Intracellular Cytidine Deaminase Regulates Gemcitabine Metabolism in Pancreatic Cancer Cell Lines

Tormod K. Bjånes, Lars Petter Jordheim, Jan Schjøtt, Tina Kamceva, Emeline Cros-Perrial, Anika Langer, Gorka Ruiz de Garibay, Spiros Kotopoulis, Emmet McCormack, and Bettina Riedel

Section of Clinical Pharmacology, Department of Medical Biochemistry and Pharmacology (T.K.B., J.S., T.K., B.R.) and National Centre for Ultrasound in Gastroenterology (S.K.), Haukeland University Hospital, Bergen, Norway; Department of Clinical Science, Faculty of Medicine (T.K.B., J.S., A.L., G.R.G., E.M., B.R.), Centre for Cancer Biomarkers, Department of Clinical Science (A.L., G.R.G., E.M.), and Department of Clinical Science (S.K.), University of Bergen, Bergen, Norway; Université Claude Bernard Lyon 1, INSERM 1052, CNRS 5286, Centre Léon Bérard, Centre de Recherche en Cancérologie de Lyon, Lyon, France (L.P.J., E.C.-P.); and Phoenix Solutions AS, Oslo, Norway (S.K.)

Received September 13, 2019; accepted December 11, 2019

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, 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 versus 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.

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.

Introduction

Gemcitabine (2′,2′-difluoro-2′-deoxycytidine [dFdC]) is a nucleoside analog used either alone or in combination with other cytostatic agents for treatment of inoperable pancreatic ductal adenocarcinoma (PDAC) and several other human cancers (https://www.legemiddelsok.no/). 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 favor the outcome of gemcitabine treatment (Maréchal 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., 2019). 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 with 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 has not been elucidated. We hypothesized that CDA regulates gemcitabine flux through its inactivation and activation pathways in PDAC cell lines.

This study was funded by the Western Health Board of Norway [Grant 912146].

E.M. and B.R. contributed equally to this work.

https://doi.org/10.1124/dmd.119.089334.

This article has supplemental material available at dmd.aspetjournals.org.

ABBREVIATIONS: CDA, cytidine deaminase; dCK, deoxycytidine kinase; dFdC, 2′,2′-difluoro-2′-deoxycytidine; dFdCMP, gemcitabine monophosphate; dFdCTP, gemcitabine triphosphate; dFdU, 2′,2′-difluoro-2′-deoxyuridine; DMEM, Dulbecco’s modified Eagle’s medium; hENT1, equilibrative nucleoside transporter 1; PDAC, pancreatic ductal adenocarcinoma; THU, tetrahydrouridine.
metabolism in cancer cells is less studied (Morita et al., 2003; Vande Voorde et al., 2014).

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; Giovannetti et al., 2007; Yoshida et al., 2010; Peter 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; Olhmne 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 tetrahydroidourine (THU). We also determined basal mRNA and protein expression profiles of CDA and other main proteins involved in the transport and metabolism of gemcitabine.

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 (Osw, Norway), centrifuge tubes from Sarstedt (Osw, Norway), and THU from AH diagnostics (Oslo, Norway). All other reagents and equipment used for liquid chromatography tandem mass spectrometry methods have been described previously (Bjånes 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-cm² flasks in a humidified atmosphere with 5% CO₂ at 37°C and subcultured twice weekly. BxPC-3 cells were cultured in RPMI 1640 medium. MIA PaCa-2 and PANC-1 were cultured in Dulbecco’s modified Eagle’s medium D5671 (DMEM). All media were supplemented with 10% FBS, 4 mM sodium pyruvate, and 2 mM L-glutamine. The medium used for MIA PaCa-2 was additionally supplemented with horse serum (2.5%) as recommended by the manufacturer. No antibiotics were used. Mycoplasma tests performed on a regular basis were negative.

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. One mL of cell-free media samples was incubated with 200 μM THU for 60 minutes at 37°C. At ASPET Journals on October 27, 2023 dmd.aspetjournals.org Downloaded from
dFdU concentrations were 17.7, 3.7, and 0.2 μM (Supplemental Fig. 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 coincubation 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 (Fig. 1B). 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 versus 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. Sixty minutes incubation with 100 μM gemcitabine did not result in significantly higher dFdCTP concentrations in any of the three cell lines. Also, CDA inhibition had no effect on dFdCTP concentrations at both gemcitabine concentrations under these experimental conditions (Supplemental Fig. 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 Fig. 2, respectively. Original Western blots can be seen in Supplemental Fig. 3. CDA showed highest mRNA and protein expression in BxPC-3. Lower CDA mRNA expression (Fig. 2A) and zero protein expression (Fig. 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.

**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 et al. (2009) in ex vivo whole blood from healthy volunteers, as 50% after 5 hours incubation and close to 100% after 24 hours. In accordance with other publications (Funamizu et al., 2012a,b), we also found that CDA displayed the highest mRNA (Fig. 2A) and protein expression (Fig. 2B) in BxPC-3 compared with 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 (Fig. 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 Fig. 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 (Fig. 1A). The finding of no detectable CDA protein expression (Fig. 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 Fig. 3) because both cell lines expressed CDA mRNA (Fig. 2A). Moreover, it has been suggested that transcriptional, posttranscriptional (Mameri et al., 2017), and posttranslational (Fresse 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 (Bjånes 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 coincubation 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 with the two other cell lines (Fig. 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 because of extensive conversion of gemcitabine to dFdU (Fig. 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 (Fig. 1B) or by inhibiting CDA (Fig. 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 others (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 (Supplemental Fig. 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 versus 24 hours incubation without THU was 77% versus <5% in BxPC-3, 92% versus 66% in MIA PaCa-2, and >98% versus 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 not previously 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, 2012a; Paproski et al., 2010; 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 Bardenheuer et al. (2005) and Mameri et al. (2017).

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 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

Fig. 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 (micromolars) and intracellular dFdCTP (picomoles per 10^6), respectively. Insert in (A) shows 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.

Fig. 2. (A) 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 is highlighted (red rectangle). Data are displayed as means of four independent samples studied in triplicate, and error bars are S.D. SLC28A1*: hCNT1, concentrative nucleoside transporter 1; SLC29A1: hENT1, equilibrative nucleoside transporter 1; SLC29A2: CMPK1, uridine/cytosine monophosphate kinase; dCK, deoxycytidine kinase; hCNT2, equilibrative nucleoside transporter 2; NME2: CDA, cytidine deaminase; dCTD, deoxycytidine monophosphate deaminase; NdPK, nucleoside diphosphate kinase; NT5C2: cN-II, cytosolic 5'-nucleotidase II; NT5C3: cN-IIA, cytosolic 5'-nucleotidase III A; NT5M: CTPS1*, cytidine triphosphate synthase 1; DCTPP1, deoxycytidine triphosphate pyrophosphatase 1; mdN, mitochondrial 5'-deoxyribonucleotidase; POLA1, deoxyribonucleic acid polymerase alpha; RRM1, large subunit of ribonucleotide reductase; RRM2, small subunit of ribonucleotide reductase. mRNA expression of SLC28A1 not detectable. (B) 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 three independent samples, and error bars are S.D. Raw data are available in Supplemental Fig. 3. cN-II, cytosolic 5'-nucleotidase II (NT5C2); cN-IIA, 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.
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 (Fig. 2B), particularly 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 Fig. 2, 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 (Fig. 1), combined with CDA inhibition, provided insight into differential CDA activities that could not be revealed by expression analyses alone (Fig. 2). In a recent commentary by Peters et al. (2019), phenotyping with cytidine or gemcitabine was also recommended over genotyping for pretreatment assessment of in vivo CDA activity in patients. Hodge et al. (2011a,b) 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 contribute to 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. However, the production rate of dFdCTP was 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 Fig. 2. Based on in silico simulations, Battaglia and Parker (2011) suggested that the rate of DNA incorporation in general is a slow process when studying cellular regulation of gemcitabine transport (Fig. 2). Based on intracellular enzyme expressions, illustrated by our own results in Fig. 2. Based on in silico simulations, Battaglia and Parker (2011) suggested that the rate of DNA incorporation in general is a slow process when studying cellular regulation of gemcitabine transport (Fig. 2).

In general, data from in vitro experiments should be interpreted with caution in terms of in vivo relevance. However, our finding 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 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 dFdCCTP accumulation. However, low CDA activity may not necessarily result in increased dFdCCTP 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.

**Acknowledgments**

The authors would like to thank Elisa Thodesen Murvold and Philip Webber for valuable technical support as well as Anders Molven for generously providing the PDAC cell lines.

**Authorship Contributions**

**Participated in research design:** Bjånes, Jordheim, Schjott, Riedel. **Conducted experiments:** Bjånes, Kamecva, Cros-Perrial. **Contributed new reagents or analytic tools:** Bjånes, Jordheim, Schjott, McCormack. **Performed data analysis:** Bjånes, Jordheim, Schjott, Cros-Perrial, Langer, Ruiz de Garibay. **Wrote or contributed to the writing of the manuscript:** Bjånes, Jordheim, Schjott, Kamecva, Cros-Perrial, Langer, Ruiz de Garibay, Kotopoulis, McCormack, Riedel.

**References**


Address correspondence to: Tormod K. Bjanes, Section of Clinical Pharmacology, Department of Medical Biochemistry and Pharmacology, Haukeland University Hospital, Haukelandsveien 22, 5021 Bergen, Norway. E-mail: tormod.karlsen.bjanes@helse-bergen.no