

Original Article

A pilot clinical trial of the cytidine deaminase inhibitor tetrahydrouridine combined with decitabine to target DNMT1 in advanced, chemorefractory pancreatic cancer

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Abstract: DNA methyltransferase 1 (DNMT1) is scientifically validated as a molecular target to treat chemo-resistant pancreatic ductal adenocarcinoma (PDAC). Results of clinical studies of the pyrimidine nucleoside analog decitabine to target DNMT1 in PDAC have, however, disappointed. One reason is high expression in PDAC of the enzyme cytidine deaminase (CDA), which catabolizes decitabine within minutes. We therefore added tetrahydrouridine (THU) to inhibit CDA with decitabine. In this pilot clinical trial, patients with advanced chemorefractory PDAC ingested oral THU ~10 mg/kg/day combined with oral decitabine ~0.2 mg/kg/day, for 5 consecutive days, then 2X/week. We treated 13 patients with extensively metastatic chemo-resistant PDAC, including 8 patients (62%) with ascites: all had received ≥ 1 prior therapies including gemcitabine/nab-paclitaxel in 9 (69%) and FOLFIRINOX in 12 (92%). Median time on THU/decitabine treatment was 35 days (range 4-63). The most frequent treatment-attributable adverse event was anemia (n=5). No deaths were attributed to THU/decitabine. Five patients had clinical progressive disease (PD) prior to week 8. Eight patients had week 8 evaluation scans: 1 had stable disease and 7 PD. Median overall survival was 3.1 months. Decitabine systemic exposure is expected to decrease neutrophil counts; however, neutropenia was unexpectedly mild. To identify reasons for limited systemic decitabine effect, we measured plasma CDA enzyme activity in PDAC patients, and found a > 10-fold increase in those with metastatic vs resectable PDAC. We concluded that CDA activity is increased not just locally but also systemically in metastatic PDAC, suggesting a need for even higher CDA-inhibitor doses than used here.

Keywords: Pancreatic cancer, clinical trial, deaminase inhibitor

Introduction

Pancreatic ductal adenocarcinoma (PDAC), commonly referred to as pancreatic cancer, is a recalcitrant, deadly problem [1]. Most patients have advanced metastatic disease at presentation, and therefore receive aggressive multi-agent chemotherapy, either combinations of 5-fluorouracil, irinotecan and oxaliplatin or gemcitabine and nab-paclitaxel. Despite the substantial intensity and toxicity of such treat-

ments, Median overall survival is < 1 year [2-4]. Several reasons for limited chemotherapy responsiveness have been proposed, but one well-established basis is mutation and deletion in most PDAC of the master regulator of apoptosis p53 [5, 6]: absence of p53 undermines the ability of cytotoxic (apoptosis-intending) agents - all the agents listed above - to achieve tumor kill by apoptosis induction [7, 8]. Meanwhile, normal dividing cells, with intact p53, are killed, causing onerous toxicity profiles [3, 9]. Thus,

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alternative treatment modalities that do not rely on cell cycle exits via the p53-apoptosis pathway are needed.

Tumor cytoreduction despite absence of p53, e.g., via activation of terminal epithelial programs, can be achieved by inhibiting/deleting the key epigenetic regulator DNA methyltransferase 1 (DNMT1) that mediates repression of these programs in cancer cells [10-16]. Moreover, DNMT1 can be inhibited/depleted by the pyrimidine nucleoside analog pro-drugs decitabine or 5-azacytidine that are approved by the Food and Drug Administration (FDA) to treat myeloid cancers. In fact, successful pre-clinical validation of DNMT1 as a molecular target for treating PDAC has motivated several clinical trials of decitabine, 5-azacytidine or analogs thereof to treat PDAC. Several of these trials have been completed, and more are ongoing as of April 2019 (Clinicaltrials.gov identifiers NCT02959164, NCT03257761, NCT03264404, NCT02961101) [17-29] (reviewed in [30]). Results from completed trials, however, have been discouraging [17-30]. Correlative studies in one prior trial of decitabine suggested a basis for disappointing clinical results: analyses of cancer tissue resected post-treatment revealed decitabine pharmacodynamic effect in solid cancer tissue in < 25% of the treated patients, despite continuous infusion of decitabine that sustained plasma C_{max} > 40 nM for hours, enough to cause grade 3/4 myelotoxicity (particularly neutropenia) [31, 32]. Decitabine and 5-azacytidine are pro-drugs that must be processed by pyrimidine metabolism in order to deplete DNMT1, and accordingly, tissue-differences in expression of key pyrimidine metabolism enzymes have been shown to underlie tissue-differences in pharmacodynamic effect. One key enzyme is cytidine deaminase (CDA) which rapidly catabolizes decitabine and 5-azacytidine into uridine counterparts that cannot deplete DNMT1 [33]. Naturally high CDA expression in gastro-intestinal tissues is the reason decitabine, 5-azacytidine and gemcitabine (first-line treatment for PDAC that is also a pyrimidine nucleoside analog) are not administered orally, and CDA is why the plasma half-lives of decitabine, 5-azacytidine or gemcitabine are ~15 minutes after parenteral administration [34, 35]. Accordingly, the addition of a CDA inhibitor tetrahydrouridine (THU) to oral decitabine produced decitabine plasma pharmacokinetics comparable to con-

tinuous intravenous infusion of decitabine alone, to produce DNMT1-depleting pharmacodynamic effect in the myeloid compartment that was substantial and clinically significant [17, 27, 29].

Here, we examined contributions of CDA to PDAC resistance to decitabine and evaluated the use of THU to reverse this mechanism-of-resistance. We found that gemcitabine-resistance PDAC cells have upregulated CDA (observed also by others [36-39]), and that such cells are also resistant to decitabine. In pre-clinical in vitro and in vivo models, the addition of THU to inhibit CDA overcame this basis for resistance. Then, to translate the pre-clinical observations, we conducted a pilot clinical trial in 13 patients with advanced chemorefractory PDAC. The doses of THU and decitabine were derived from prior clinical studies in patients without PDAC. The frequent and distributed schedule of administration of THU/decitabine was intended to increase possibilities of overlap between malignant S-phase entries and drug exposures, needed for S-phase dependent DNMT1-depletion in PDAC tissue. Unfortunately, there was no clinical benefit, but correlative observations suggested why: there was surprisingly little neutropenia in this clinical trial, a sensitive indicator of decitabine systemic activity (the myeloid compartment is the most sensitive tissue compartment to systemic decitabine exposures [34, 35, 40-43]), and showing why, plasma CDA enzyme activity was > 10-fold higher in patients with metastatic vs resectable PDAC. That is, CDA activity was increased not just within PDAC tissue, but also systemically, and the THU dosages used, enough for CDA inhibition and decitabine exposure in patients without PDAC, were insufficient in patients with advanced PDAC. Since CDA contributes to resistance not just to decitabine/5-azacytidine, but also to resistance to standard first-line PDAC therapy with gemcitabine [38, 39, 44, 45], this information is pertinent to future clinical evaluations of decitabine, 5-azacytidine or gemcitabine to treat PDAC.

Patients and methods

Study design and patients

This was an open-label, single-arm pilot clinical trial in patients with metastatic PDAC that had

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progressed on prior chemotherapy. The study was reviewed and approved by the Case Comprehensive Cancer Center Protocol Review and Monitoring Committee, and the Cleveland Clinic Institutional Review Board. The study was registered on clinicaltrials.gov (NCT028-47000). Patients were enrolled at Cleveland Clinic and University Hospitals of the Case Western Reserve University from April to August 2017. Key inclusion criteria were: a confirmed pathologic diagnosis of pancreatic cancer (histologies other than carcinoma or adenocarcinoma were not eligible); one or more prior systemic chemotherapy regimens with treatment cessation due to disease progression or intolerable toxicity; adult patients with Eastern Cooperative Oncology Group (ECOG) Performance Score (PS) of 0, 1, or 2; with measurable disease per RECIST 1.1 criteria; adequate organ function (hemoglobin \geq 8 g/dl; absolute neutrophil count (ANC) \geq 1500/ μ l; platelets \geq 75000/ μ l; serum creatinine \leq 2.9 mg/dl; serum total bilirubin \leq 2 times upper limit of laboratory normal; serum AST/ALT \leq 2.5 times upper limit of laboratory normal; serum calcium \leq 12 mg/dl); eligible and agreeable for baseline and week 16 percutaneous tumor biopsies. All prior cancer-directed therapies had to be completed/stopped at least 14 days prior to enrollment. Any uncontrolled comorbidities were exclusionary.

Study procedures

After written informed consent, patients underwent baseline screening, including a complete history and physical exam, laboratory evaluation (including organ function assessment, pregnancy test as applicable, CA19.9 levels), tumor imaging using CT or MRI scans, and a baseline tumor biopsy. After confirmation of eligibility, patients were started on treatment.

The doses were as follows: THU was supplied as 250 mg/capsules, and decitabine as 5 mg/capsules. Starting doses were by weight: Weight 40-60 kg: 2 capsules of each drug. Weight 61-80 kg: 3 capsules of each drug. Weight 81 kg or higher: 4 capsules of each drug. Patients were instructed to take the decitabine capsules ~60 minutes after taking the THU capsules, to generate sufficient time for the intended biological effect of THU of systemic CDA-inhibition.

The treatment schedule consisted of an induction cycle (4 weeks) and maintenance thereafter. During the induction cycle, the drugs were taken for 5 consecutive days (typically, Monday-Friday) in week 1. In week 2, the same doses were used if no grade 3 or higher hematologic toxicities were noted. Based on the absolute neutrophil count (ANC) at the start of week 2, treatments during this week were given for 2, 3, 4, or 5 consecutive days. During weeks 3 and 4, treatments were given for 2 consecutive days (typically, Monday and Tuesday) at the same doses, if no grade 3 or higher hematologic toxicities were noted. After the induction cycle, a maintenance phase was introduced, where treatments were given for 2 consecutive days (typically, Monday and Tuesday) of each week, for up to 52 weeks.

If hematologic toxicities precluded the above plan, then dose and schedule modifications were prespecified in the study protocol. If stable disease with absence of at least partial response by RECIST1.1 at week 8 or 16 staging scans was noted, and no treatment-related toxicities were seen, an increase in THU and Dec dosage by one capsule each was allowed.

Endpoints and statistics

The primary endpoint of this pilot study was to detect decitabine therapy-induced DNMT1 protein level decrease in tumor tissue. DNMT1 levels were to be assessed using quantitative immunofluorescence, on baseline tumor biopsy and the week 16 biopsy. We chose an effect size of 1, assessed using a paired t-test and $\alpha = 0.05$. The effect size was defined as the difference in mean DNMT1-protein levels between post-treatment and pre-treatment divided by the standard deviation and was thus a metric of change in the natural units of the distribution, its standard deviation. Our goal was thus to detect drops in DNMT1 of at least one standard deviation. To have 90% power to detect this change, a sample size of 12 patients was required.

Secondary endpoints included safety of the treatment regimen, response rate per RECIST 1.1 criteria, and overall survival, calculated from date of patient registration on study to date of death.

All patients enrolled into the study are included in the analyses.

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Plasma CDA enzyme activity assay

Conversion of cytidine into uridine by plasma at 37°C was measured by high performance liquid chromatography (HPLC) based on published methods [46]. Reaction buffer 0.1 M Tris/HCL pH 7.5 (265 µl) was added to 25 µl of human plasma followed by addition of cytidine (Sigma-Aldrich C122106) to a final concentration of 4.1 mM and 5-fluorouridine (Sigma-Aldrich F5130) 0.381 mM (not metabolized by CDA) as an internal control. After incubation at 37°C for 4 hours the reaction was terminated with 50 µl of hydrochloric acid 1 N. Blanks used in calculations consisted of the above but with cytidine substrate added at the end of the 60 minute incubation. After reaction termination, protein was precipitated with perchloric acid (TCA, 2%). 100 µl of supernatant was injected for HPLC using ammonium acetate (5 mM) as the mobile phase with a flow rate of 0.35 ml/min through ZORBAX eclipse XDB 80°A C18, 4.6 × 150 mm, 3.5 µM (Agilent 963967-902) High Performance Liquid Chromatography (HPLC) column on Dionex UltiMate 3000 µ-HPLC system supported with Chromeleon 7.1 data system (Dionex Corporation, Sunnyvale, CA). Retention time and peak area of uridine at 260 nm were compared to the internal control for each injection. The average net uridine peak area of test minus blank was calculated for each test sample. Known concentrations of uridine (0.0 to 95.8 µM) (Sigma-Aldrich U3750) were used to construct a standard curve to calculate the amount of uridine based on the net uridine peak area normalized to the net peak area of the internal standard 5-fluorouridine. One unit (U) of CDA enzyme activity is defined as the amount of enzyme needed to produce 1 µmole of uridine in 1 minute. Multiple runs with known concentrations of uridine were used to confirm accuracy and precision: between run variability was < 5%.

Pre-clinical in vitro studies of gemcitabine-resistant PDAC

MIA PaCa2 parental, MIA PaCa2 gemcitabine resistant and PANC-1 cells were a gift from John Pink (Case Western Reserve University). Gemcitabine resistant cells were kept under drug selection at 2 µM gemcitabine. The pancreatic cell lines and K562 cells (ATCC) were cultured in RPMI-1640 with 10% FBS at 37°C in a humidified atmosphere with 5% CO₂ in air.

Decitabine stock solution (5 mM) was generated by reconstituting lyophilized decitabine in 100% DMSO. Stock solution aliquots were stored at -80°C for up to 3 weeks. Working solution was generated by diluting the stock solution 1:100 in phosphate-buffered saline (PBS) immediately before addition to the cells at a further dilution as per the intended final concentration. Similar amounts of PBS are added to untreated control cells. Cells were treated with increasing concentrations of decitabine (0-100 µM) to generate a dose-response curve or with 0.2 µM decitabine on day 1 and 2 of culture for the rest of the experiments. THU stock solution 12 mM was generated by reconstituting THU in 100% PBS. Cells were treated with 1 µM THU.

Approximately 60 µg of protein extracts, together with molecular weight markers, were subjected to 1D SDS-PAGE on 4-12% gradient gels (Invitrogen). After electrophoresis per manufacturer's manual (Invitrogen), proteins were transferred to PVDF membranes (Millipore) at a constant voltage for 1 hour using Invitrogen's semidry blotting apparatus. Western analyses of PVDF membranes utilized established protocols and antibodies for DNMT1 (Cell Signaling, #5032S), cMYC (Cell Signaling, #D84C12), and anti-β-Actin peroxidase (Sigma-Aldrich, #A3854).

mRNA levels were assayed by QRT-PCR using standard methods. Briefly, GAPDH was amplified as control. Primer sequences were as follows: CDA Forward 5'-AAGGGTACAAGGATTTCAGGG-3' and CDA Reverse 5'-ACAATATACGTACCATCCGGC-3'. Real-time detection of the emission intensity of SYBR Green bound to double-stranded DNA was detected using the iCycler instrument (Bio-Rad). Data is reported as 'relative expression value' which was determined by raising 2 to the power of the negative value of delta-delta CT for each sample.

Cytospins of cells were fixed for 2 minutes in methanol, air-dried, and stained for 20 minutes with filtered modified solution of Giemsa stain (Sigma Aldrich, Cat #48900, St Louis, MO), diluted (1:20) with buffer solution pH6.5, rinsed with distilled water, air-dried and examined using low and high magnifications with a Leica DMR microscope (Leica Microsystems, Wetzlar GmbH, Germany) connected to Nuance multi-spectral imaging system FX using Nuance ver-

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sion 3.0.2 software (PerkinElmer, Inc., Hopkinton, MA).

Pre-clinical in vivo studies of gemcitabine-resistant PDAC

All experiments were approved by the Cleveland Clinic IACUC and followed approved procedures. Gemcitabine Resistant MIA PaCa2 cells were subcutaneously injected into the right and left flanks of 6- to 8-week-old immunocompromised male BALB/c nu/nu mice (2 million cells per injection in 200 μ l sterile control). When tumor volumes reached an average of 100 mm³, mice were randomized to the treatment groups, and tumor volume was assessed by caliper measurement twice weekly throughout the study using the equation: volume (mm³) = long (mm) \times wide² (mm)/2 (at least four mice with two tumors each for adequate power > 0.08). Mice were treated subcutaneously twice a week with vehicle control, twice a week with 60 mg/kg gemcitabine for two weeks followed by a week off, or a combination of THU (10 mg/kg) and decitabine (0.1 mg/kg) twice a week. The reagents were formulated in a PBS solution. Mice body weights were recorded weekly and percentage of mice body weights during treatment was calculated as: weight at each time point/initial weight \times 100. Animals were observed for signs of toxicity (mucous diarrhea, abdominal stiffness, and weight loss) and were euthanized when tumor volume reached 1000 mm³ using CO₂ inhalation and followed by cervical dislocation. Statistical analysis was performed using ANOVA with significance reached at $P < 0.05$.

Results

PDAC cells resist gemcitabine and decitabine by upregulating CDA

Clinical trials are typically conducted in patients with PDAC that has progressed through earlier therapy with gemcitabine. Therefore, we evaluated sensitivity of gemcitabine-resistant patient-derived PDAC cells (MiaPaCa [37]) to decitabine 0-50 μ M: the gemcitabine-resistant PDAC cells were also resistant to high concentrations of decitabine - the concentration of decitabine needed to produce 50% growth inhibition of the cells (IC₅₀) was ~40 μ M (**Figure 1A**). Gemcitabine and decitabine are both inactivated by CDA [47, 48], and CDA expres-

sion was > 100-fold higher in gemcitabine-resistant PDAC cells compared to decitabine and gemcitabine-sensitive K562 cells, a model of decitabine-sensitive myeloid malignancy (**Figure 1B**). We also compared expression of CDA in several PDAC cancer cell lines (n=19) vs other cancer cell types (n=351) in the Cancer Cell Line Encyclopedia (CCLE): PDAC cells inherently express higher levels of CDA than almost any other cancer cell type (public RNA sequencing data) (**Figure 1C**).

We reasoned that if decitabine-resistance of the PDAC cells was mediated by CDA, then addition of the CDA-inhibitor THU might confer sensitivity to decitabine: addition of THU 1 μ M enabled a clinically relevant concentration of decitabine 0.2 μ M to deplete DNMT1 from the gemcitabine-resistant PDAC cells, decrease levels of the master regulator of proliferation MYC (**Figure 1D**), and substantially decrease proliferation of the gemcitabine-resistant PDAC cells (**Figure 1E**), accompanied by morphologic changes (e.g., decreased nuclear/cytoplasmic ratio) (**Figure 1F**). We then engrafted mice with gemcitabine-sensitive or gemcitabine-resistant PDAC cells and compared cytotoxic gemcitabine therapy with non-cytotoxic DNMT1-targeting regimens of decitabine: both a cytotoxic regimen of gemcitabine and non-cytotoxic, DNMT1-targeting regimens of decitabine or THU/decitabine controlled gemcitabine-sensitive PDAC tumor growth (Panc-1) (the non-cytotoxic, DNMT1-targeting properties of the decitabine regimens were documented previously [33, 49, 50]) (**Figure 2A, 2B**). However, the gemcitabine regimen was not able to control in vivo growth of gemcitabine-resistant PDAC cells (gemcitabine-resistant MiaPaCa-2 that were also resistant to decitabine), while the THU/decitabine regimen suppressed growth of these tumors (**Figure 2C, 2D**).

Clinical trial study population

To translate pre-clinical observations into the clinic, we conducted a pilot clinical trial of THU/decitabine to treat advanced, chemorefractory PDAC. From April to August 2017, we enrolled 13 patients. Their median age was 65 years (range 44-74), and 7 were male (54%) (**Table 1**). The primary site of PDA within the pancreas was the head/neck in 4 (31%) and body/tail in 9 (69%) (**Table 1**). The median time from diagnosis was 13 months (range 3.9-53.5) (**Table 1**). Baseline ECOG PS was 0 or 1 in 12 (92%) of

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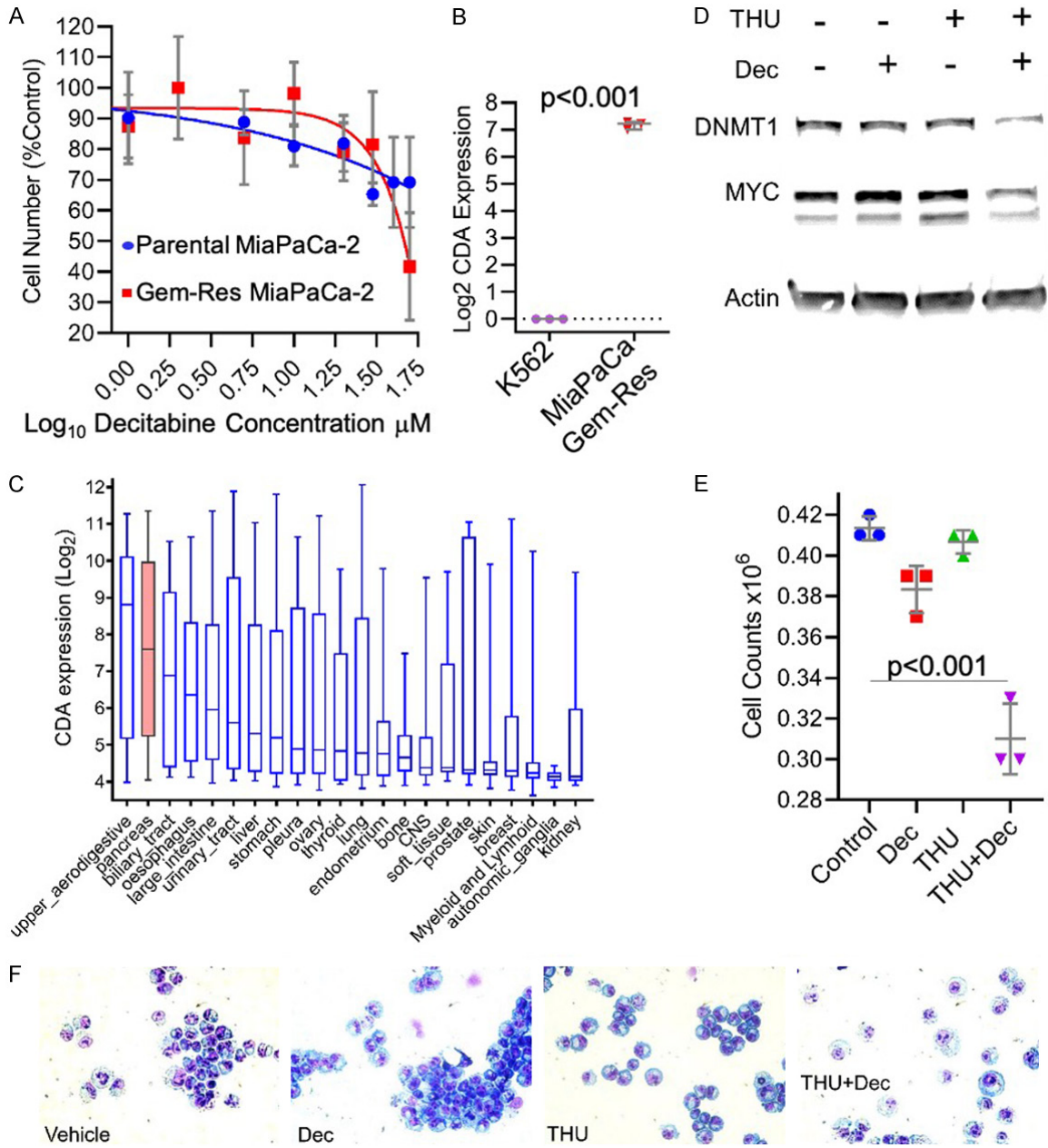


Figure 1. The addition of a CDA-inhibitor to decitabine enabled DNMT1-depletion by decitabine and hence cyto-reduction of gemcitabine-resistant PDAC cells. **A.** Concentrations of decitabine needed to produce 50% growth inhibition (GI₅₀) in parental and gemcitabine-resistant PDAC cells (MiaPaCa-2). Mean ± SD. **B.** CDA expression in gemcitabine-resistant PDAC cells (MiaPaCa-2) vs decitabine- and gemcitabine-resistant myeloid leukemia cells (K562). QRT-PCR. Mean ± SD. *P*-value 2-sided unpaired t-test. **C.** CDA expression is inherently higher in pancreatic cancer vs other cancers. CDA expression in cancer cell lines (n=370) grouped by histology of origin and ordered by median CDA expression level (analysis of public RNA-sequencing data from CCLE). Box-plots median ± IQR, whiskers range. **D.** The addition of the CDA-inhibitor THU (1 mM) to a clinically-relevant concentration of decitabine (0.2 mM) enabled depletion of DNMT1 and MYC from gemcitabine-resistant PDAC cells (MiaPaCa-2). Western blots 72 hours after a single addition of decitabine and/or THU. **E.** Cell counts of the cells 72 hours after a single addition of decitabine and/or THU as per panel D. Mean ± SD. *P*-value 2-sided unpaired t-test. **F.** Giemsa-stained cytopsin preparations of the cells 72 hours after a single addition of decitabine and/or THU as per panel D.

the patients. They had received a median of 2 prior lines of therapy (range 1-3), including

FOLFIRINOX or its variants in 12 (92%) and gemcitabine/nab-paclitaxel in 9 (69%) patients.

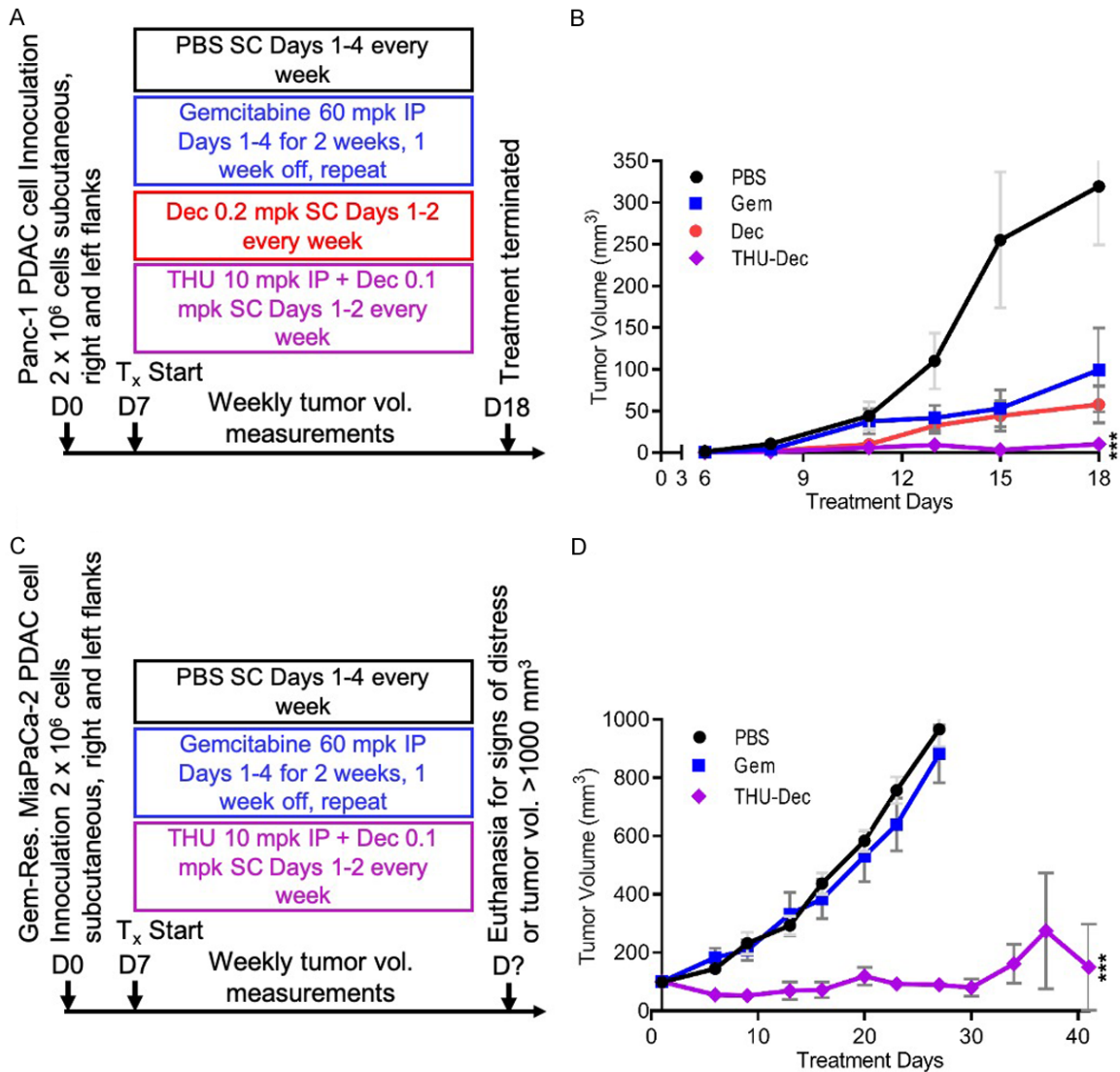


Figure 2. Combination THU+Decitabine significantly cytoreduced gemcitabine-resistant PDAC in vivo. **A.** Experiment schema gemcitabine-sensitive Panc-1 PDAC cells: Once tumor volumes reached 100 mm³ (~Day 7), mice were randomized to the indicated treatments. The experiment was terminated on Day 18 for analysis. Mpk = mg/kg; SC = subcutaneous; IP = intra-peritoneal; Dec = decitabine. n=8/treatment group. **B.** Tumor volume measurements. Mean \pm SE. ***P < 0.001 vs gemcitabine-treatment, 2-sided t-test. **C.** Experiment schema gemcitabine-resistant MiaPaCa-2 PDAC cells: Once tumor volumes reached 100 mm³ (~Day 7), mice were randomized to the indicated treatments. Mice were euthanized when their tumor volume measurement reached 1000 mm³ or when they exhibited signs of distress as defined in the Animal Protocol; **D.** Tumor volume measurements. Mean \pm SE. ***P < 0.001 vs gemcitabine-treatment, 2-sided t-test.

All patients had multiple sites of metastases, including 8 (62%) with ascites. Baseline CA19.9 levels were > 1000 U/ml in 8 (67%) patients (Table 1).

Clinical safety

All enrolled patients started planned treatment. Median time on treatment was 35 days (range

4-63); median time on study was 72 days (range 25-105). The most frequent adverse events deemed to be possibly, probably, or definitely related to treatment were anemia (n=5), and in 3 patients each, increased alkaline phosphatase, anorexia, dehydration, nausea, fatigue, febrile neutropenia, decreased lymphocyte count, hyponatremia, and hypokalemia (Table 2). There were no deaths (grade 5 toxic-

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Table 1. Baseline Characteristics of Study Patients

Patient #	Age, Sex	ECOG PS	# Prior Rx*	Serum CA19.9 Level	Liver Lesions	Lung Lesions	Peritoneal Disease/Ascites
1	44M	0	3	9,187	✓	✓	
2	65M	1	3	NL	✓		✓
3	69M	1	1	351	✓		✓
4	59F	1	2	1,699	✓	✓	
5	59F	0	2	171,630	✓		✓
6	74M	1	2	9,747	✓	✓	✓
7	69F	0	1	376		✓	
8	65F	1	1	NL	✓	✓	✓
9	64M	1	2	618	✓	✓	✓
10	44F	2	2	2,864	✓	✓	
11	67M	1	2	3,824	✓		✓
12	67F	1	2	12,668	✓	✓	✓
13	60F	1	1	3,707	✓		✓

Notes: ECOG PS: ECOG Performance Score (scale from 0 to 4; 0= best, with no impairments; 4= bedridden); # Prior Rx = number of prior lines of therapy; *prior therapy includes radiation if given separately from chemotherapy; NL = normal; NA = not available).

Table 2. Most Frequent Treatment-Related Adverse Events (N=13)

	Grade 1 or 2	Grade 3 or 4	Total
Anemia	4	1	5
Febrile neutropenia	0	3	3
Decreased lymphocyte count	1	2	3
Fatigue	3	0	3
Nausea	3	0	3
Anorexia	2	1	3
Dehydration	1	2	3
Increased alkaline phosphatase	2	1	3
Hyponatremia	1	2	3
Hyperkalemia	3	0	3

ity) attributed to the study drugs. Six patients required study drug holds per protocol for laboratory abnormalities (grade 3 alkaline phosphatase rise, n=2; grade 3 hyperbilirubinemia, n=1; thrombocytopenia, n=1). One patient required study drug hold for grade 3 nausea and vomiting, and another for atrial fibrillation (n=1).

Clinical efficacy

Eight (62%) patients underwent evaluation scans at week 8. The best response was stable disease in 1 patient; the other 7 had progressive disease. Reasons for coming off of study drug treatment before or after week 8 were progressive disease (n=6), treating physician discretion (clinical progressive disease) (n=3), ad-

verse events (n=2), and others (n=2). During follow-up, 6 patients died. Median overall survival in this cohort of patients from time of enrollment was 3.1 months.

Limited systemic pharmacodynamic effect of the THU/decitabine therapy could be from systemically elevated CDA enzyme activity

Toxicological studies have demonstrated that the myeloid compartment is the most sensitive tissue compartment to decitabine (with or without THU) systemic effect - neutropenia concurrent with thrombocytosis are expected side-effects of non-cytotoxic DNMT1-targeting regimens [34, 35, 40, 42, 43]. Most of the patients in this study, however, developed neither neutropenia nor thrombocytosis (**Figure 3A**) [34, 35, 42, 43]. To identify a possible reason for limited systemic decitabine effect, we compared plasma CDA enzyme activity in patients with resectable vs advanced/metastatic PDAC, and found a significant increase in the patients with advanced disease (**Figure 3B**). Moreover, PDAC metastatic to liver or peritoneum expresses significantly higher (~2-fold) CDA levels than primary PDAC or normal pancreas tissue (analysis of public data [51] (**Figure 3C**).

Discussion

DNMT1 has been scientifically validated by several research groups as a molecular target for cyto-reduction of PDAC, importantly, offering a

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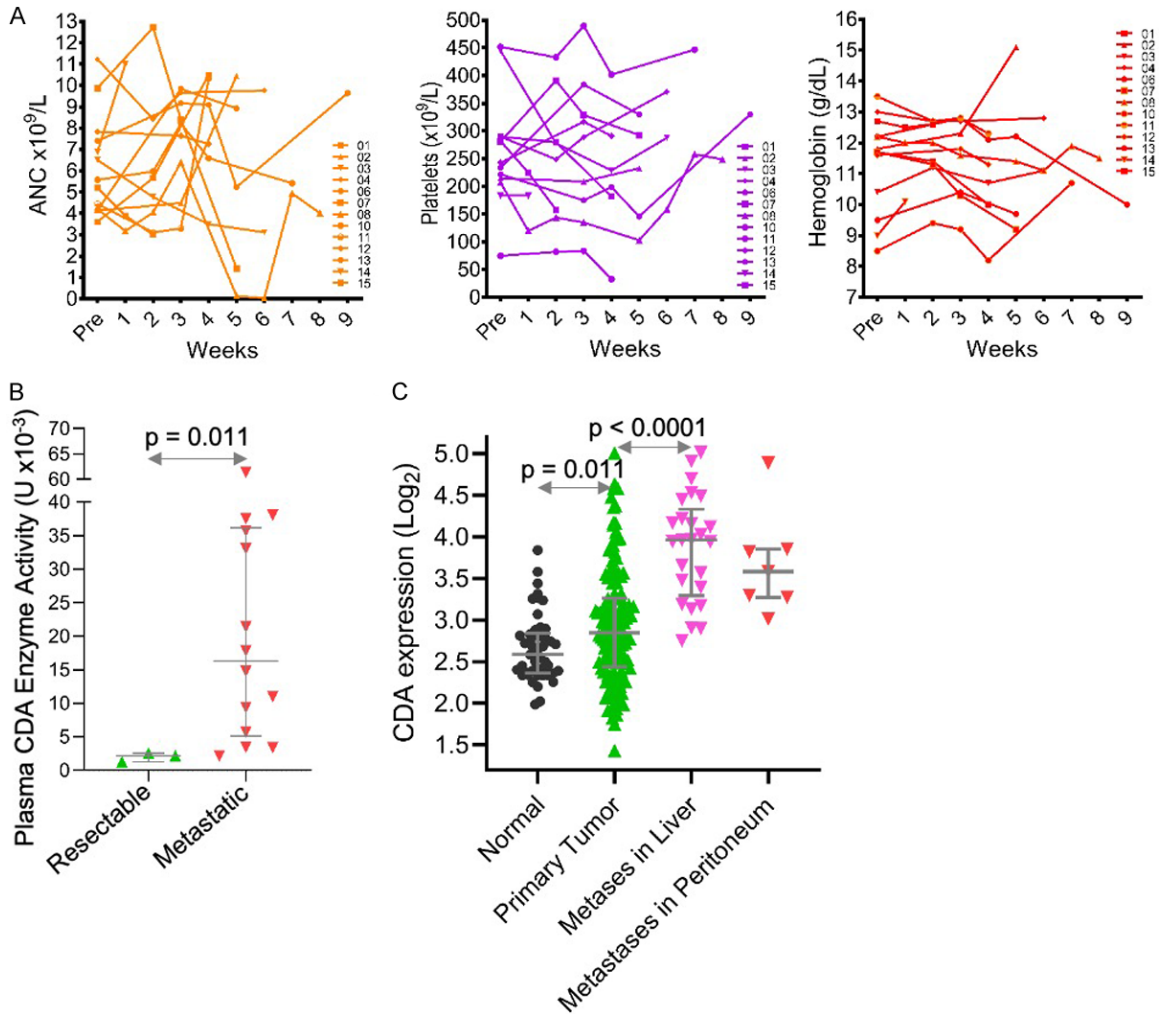


Figure 3. The THU/decitabine therapy had minimal effects on blood counts in most patients, most likely because of systemically elevated CDA enzyme activity in patients with advanced PDAC. A. Neutropenia and increase platelet counts expected with systemic decitabine exposure and DNMT1-depletion mostly did not occur. Serial blood counts during therapy. B. Plasma CDA enzyme activity is increased in patients with metastatic vs resectable PDAC. Plasma CDA enzyme activity was measured using an HPLC-based assay in a separate cohort of patients with PDAC enrolled in a sample collection protocol, grouped by resectable vs metastatic disease (n=17). Median ± IQR, Mann-Whitney test, 2-sided. C. CDA expression is significantly higher in PDAC metastases in the liver and peritoneum than in primary tumor. Analysis of public gene expression data GeoDatabase GSE71729, gene expression by microarray (n=223). Median ± IQR, Mann-Whitney test, 2-sided.

p53-independent pathway for cell cycle exits, distinct from that used by intensive chemotherapy. Encouraged by pre-clinical science, several clinical trials have been conducted with the pyrimidine nucleoside analog decitabine to target DNMT1 in PDAC, but with disappointing results. The pyrimidine metabolism enzyme CDA rapidly catabolizes decitabine into a uridine analog that does not deplete DNMT1. CDA also catabolizes the pyrimidine nucleoside analog gemcitabine that is a first-line agent for treatment of PDAC, and CDA in PDAC cells [37-39],

or from other cells, and even from bacteria in the PDAC micro-environment [36, 52], has been implicated as in PDAC resistance to first-line gemcitabine therapy. As expected with CDA as the cause of resistance, we found here that PDAC cells resistant to gemcitabine were also resistant to decitabine. Accordingly, the addition of a CDA-inhibitor THU to clinically relevant concentrations/doses of decitabine enabled cyto-reduction of gemcitabine/decitabine-resistant PDAC in pre-clinical models. To translate these observations, we used doses of oral THU

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and oral decitabine that we had previously identified as being sufficient for clinically meaningful engagement of the DNMT1-target in the myeloid compartment in human beings without PDAC [35]: that is, these doses of oral decitabine, administered after oral THU, successfully distributed through CDA-enriched intestines and liver in order to produce clinically significant pharmacodynamic effect in the myeloid compartment. Since the oral decitabine (administered with THU) successfully distributed through CDA-rich intestines and liver, we inferred that the decitabine could also distribute through CDA-enriched PDAC. To increase the fraction of PDAC subject to S-phase dependent DNMT1-depletion, these doses of oral THU/decitabine were administered frequently and in distributed fashion (for 5 consecutive days then 2X/week). Unfortunately, no clinical benefit was observed. Neither did we observe neutropenia concurrent with an increase in the platelet count, the side-effect expected with systemic exposure to non-cytotoxic, DNMT1-depleting concentrations of decitabine (the myeloid compartment is the most sensitive compartment to systemic decitabine exposures [35, 40, 42]). A reason for this was indicated by measurements of serum CDA enzyme activity: in 17 PDAC patients, we found ~10-fold higher levels in those with advanced versus resectable PDAC. Additionally, in analyses of public datasets, we found that PDAC cells express higher CDA levels than almost every other type of cancer, and CDA expression is especially high in PDAC metastatic to liver or peritoneum vs primary PDAC. Corroborating our findings are published observations from others: in 40 patients with PDAC, serum CDA activity was more than 2-fold increased in those with progressive vs controlled disease [45]; differences in systemic CDA activity between individual patients [53] has been implicated as contributing to PDAC resistance to first-line gemcitabine; and generally higher serum CDA enzyme activity in solid tumor versus other patients has been documented [53, 54). Also worth noting, decitabine is used at several-fold lower doses than gemcitabine (5-20 mg/m²/day versus 1000 mg/m²/day), suggesting it would be even more vulnerable to CDA upregulation [55].

Deoxycytidine kinase (DCK), which executes the initial, rate-limiting modification of decitabine towards the nucleotide form that

depletes DNMT1, is another pyrimidine metabolism enzyme essential to DNMT1-depletion by decitabine. DCK also executes the initial rate-limiting modification of gemcitabine, and thus, gemcitabine-therapy can select for DCK-low as well as CDA-high PDAC cells [56]. Thus, it is conceivable that even with adequate CDA inhibition to permit decitabine distribution through PDAC tissue, that DNMT1-depletion may not be achieved if the gemcitabine-resistant PDAC does not express sufficient DCK. Nevertheless, inhibiting CDA to prevent rapid systemic catabolism of decitabine is a needed first-step for decitabine to have even the possibility of being captured by DCK in PDAC cells.

In sum, CDA activity is increased not just within PDAC tissue, but also systemically in patients with advanced PDAC, likely requiring higher THU dosages than found enough for CDA inhibition in other clinical contexts. Since CDA contributes to resistance not just to decitabine/5-azacytidine, but also to standard first-line PDAC therapy gemcitabine [38, 39, 44, 45], this information is pertinent to future clinical evaluations of decitabine, 5-azacytidine or gemcitabine to treat PDAC.

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Disclosure of conflict of interest

None.

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