahydrouridine (THU) from AH diagnostics (Oslo, Norway). All other reagents and equipment used for Liquid chromatography tandem mass spectrometry (LC-MS/MS) methods have been described previously (Bjanes et al., 2015; Kamceva et al., 2015).

Cell culture

Three human PDAC cell lines, BxPC-3, MIA PaCa-2 and PANC-1, authenticated and generously provided by Prof. Anders Molven (University of Bergen), were cultured in 75 cm^2 flasks in a humidified atmosphere with 5 % CO_2 at 37 °C, and sub-cultured twice weekly. BxPC-3 cells were cultured in Roswell Park Memorial Institute 1640 medium (RPMI). MIA PaCa-2 and PANC-1 were cultured in Dulbecco's Modified Eagles Medium D5671 (DMEM). All media were supplemented with 10 % fetal bovine serum (FBS), 4 mM sodium pyruvate and 2 mM L-glutamine. The medium used for MIA PaCa-2 was additionally supplied with horse serum (2.5 %), as recommended by the manufacturer. No antibiotics were used. Mycoplasma tests performed on a regular basis were negative.

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Gemcitabine incubation

Cell-free media (RPMI, DMEM, and DMEM with horse serum) were spiked with 10 or 100 µM gemcitabine. Resulting spiked medium samples were aliquoted and stored in 1.5 mL Eppendorf tubes at 4 °C, room temperature (RT) and 37 °C for up to seven days, and subsequently stored at -80°C until the entire batch was analysed concurrently. The concentration ratios of dFdU over the sum of gemcitabine and dFdU in each sample, dFdU/(gemcitabine+dFdU) (%), was used as an indicator of CDA activity.

PDAC cell lines (0.25 – 0.4 x 10^6 cells per well in 2 mL culture medium) were seeded in six-well plates 48 hours prior to gemcitabine incubation. Culture media was removed and replaced with freshly prepared drug-supplemented media at initiation of the experiments. The cells were incubated in quadruplicate for a) 24 hours with 10 or 100 µM gemcitabine, with or without 200 µM THU or b) 60 minutes with 10 or 100 µM gemcitabine with or without 200 µM THU. The two different durations of gemcitabine incubation were chosen based on a) that 24 hours is within a typical range applied in *in vitro* cytotoxicity experiments (Giovannetti et al., 2007; Yoshida et al., 2010; Mameri et al., 2017) and b) that 60 minutes *in vitro* incubation reflects a comparable exposure to *in vivo* gemcitabine treatment (Gusella et al., 2011). .

Following gemcitabine incubation, media was collected, transferred to cryotubes and stored at -80 °C until quantification of extracellular gemcitabine and dFdU. Wells were rinsed twice with PBS, and cells were subsequently trypsinized for five to eight minutes, harvested and gently re-suspended in cold culture medium. Manual cell counting was performed on a representative sample of the suspension. Cell suspensions were centrifuged for five minutes. Supernatant was discarded and the cell pellets were dissolved in cold 60 % methanol, transferred to cryotubes, vortexed for 20 seconds and snap frozen on liquid nitrogen. All samples were stored at -80 °C until quantification of intracellular dFdCTP.

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Gemcitabine and -metabolite quantification

Quantification of gemcitabine and its metabolites was performed using an Agilent 1200 series HPLC-system (Agilent Technologies, Waldbronn, Germany) for chromatographic separation and an Agilent 6410 triple-quad mass spectrometer for mass detection. Gemcitabine and dFdU in culture media samples were quantified as described previously (Bjanes et al., 2015), optimized with lower limits of quantitation of 0.1 µM for both gemcitabine and dFdU. Gemcitabine triphosphate (dFdCTP) was analysed in cell lysates with a modified version of our previously published method (Kamceva et al., 2015). Modification consisted in shorter analysis time and with the mass spectrometer operating in positive ionization mode, since we were only interested in quantification of dFdCTP and not in the endogenous nucleosides that eluted later. dCTP was used as internal standard due to its similar structure and retention time with dFdCTP. Concentrations above the lower limit of quantitation of 0.05 µM were normalized to the cell count in each sample and expressed as *pmol per* 10^6 *cells* (abbreviated to *pmol/*10^6 throughout the manuscript).

mRNA and protein expression

Extraction of mRNA was performed on cell pellets from each cell line, in quadruplicate, using the Qiagen column extraction kit. Two µg of mRNA was used for reverse transcription with M-MLV reverse transcriptase (InVitrogen). cDNA was diluted, and relative gene expression determined by PCR in a final volume of 6.67 µL with Takyon NoRox SYBR MasterMix blue dTTP (Eurogentec). Triplicate runs were performed on a Lightcycler (LC480, Roche Life Science). Relative quantification was performed by the ΔΔCT method using 28S mRNA expression as a housekeeping gene and mean CT values as reference. Primers used for each gene are given in Supplemental table 1A.

Total proteins were extracted using cold buffer (20 mM Tris-HCl pH 6.8, 1 mM $MgCl_2$, 2 mM EGTA, 0.5% NP40 and phosphatase inhibitor cocktails) with 60 minutes incubation on ice,

followed by centrifugation (15 minutes, 12 000 g, 4°C). Proteins were separated by SDS-PAGE and transferred onto PVDF membranes using the iBlot® system (Life Technologies). Membranes were incubated with specific antibodies, as shown in Supplemental table 1B. Protein expression was visualized using the Odyssey infrared system (LI-COR Biosciences). Protein bands were quantified using the Odyssey system, subtracting background noise from a similarly sized area just below the band, and presented as ratio of the expression of proteins of interest versus beta-actin expression.

Data processing and statistics

Quantitative data were analysed with SPSS Statistics 24.0 (IBM Inc., Armon, NY, USA) and GraphPad Prism 8 (San Diego, CA, USA) for Windows. Results were expressed as means ± standard deviations (SD) or as concentration ratios between analytes (%). A two-sided student's *t*-test was used to compare results in individual cell lines under different experimental conditions. One-way analysis of variance (ANOVA) with Bonferroni post hoc test was used to compare results in different cell lines. A *p*-value of less than 0.05 was considered significant.

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Results

CDA activity in cell-free culture media

We investigated whether cell-free culture media had any CDA activity, which would be of importance in the subsequent interpretation of data from cell lines incubated with gemcitabine. We found CDA activity only in DMEM supplemented with horse serum, used for culturing MIA PaCa-2 cells. Within the maximum duration of our cell experiments (24 hours), the highest dFdU/(gemcitabine+dFdU) ratio at both gemcitabine concentrations was 6.3 % at 37 °C (Supplemental Figure 1). No CDA activity was found in either RPMI or DMEM media without horse serum.

Accumulation of inactive gemcitabine metabolite in culture media

To quantify inactivation of gemcitabine in PDAC cells, we measured extracellular dFdU concentrations after incubation with 10 and 100 µM gemcitabine for 60 minutes or 24 hours, with or without inhibition of CDA. After 24 hours incubation of BxPC-3, MIA PaCa-2 and PANC-1 with 100 µM gemcitabine, mean dFdU concentrations were 86.3, 23.5 and 7.3 µM, respectively (Figure 1A). After 60 minutes incubation with 100 µM gemcitabine, the corresponding dFdU concentrations were 17.7, 3.7 and 0.2 µM (Supplemental Figure 2A). The percentage conversion of gemcitabine to dFdU was similar when cells had been incubated with 10 µM gemcitabine, both after 60 minutes and 24 hours. After co-incubation with gemcitabine and THU, dFdU was low or undetectable in medium from all three cell lines both after 60 minutes and 24 hours.

Intracellular accumulation of active gemcitabine metabolite

After 24 hours incubation of BxPC-3, MIA PaCa-2 and PANC-1 with 10 µM gemcitabine, mean dFdCTP concentrations were 210, 1466 and 955 pmol/10^6, respectively (Figure 1B).

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After 24 hours incubation with 100 µM gemcitabine, dFdCTP concentrations in BxPC-3 were significantly higher (851 pmol/10^6; $p<0.001$) than with 10 µM gemcitabine incubation. In MIA PaCa-2, dFdCTP concentrations were not significantly different between the two gemcitabine concentrations ($p = 0.12$), whereas in PANC-1 they were significantly lower at 100 µM gemcitabine (662 pmol/10^6; $p<0.05$). CDA-inhibition resulted in significantly higher dFdCTP concentrations in BxPC-3, with mean concentrations of 1370 ($p<0.01$) and 1368 pmol/10^6 ($p<0.05$) at 10 and 100 µM gemcitabine, respectively. In MIA PaCa-2 or PANC-1, dFdCTP concentrations were not significantly different with vs without CDA-inhibition.

After 60 minutes incubation with 10 µM gemcitabine, mean dFdCTP concentrations were 92, 80 and 110 pmol/10^6 in BxPC-3, MIA PaCa-2 and PANC-1, respectively. 60 minutes incubation with 100 µM gemcitabine did not result in significantly higher dFdCTP concentrations in any of the three cell line. Also, CDA-inhibition had no effect on dFdCTP concentrations at both gemcitabine concentrations under these experimental conditions (Supplemental Figure 2B).

Basal mRNA and protein expression

We assessed basal mRNA and protein expression of selected transporters and enzymes involved in gemcitabine uptake, metabolism and activity, in gemcitabine-untreated cell lines. Relative expression of mRNA and proteins are given in Figure 2A and Figure 2B, respectively. Original Western blots can be seen in Supplemental Figure 3. CDA showed highest mRNA and protein expression in BxPC-3. Lower CDA mRNA expression (Figure 2A) and zero protein expression (Figure 2B) was detected in both MIA PaCa-2 and PANC-1. The majority of the other transporters and enzymes revealed highest mRNA and protein expressions in PANC-1.

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Discussion

Our overall finding was that intracellular cytidine deaminase plays a regulatory role for gemcitabine activation in PDAC cells, hence confirming our hypothesis.

Gemcitabine inactivation

Almost all gemcitabine added to the culture medium was converted to dFdU during 24 hours gemcitabine incubation of BxPC-3, highlighting the extensive CDA activity in this cell line. A comparable extent of gemcitabine conversion was reported by Bowen and co-workers (Bowen et al., 2009) in *ex vivo* whole blood from healthy volunteers; 50 % after five hours incubation and close to 100 % after 24 hours. In accordance with other publications (Funamizu et al., 2012a; Funamizu et al., 2012b), we also found that CDA displayed the highest mRNA (Figure 2A) and protein expression (Figure 2B) in BxPC-3, compared to MIA PaCa-2 and PANC-1.

Based on the pre-experimental stability assessments in cell-free culture media, all dFdU in BxPC-3 experiments was a result of cellular uptake, intracellular conversion and subsequent efflux into the culture medium. In MIA PaCa-2 and PANC-1, respectively, the extent of gemcitabine conversion to dFdU was 20–30% and <10 % of BxPC-3 (Figure 1A). This indicated that CDA-activities were lower in MIA PaCa-2 and PANC-1. Gemcitabine was also to some extent converted to dFdU in the medium used for culturing MIA PaCa-2 (Supplemental Figure 1). However, the conversion in cell-free medium only accounted for 20-30 % of the total amount found after 24 hours gemcitabine incubation of MIA PaCa-2 cells (Figure 1A). The finding of no detectable CDA protein expression (Figure 2B) in MIA PaCa-2 and PANC-1 did not fit with the appearance of dFdU following 24 hours gemcitabine incubation. These inconsistencies could preferably be explained by lack of sensitivity in the protein expression assay (Supplemental Figure 3), since both cell lines expressed CDA mRNA (Figure 2A). Moreover, it has been suggested that transcriptional, posttranscriptional

(Mameri et al., 2017) and posttranslational (Frese et al., 2012) modulations could blur the relationship between mRNA and protein expression and the observed CDA phenotype.

In all cell lines, a long-lasting and strong inhibition of gemcitabine inactivation was achieved with 200 µM THU even at the highest gemcitabine concentrations, and at both incubation durations. This is in line with previous studies in human blood performed by our own group (Bjanes et al., 2015) and other researchers (Bowen et al., 2009). dFdU could otherwise be assumed to be derived from the deamination of dFdCMP (Wong et al., 2009), but THU is not known to inhibit gemcitabine inactivating enzymes other than CDA (Heinemann and Plunkett, 1989). The fact that co-incubation of the cell lines with THU inhibited the formation of dFdU effectively underscores that direct gemcitabine deamination through CDA was the main source of dFdU in our experiments.

Gemcitabine activation

Without CDA-inhibition, BxPC-3 accumulated significantly less dFdCTP over 24 hours compared to the two other cell lines (Figure 1B). A probable explanation, in line with previous theories (Riva et al., 1992; Bardenheuer et al., 2005), was that the supply into the activation pathway was limited due to extensive conversion of gemcitabine to dFdU (Figure 1A). This notion was supported by the observation that dFdCTP concentrations in BxPC-3 were significantly higher when gemcitabine exposure was increased, either by increasing gemcitabine concentrations from 10 to 100 µM (Figure 1B), or by inhibiting CDA (Figure 1B). No increase in dFdCTP concentrations was seen with increasing gemcitabine concentrations in MIA PaCa-2 or PANC-1, although baseline CDA-activities were low. The same was true in BxPC-3 when CDA was inhibited. These findings were consistent with saturation kinetics of dCK, as previously described by other authors (Grunewald et al., 1991; Wong et al., 2009).

Despite the distinct effects after 24 hours incubation in BxPC-3, CDA inhibition had no effect on dFdCTP concentrations in any of the three cell lines when incubated for 60 minutes

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(Supplemental Figure 2B). These findings could preferably be explained by sufficient concentrations of gemcitabine still available for the activation pathway in all three cell lines, but with dCK operating close to its saturation limit. This view is supported by the fact that the mean percentage gemcitabine remaining in the medium after 60 minutes vs. 24 hours incubation without THU, was 77 vs. <5% in BxPC-3, 92 vs. 66% in MIA PaCa-2 and >98 vs. 80% in PANC-1.

Overall perspective

Studies have highlighted the importance of CDA with respect to *in vivo* gemcitabine systemic pharmacokinetics (Sugiyama et al., 2007; Ciccolini et al., 2010; Gusella et al., 2011), and *in vitro* drug sensitivity (Yoshida et al., 2010; Funamizu et al., 2012b; Vande Voorde et al., 2014; Mameri et al., 2017), but the quantitative aspects of intracellular gemcitabine metabolism in PDAC cells has previously not been examined. We found that concentrations of both dFdU and dFdCTP after incubation with gemcitabine varied considerably between the PDAC cell lines, depending on CDA-activity. As all three cell lines in this study are frequently used in *in vitro* PDAC studies (Funamizu et al., 2010; Paproski et al., 2010; Funamizu et al., 2012a; Mariglia et al., 2018), the observed metabolic variability may be important to take into account when interpreting results from gemcitabine incubation experiments. Moreover, the quantitative contribution of intracellular CDA in gemcitabine metabolism may provide a mechanistic explanation by which manipulating CDA-activity could modify cellular gemcitabine sensitivity, as demonstrated by Mameri and co-workers (Mameri et al., 2017) and Bardenheuer and co-workers (Bardenheuer et al., 2005).

By incubating the cell lines with gemcitabine with and without THU, we demonstrated that an extensive CDA-mediated gemcitabine conversion to dFdU in BxPC-3 was associated with less accumulation of the active metabolite dFdCTP. This was evident after 24 hours incubation, but not after 60 minutes, indicating that a balanced substrate supply to dCK was an important factor for the accumulation of dFdCTP. In MIA PaCa-2 and PANC-1 no such

effect of CDA-inhibition on the gemcitabine activation pathway was seen, which was consistent with their *a priori* low CDA activities. This supports the idea that CDA activity may be a predictor for gemcitabine activation by regulating intracellular gemcitabine metabolism (Bardenheuer et al., 2005; Tibaldi et al., 2018). The observation that MIA PaCa-2 cells produced both more dFdU and dFdCTP than PANC-1 cells following 24 hours gemcitabine incubation, could be explained by the higher expression of 5'-nucleotidases in PANC-1 (Figure 2B), in particular cN-IIIA. Indeed, this enzyme has been suggested to dephosphorylate dFdCMP and thus oppose the accumulation of dFdCTP (Li et al., 2008; Aksoy et al., 2009). To decipher the exact mechanisms of these differences and the involvement of each of the other proteins shown in Figure 2A-B, it would be necessary to develop additional tools (protein-deficient cells, specific inhibitors etc.) that are outside the scope of this work.

Direct quantification of gemcitabine and its metabolites (Figure 1A-B), combined with CDA-inhibition, provided insight into differential CDA-activities that could not be revealed by expression-analyses alone (Figure 2A-B). In a recent commentary by Peters and co-workers (Peters et al., 2019), phenotyping with cytidine or gemcitabine was also recommended over genotyping for pre-treatment assessment of *in vivo* CDA-activity in patients. Hodge and co-workers (Hodge et al., 2011a; Hodge et al., 2011b) also demonstrated the value of applying different drug concentrations and duration of incubations, combined with enzyme-inhibition, when studying cellular regulation of gemcitabine transport (Hodge et al., 2011b) and metabolic (Hodge et al., 2011a) pathways.

In our experiments, we measured the free dFdCTP concentrations, and did not have a measure of the total intracellular amount comprising both free and DNA-bound gemcitabine that might correlate better with cytotoxicity (Gandhi et al., 1991). Indeed, using the AnnexinV -PI assay, no additional effect of CDA-inhibition was observed in any of the cell lines (Supplemental Figure 4), underscoring that free dFdCTP is not the only determinant of gemcitabine efficacy. The ratio between free and total dFdCTP is expected to change over

time during and after gemcitabine incubation, and cell lines might also behave differently based on intracellular enzyme expressions, illustrated by our own results in Figure 2A and Figure 2B. Based on *in silico* simulations, Battaglia and co-workers suggested that the rate of DNA-incorporation in general is a slow process compared to the production rate of dFdCTP (Battaglia and Parker, 2011). Hence, quantification of free dFdCTP could therefore be a better measure of cellular uptake and metabolism of gemcitabine following 60 minutes incubation, compared to 24 hours incubation. Incubation for 60 minutes with 10 – 100 µM gemcitabine *in vitro* might also more accurately represent the *in vivo* drug exposure during and after clinically applied 30-minutes gemcitabine infusions of 1000 mg/m^2, with a comparable concentration-time-product (AUC) of 41 ± 12 µM*h (Gusella et al., 2011). We calculated that 60 minutes or 24 hours *in vitro* incubation with 10 µM gemcitabine render AUCs of 10 or 240 µM*h, respectively.

In general, data from *in vitro* experiments should be interpreted with caution in terms of *in vivo* relevance. However, our findings that increased gemcitabine exposure does not necessarily lead to an increase in the intracellular active metabolite concentrations are in line with observations from *in vivo* studies, as illustrated by Hessmann and co-workers (Hessmann et al., 2018).

Conclusion

Our findings reveal quantitative aspects of gemcitabine intracellular metabolism in PDAC cell lines. The data support the notion that high CDA-activity limits intracellular dFdCTP accumulation. However, low CDA activity may not necessarily result in increased dFdCTP accumulation and decreased cell viability. Both CDA activity and the cellular ability to synthesize active metabolites should be taken into consideration in future studies of gemcitabine delivery to pancreatic cancer cells.

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Authorship contributions

Participated in research design: Bjånes, Jordheim, Schjøtt and Riedel

Conducted experiments: Bjånes, Kamceva and Cros-Perrial

Contributed new reagents or analytic tools: Bjånes, Jordheim, Schjøtt, McCormack

Performed data analysis: Bjånes, Jordheim, Schjøtt, Cros-Perrial, Langer, Garibay

Wrote or contributed to the writing of the manuscript: Bjånes, Jordheim, Schjøtt, Kamceva, Cros-Perrial, Langer, Garibay, Kotopoulis, McCormack, Riedel

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Footnotes

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Legends for figures

Figure 1. Concentrations of gemcitabine metabolites following 24 hours incubation with 10 or 100 µM gemcitabine ± 200 µM tetrahydrouridine (THU), a cytidine deaminase inhibitor. **A and B** show extracellular dFdU***** (µM) and intracellular dFdCTP (pmol/10^6), respectively. **Insert** in Figure 1A: Data from 10 µM gemcitabine incubations in greater detail, with a differently scaled Y-axis. Data are displayed as means (n = 4 – 8). Error bars excluded from view for clarity. Original data are shown in **Supplemental Table 2**.

*****dFdCTP concentrations in PANC-1 incubated with 10 µM gemcitabine with or without THU are overlapping, and therefore appear as a single symbol.

Figure 2A. Relative mRNA expression of selected proteins involved in the transport and metabolism of gemcitabine in BxPC-3, MIA PaCa-2 and PANC-1. Cytidine deaminase highlighted (red rectangle). Data are displayed as means of 4 independent samples studied in triplicate, and error bars are standard deviations.

SLC28A1*****: Concentrative nucleoside transporter 1 (hCNT1); SLC29A1: Equlibrative nucleoside transporter 1 (hENT1); SLC29A2: Equlibrative nucleoside transporter 2 (hENT2); dCK: deoxycytidine kinase; CMPK1: uridine/cytosine monophosphate kinase; NME2: nucleoside diphosphate kinase (NdPK); CDA: Cytidine deaminase; dCTD: deoxycytidine monophosphate deaminase; NT5C: cytosolic 5'(3')-deoxyribonucleotidase (cdN); NT5C2: cytosolic 5'-nucleotidase II (cN-II); NT5C3: cytosolic 5'-nucleotidase III A (cN-IIIA); NT5M: mitochondrial 5'(3')-deoxyribonucleotidase (mdN); RRM1: Large subunit of ribonucleotide reductase; RRM2: Small subunit of ribonucleotide reductase; DCTPP1: deoxycytidine triphosphate pyrophosphatase 1; CTPS1*****: cytidine triphosphate synthase 1; POLA1: deoxyribonucleic acid polymerase alpha

*mRNA expression of SLC28A1 not detectable.

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Figure 2B. Relative protein expression of selected proteins involved in the transport* and metabolism of gemcitabine in BxPC-3, MIA PaCa-2 and PANC-1. Cytidine deaminase highlighted (red rectangle). Data are displayed as means of 3 independent samples, and error bars are standard deviations. Raw data are available in Supplemental Figure 3.

dCK: deoxycytidine kinase; CDA: Cytidine deaminase; cN-II: cytosolic 5'-nucleotidase II (NT5C2); cN-IIIA: cytosolic 5'-nucleotidase III A (NT5C3); RRM1: Large subunit of ribonucleotide reductase; RRM2: Small subunit of ribonucleotide reductase

*Antibodies against transporter proteins (hCNT and hENT) not available

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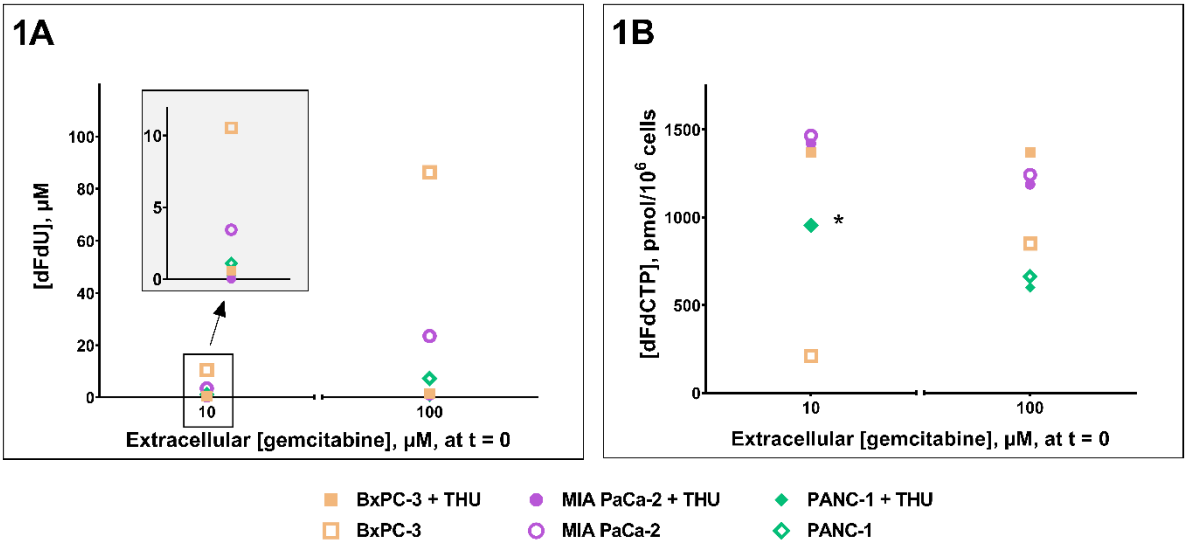
Figures



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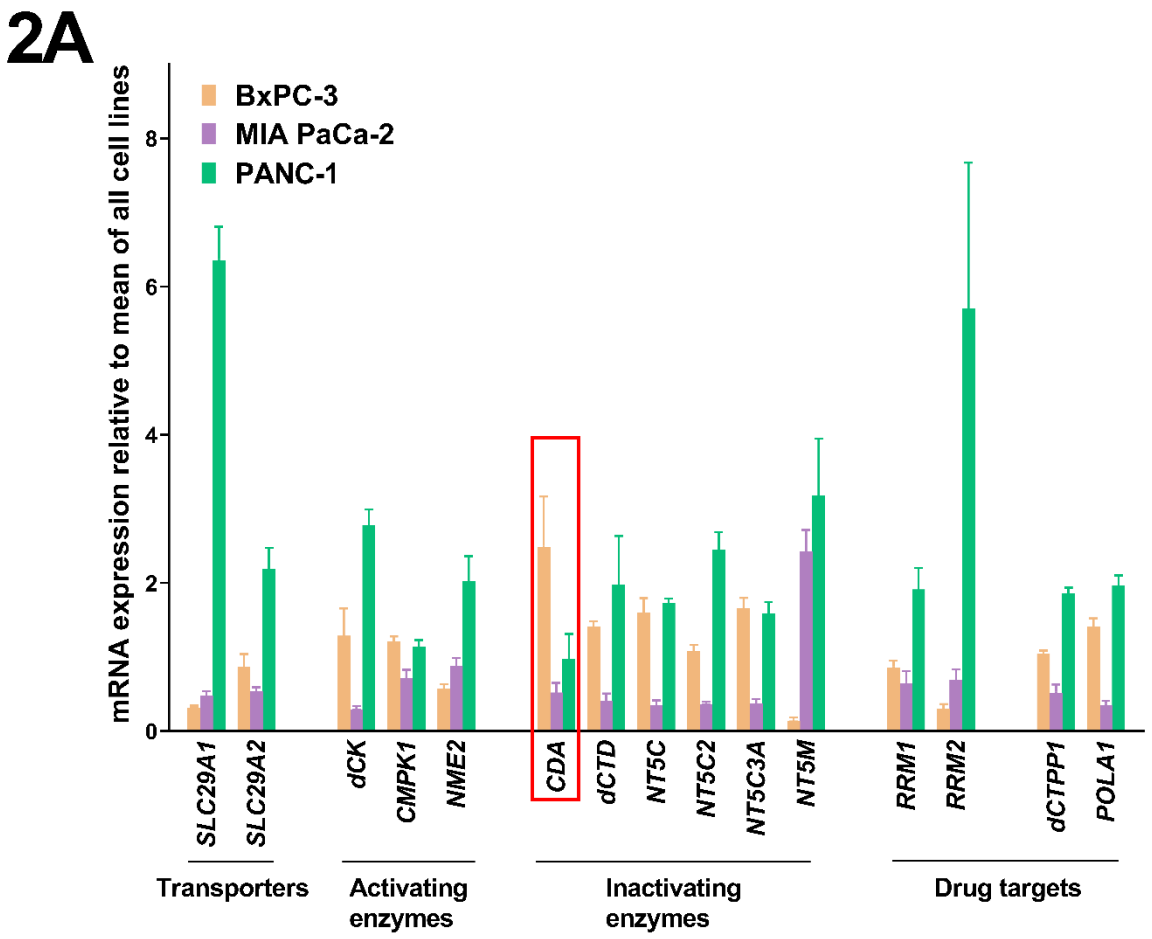

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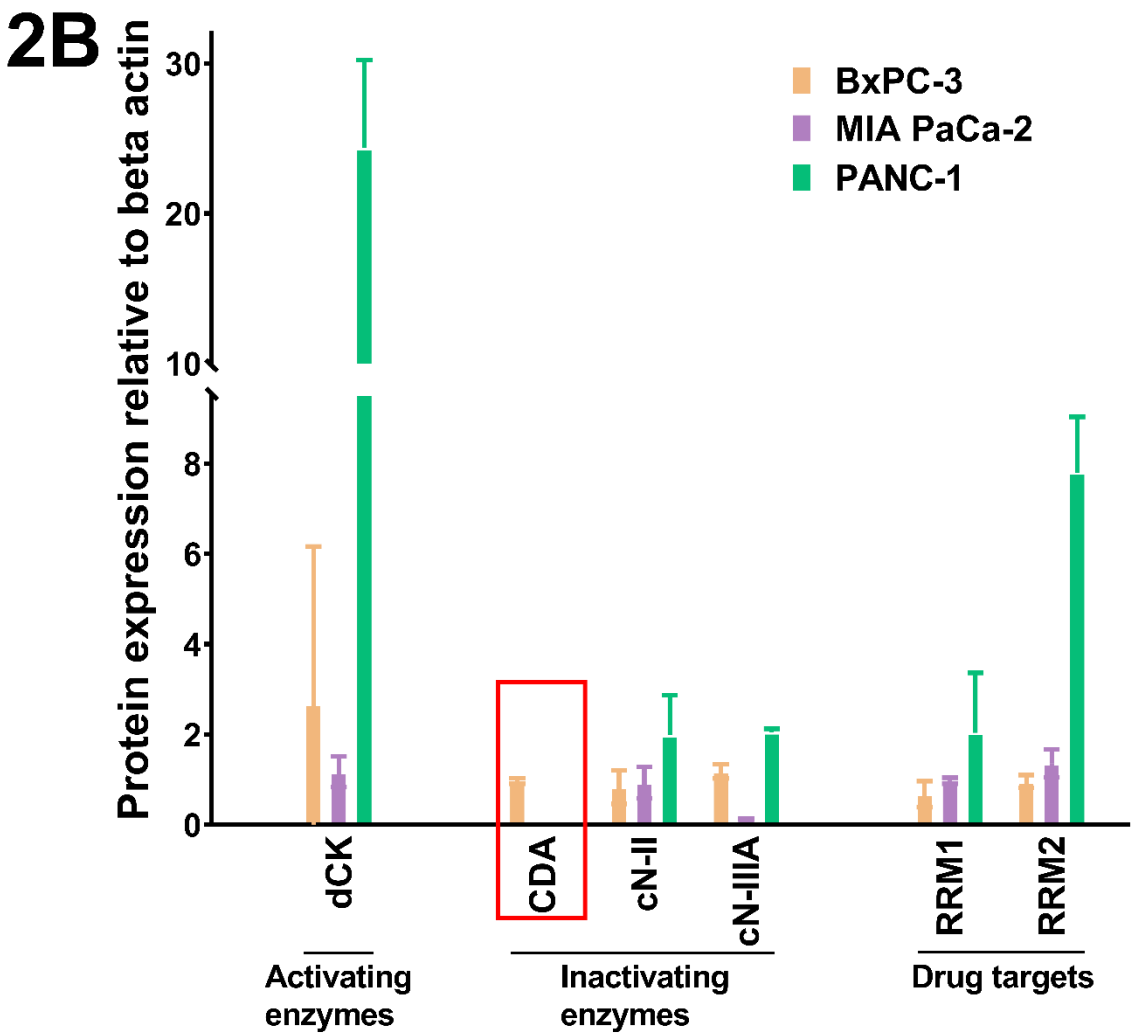

Intracellular cytidine deaminase regulates gemcitabine metabolism in pancreatic cancer cell lines

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*Joint senior authorship

Supplemental table 1A. Primer sequences for RT-qPCR.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
RPS28	CGATCCATCATCCGCAATG	AGCCAAGCTCAGCGCAAC
SLC28A1	TCTGTGGATTTGCCAATTTCAG	CGGAGCACTATCTGGGAGAAGT
SLC29A1	GCTGGGTCTGACCGTCGTAT	CGTTACAGGGTGCATGATGG
SLC29A2	ATGAGAACGGGATTCCCAGTAG	GCTCTGATTCCGGCTCCTT
DCK	AAACCTGAACGATGGTCTTTTACC	CTTTGAGCTTGCCATTCAGAGA
CMPK1	GGGCATATTCTTTGCTTCCA	TGCATTTCAAGGTTCCACTG
NME2	ATGCAGTGCGGCCTGGTGGG	GACCCAGTCATGAGCACAAGAC
CDA	GAGCTGCAATCGTGTCTGG	CAGAGCAGCGGGAAACAG
DCTD	GTCGCCTTGTTCCCTTGTAA	TCTTGCTGCACTTCGGTATG
NT5C	GGACACGCAGGTCTTCATCTG	GCGGTACTTCTCACCCACACA
NT5C2	ACCTGCTGTATTACCCTTTCAGCTA	GCTCCACCGTTGATTCATGA
NT5C3A	AATCGGCGATGTACTAGAG	CATCTGCCATTCTTAAGTCTC
NT5M	CATCAGCATTTGGGAGTCAA	CGACACAATCTGCTCCAGAA
DCTPP1	AAATGGACATCAACCGGCGA	AGTCACAGGGAATGTCCGCA
CTPS1	GTGGCGAAATACACCGAGTT	TCCTCGAACACCAAATCCTC
POLA1	AGCTTGACCTGATTGCTGTC	ATGACGGGACAAAGACAAGG
RRM1	GCAGCTGAGAGAGGTGCTTT	CAGGATCCACACATCAGACA
RRM2	GAGTTCCTCACTGAGGCC	TTAGAAGTCAGCATCCAAG

RPS28; Ribosomal protein S 28; SLC28A1*****: Concentrative nucleoside transporter 1 (hCNT1); SLC29A1: Equlibrative nucleoside transporter 1 (hENT1); SLC29A2: Equlibrative nucleoside transporter 2 (hENT2); dCK: deoxycytidine kinase; CMPK1: uridine/cytosine monophosphate kinase; NME2: nucleoside diphosphate kinase (NdPK); CDA: Cytidine deaminase; dCTD: deoxycytidine monophosphate deaminase; NT5C: cytosolic 5'(3')-deoxyribonucleotidase (cdN); NT5C2: cytosolic 5'-nucleotidase II (cN-II); NT5C3: cytosolic 5'-nucleotidase III A (cN-IIIA); NT5M: mitochondrial 5'(3')-deoxyribonucleotidase (mdN); RRM1: Large subunit of ribonucleotide reductase; RRM2: Small subunit of ribonucleotide reductase; DCTPP1: deoxycytidine triphosphate pyrophosphatase 1; CTPS1*****: cytidine triphosphate synthase 1; POLA1: deoxyribonucleic acid polymerase alpha.

Supplemental table 1B. **Antibodies used for Western blot.**

Protein/target*	Clone / Reference	Dilution	Primary / Secondary	Host organism	Supplier
CDA	-/ab56053	1/500	Primary	Rabbit	Abcam
cN-II	3C1/H00022978-M02	1/500	Primary	Mouse	Abnova
cN-IIIA	-/ARP32185	1/1000	Primary	Rabbit	Aviva Systems Biology
dCK	-/ab96599	1/2000	Primary	Rabbit	Abcam
RRM1	-/sc11733	1/1000	Primary	Goat	Santa Cruz Biotechnology
RRM2	-/sc10846	1/1000	Primary	Goat	Santa Cruz Biotechnology
Beta-actin	AC-15/A5441	1/5000	Primary	Mouse	Sigma
Anti-murine	-/926-32210	1/5000	Secondary	Goat	LI-COR Bioscience
Anti-rabbit	-/926-68171	1/5000	Secondary	Goat	LI-COR Bioscience
Anti-goat	-/926-32214	1/5000	Secondary	Donkey	LI-COR Bioscience

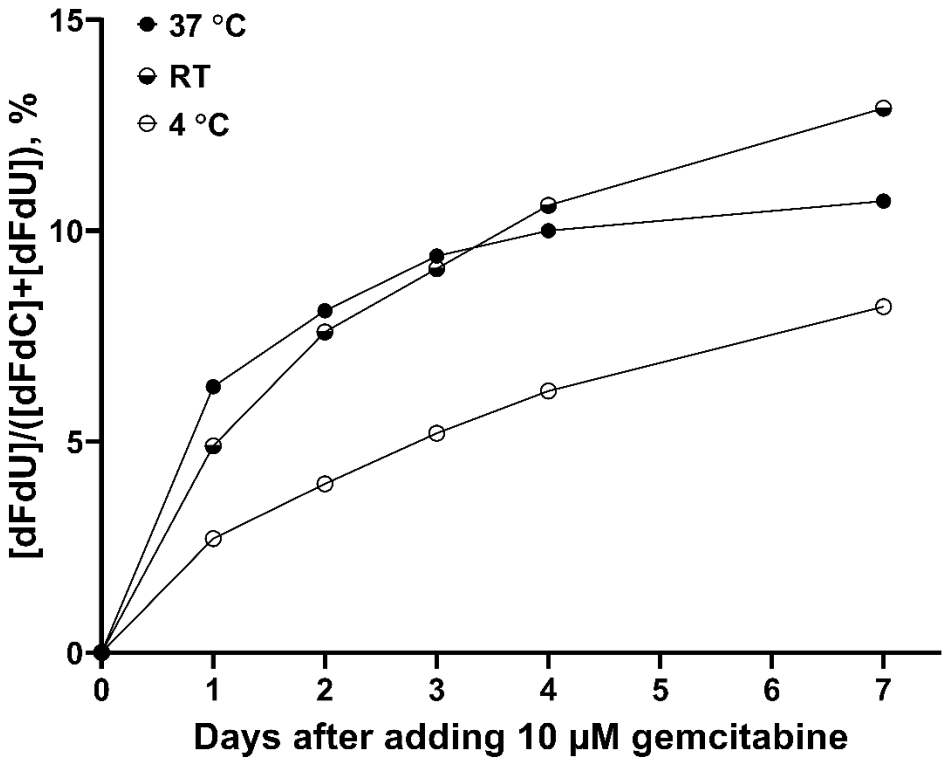
CDA: Cytidine deaminase; cN-II: cytosolic 5'-nucleotidase II (NT5C2); cN-IIIA: cytosolic 5'-nucleotidase III A (NT5C3); dCK: deoxycytidine kinase; RRM1: Large subunit of ribonucleotide reductase; RRM2: Small subunit of ribonucleotide reductase

*Antibodies against transporter proteins (hCNT and hENT) not available

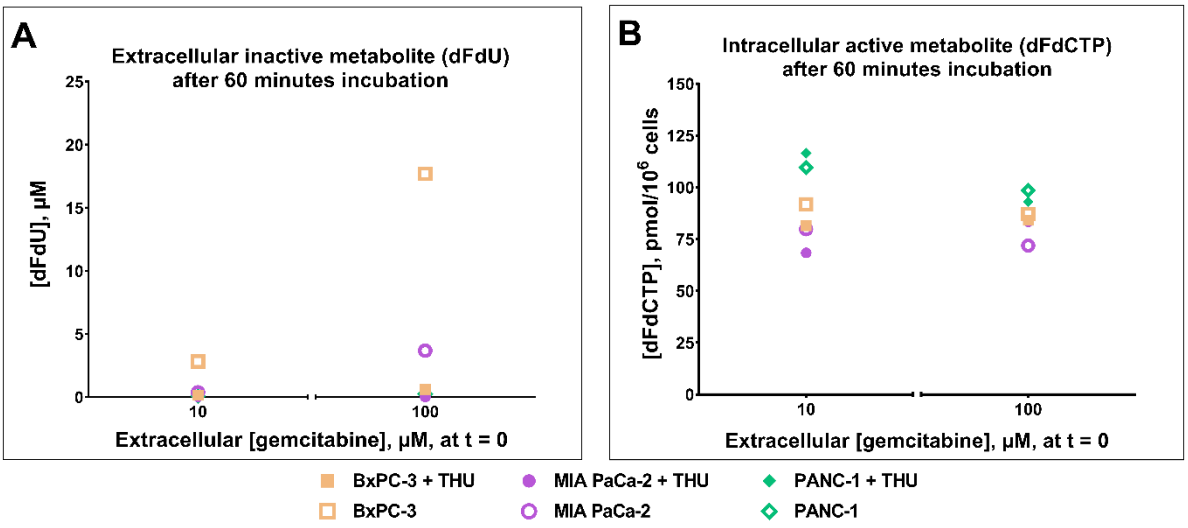
Supplemental table 2. Extracellular dFdU and intracellular dFdCTP concentrations following 24 hours gemcitabine (10 or 100 µM) incubation with or without 200 µM tetrahydrouridine. Data displayed in Figure 1A and 1B.

Cell line [Gemcitabine] ± 200 µM THU	Extracellular [dFdU], µM		Intracellular [dFdCTP], pmol/10^6	
	Mean	SD	Mean	SD
BxPC-3				
10 µM	10.5*	1.1	209.6	29.5
10 µM + THU	0.6*	0.05	1370.0	182.4
100 µM	86.3	4.1	850.5	127.1
100 µM + THU	1.5	0.02	1368.5	200.5
MIA PaCa-2				
10 µM	3.4*	0.8	1465.5	247.6
10 µM + THU	n.d.	n.d.	1420.4	95.7
100 µM	23.5*	7.1	1242.2*	197.0
100 µM + THU	0.8	0.2	1187.7*	203.6
PANC-1				
10 µM	1.1	0.0	954.7	224.7
10 µM + THU	0.2	0.0	950.9	66.8
100 µM	7.3	0.7	662.5	77.2
100 µM + THU	0.2	0.05	600.7	77.4

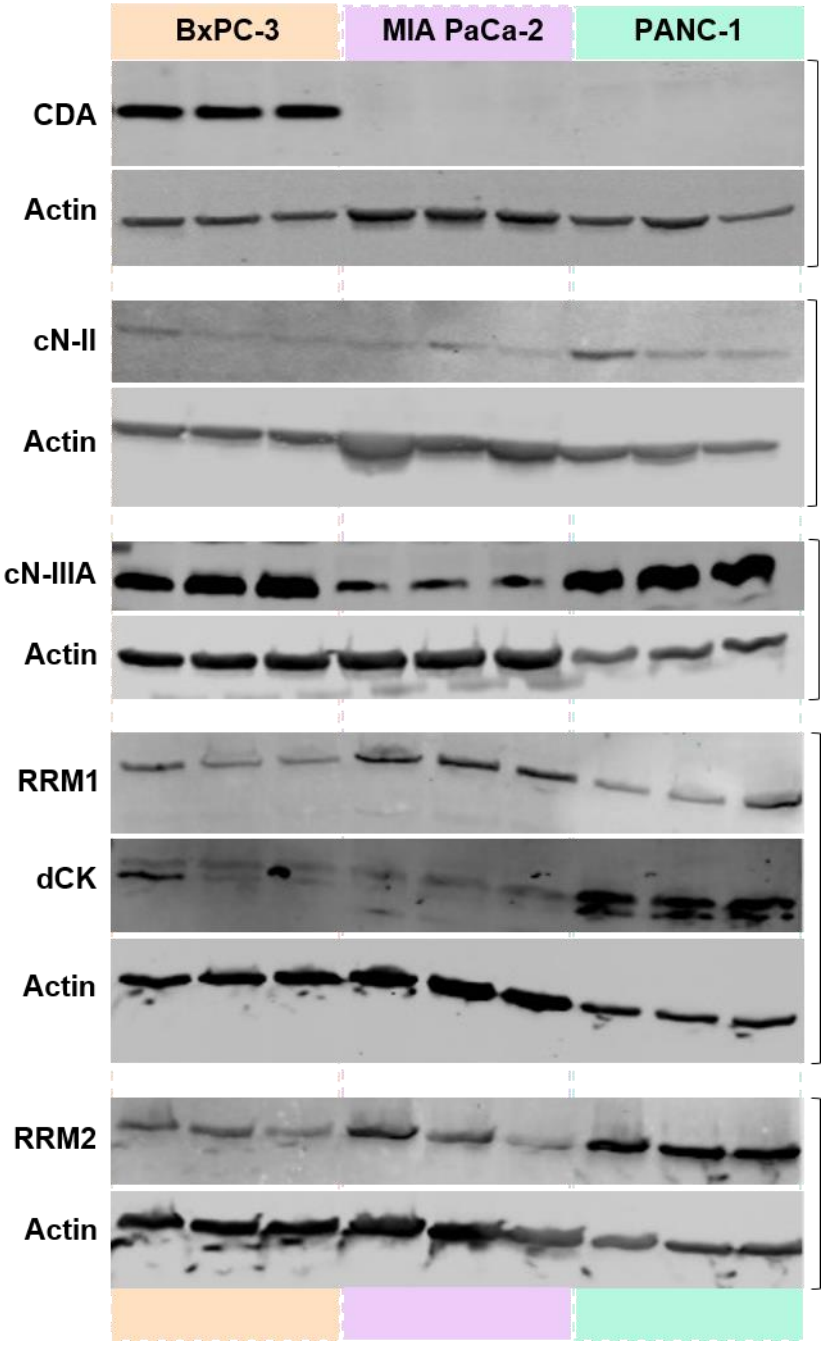
n = 4-8 per experimental condition; * experiments with n=8; n.d.: not detectable;
dFdCTP: 2',2'-difluoro-2'-deoxycytidine-5'-triphosphate; dFdU: 2',2'-difluoro-2'-deoxyuridine;
THU: tetrahydrouridine



Supplemental Figure 1. Stability of 10 µM gemcitabine (dFdC) in Dulbecco's modified Eagles medium with horse serum at 4 °C, room temperature (RT) and 37 °C. dFdU concentrations relative to the sum of dFdC and dFdU concentrations ([dFdU]/([dFdC]+[dFdU]), %), was used as a measure of CDA-activity. No CDA activity was found in either of the two other culture media; RPMI and DMEM.



Supplemental Figure 2. Concentrations of gemcitabine metabolites following 60 minutes incubation with 10 or 100 µM gemcitabine ± 200 µM tetrahydrouridine (THU), a cytidine deaminase inhibitor. **A and B** show extracellular dFdU* (µM) and intracellular dFdCTP (pmol/10^6), respectively. Data are displayed as means (n = 4 – 8). Error bars excluded from view for clarity.

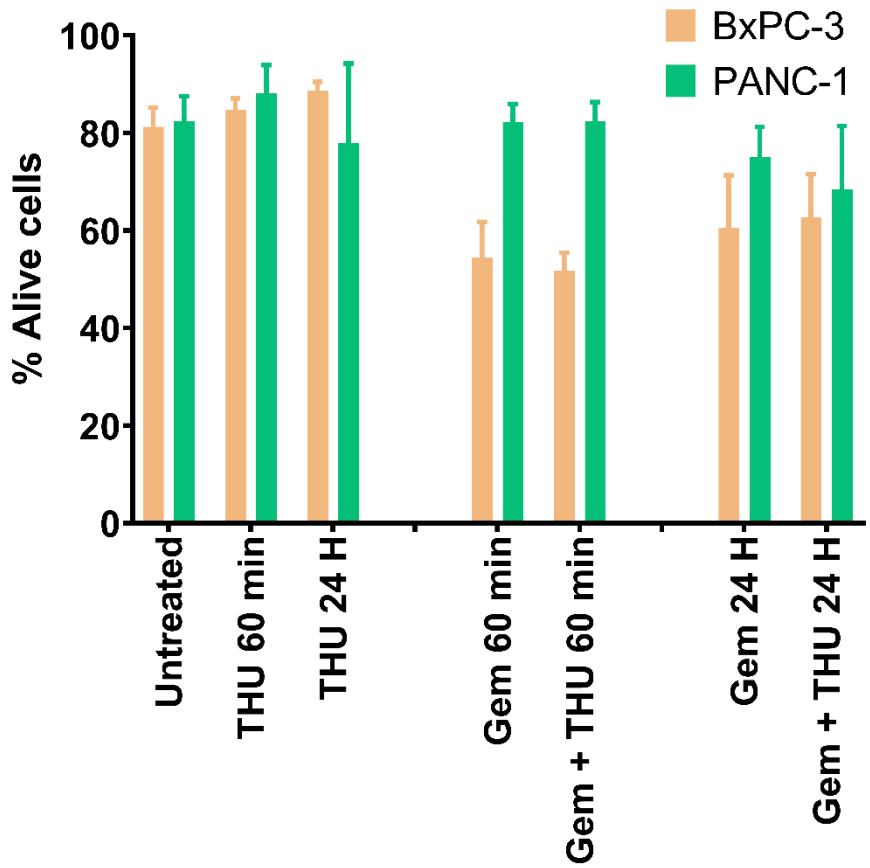


Supplemental Figure 3. Western blots of protein expression of selected proteins involved in the transport* and metabolism of gemcitabine in BxPC-3, MIA PaCa-2 and PANC-1.

Brackets indicate the individual analytical runs, with each beta-actin control included.

CDA: Cytidine deaminase; cN-II: cytosolic 5'-nucleotidase II (NT5C2); cN-IIIA: cytosolic 5'-nucleotidase IIIA (NT5C3); dCK: deoxycytidine kinase; RRM1: Large subunit of ribonucleotide reductase; RRM2: Small subunit of ribonucleotide reductase

*Antibodies against transporter proteins (hCNT and hENT) not available



Supplemental Figure 4. Viability of BxPC-3 (high CDA expression) and PANC-1 (low CDA expression) following 60 minutes or 24 hours incubation with 10 µM gemcitabine and/or 200 µM tetrahydrouridine. THU: Tetrahydrouridine 200 µM; "Gem": Gemcitabine 10 µM

Cells (100,000 per well) were seeded in 24-well plates in 1 mL complete media and left to attach before adding the various compounds. Cells were incubated with 10 µM gemcitabine and/or 200 µM THU for 60 minutes or 24 hours. Media was changed and cells were incubated for another 47 or 24 hours (total incubation 48 hours). Unexposed cells were used as controls. After harvesting by trypsinisation and centrifugation, cells were labelled with AnnexinV-Fluos Staining kit (Roche) and AnnexinV and PI staining of cells was determined on flow cytometry (Fortessa, BD Biosciences) as indicated in manufactures' instructions, and percentage alive cells were used as a measure of drug efficacy. Graph show mean values of four independent experiments performed in duplicate, and error bars are standard deviations.