Deoxycytidine Kinase Expression Underpins Response to Gemcitabine in Bladder Cancer

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Abstract

Purpose: In a recent phase II clinical trial, low-dose (100 mg/m²) gemcitabine showed promise as a radiosensitizer in bladder cancer, but underlying mechanisms lack elucidation. Here, we investigated the mechanism of radiosensitization by low-dose gemcitabine in bladder cancer cell lines.

Experimental Design: Four bladder cancer cell lines were screened for radiosensitization by low-dose gemcitabine using clonogenic assay, and gemcitabine-resistant RT112gem and CALgem cells created by exposure to increasing gemcitabine doses. Four key gemcitabine-regulatory genes were knocked down by transient siRNA. Nude mice carrying CALgem subcutaneous xenografts were exposed to 100 mg/kg gemcitabine ± ionizing radiation (IR) and response assessed by tumor growth delay.

Results: Gemcitabine was cytotoxic in the low nanomolar range (10–40 nmol/L) in four bladder cancer cell lines and radiosensitized all four lines. Sensitizer enhancement ratios at 10% survival were: RT112 1.42, CAL29 1.55, T24 1.63, and VMCUB1 1.47. Transient siRNA knockdown of deoxycytidine kinase (dCK) significantly reduced radiosensitization by gemcitabine (P = 0.02). RT112gem and CALgem cells displayed robust decreases of dCK mRNA and protein levels; reexpression of dCK restored gemcitabine sensitivity. However, CALgem xenografts responded better to combination gemcitabine/IR than either treatment alone (P < 0.001) with dCK strongly expressed in the tumor vasculature and stroma.

Conclusions: Gemcitabine resistance in bladder cancer cell lines was associated with decreased dCK expression, but gemcitabine-resistant xenografts were responsive to combination low-dose gemcitabine/IR. We propose that dCK activity in tumor vasculature renders it gemcitabine sensitive, which is sufficient to invoke a tumor response and permit tumor cell kill in gemcitabine-resistant tumors. Clin Cancer Res; 20(21); 5435–45. ©2014 AACR.

Introduction

Bladder cancer is the ninth most common cancer worldwide and muscle-invasive disease can be treated by either radical cystectomy or radiotherapy, with similar outcomes (1). Addition of neoadjuvant chemotherapy or concurrent chemoradiotherapy modestly improves survival rates, but there is an urgent need to identify predictive biomarkers of response, to identify those patients best suited for a particular therapy.

The pyrimidine antimetabolite gemcitabine, a fluorinated analogue of cytidine (2',2'-difluorodeoxycytidine; dFdC), has been used successfully as a single-agent chemotherapy to treat many tumor types, including breast (2), pancreatic (3), ovarian (4), and bladder cancer (5) and is an effective radiosensitizer clinically (6–8). The primary mode of entry for gemcitabine in cancer cells is through the human equilibrative nucleoside transporter 1SLC29A1 (9), and after entering the cell requires phosphorylation to become the active antimetabolite (10). Gemcitabine can be deaminated and subsequently excreted as dFdU by the enzyme cytidine deaminase (CDA; ref. 11); dFdU has also been reported as having radiosensitizing effects on cells (12).

The rate-limiting enzyme responsible for conversion of gemcitabine to active antimetabolite is deoxycytidine kinase (dCK). This initial phosphorylation event leads to the rapid production of gemcitabine triphosphate, which competes with endogenous deoxycytidine triphosphate for incorporation into newly synthesized DNA (13). Gemcitabine triphosphate incorporation allows the addition of one further nucleotide before the blocked DNA polymerase is unable to add more nucleotides to the DNA strand (10, 14). This stalled replication is termed...
**Translational Relevance**

Gemcitabine has efficacy as a radiosensitizer in bladder cancer but with some toxic side effects, so biomarkers need to be found to optimize patient selection for this combination. We found that gemcitabine-resistant bladder cancer cells expressed reduced levels of the metabolizing enzyme deoxycytidine kinase (dCK), associated with abrogation of the radiosensitizing effects of gemcitabine. However, in mouse, xenographs from human gemcitabine-resistant bladder cancer cells displayed significant growth delay, but with high expression of dCK retained by the tumor vasculature and stroma. We hypothesize that radiosensitization of the vasculature is sufficient to achieve tumor cell kill. This preclinical observation implies that patients may respond to gemcitabine/radiotherapy even when their tumor is gemcitabine resistant, and suggests that dCK expression in the tumor vasculature and/or stroma, rather than in the tumor, may be a biomarker predictive of treatment outcome.

masked chain termination as the gemcitabine nucleotide is not recognized as misincorporation and can lead to replication fork collapse and subsequent DNA double-strand break formation.

Gemcitabine has also been reported to inhibit ribonucleotide reductase (RNR; ref. 15), which converts nucleotide 5'-diphosphates to deoxynucleotide diphosphates, generating the nucleotides required for DNA synthesis and repair. Gemcitabine diphosphate (dFdCDP) binds irreversibly to the active site of RNR, thus inhibiting production of nucleotide di- and triphosphates, leading to a decrease in intracellular pools of endogenous nucleotides (14). This depletion feeds back to dCK and stimulates further phosphorylation of gemcitabine thus potentiating the effects of the drug.

In a recent bladder cancer phase II clinical trial, weekly intravenous low-dose gemcitabine (100 mg/m²) was given as a radiosensitizer with promising results (16). Forty-six out of 50 patients recruited tolerated the treatment well, with a 3-year cause specific survival of 82% and overall survival of 75%. However, the underlying mechanisms of radiosensitization by low-dose gemcitabine have yet to be elucidated, so here we investigated the mechanisms both in vitro and in vivo.

**Materials and Methods**

**Cell lines**

CAL29, T24, and VMUC1B1 cell lines were purchased from DSMZ-German collection of Microorganisms and Cell Cultures (ACC-515, ACC-400 and ACC-376). DSMZ authenticates lines by microsatellite short tandem repeat DNA typing (http://www.dsmz.de/catalogues/catalogue-human-and-animal-cell-lines/quality-assurance/identity-authentication-of-cell-lines.html). RT112 cells were a gift from Margaret Knowles, University of Leeds (United Kingdom) and were authenticated by extensive genomic analysis (microsatellite typing, conventional karyotypic analysis, M-FISH, and array-based copy number analysis). Human umbilical vein endothelial cells (HUV EC) were purchased from Lonza and grown in EGM-2 medium. 3T3 cells were a kind gift from Eric O’Neill and were verified by short tandem repeat profiling (DNA Diagnostics Centre). RT112 and T24 cells were grown in RPMI medium (Sigma) supplemented with 10% FBS (Invitrogen). CAL29, VMUC1B1, and 3T3 were grown in DMEM (Invitrogen), supplemented with 10% FBS. All cells were maintained at 37°C in 5% CO₂ in exponential growth phase. Resistant cell lines were generated over 3 months by gradually increasing doses of drug added to the medium, until cells reached resistance to 10 μmol/L gemcitabine. These cell lines were named RT112gem and CALgem.

**Western blot analyses**

Cells were processed for Western blotting as outlined in Supplementary Methods. Antibodies used were rabbit polyclonal RRM1-10526–1-AP (ProteinTech Group), mouse monoclonal RRM2 MCA3434Z (AbD Serotec), rabbit polyclonal dCK ab96599 (Abcam), rabbit polyclonal CDA ab82347 (Abcam), rabbit polyclonal hENT1 ab48607 (Abcam), γH2AX 05–636 (Millipore), H2AX 07–627 (Millipore), and mouse monoclonal β-actin ab8226 (Abcam).

**Clonogenic assays**

Cells (1 × 10⁵) were seeded in duplicate onto 90 mm tissue culture dishes and treated with appropriate concentrations of gemcitabine 24 hours later. After a further 24 hours, cells were trypsinized, counted, and replated onto 90 mm dishes immediately before ionizing radiation (IR) treatment. Cells were irradiated with Cs-137 at a dose of 1.7 Gy minute⁻¹ in a GSR D1 irradiator (Gamma-Service Medical GmbH). After 2 weeks, cells were washed and fixed in 93% methanol, 7% acetic acid and stained with Coomassie blue (Sigma). Colonies containing >50 cells were counted using a Colcount automated cell counter (Oxford Optronix).

**siRNA transfection**

Cells (5 × 10⁴) were plated 24 hours before treatment with 50 nmol/L siRNA (Invitrogen). BLOCK-it Fluorescent Oligo (Invitrogen) was used to assess transfection efficiency (Supplementary Fig. S1). siRNA was combined with Oligofectamine (Invitrogen) and added to serum-free medium for 4 hours before replenishment with medium containing 10% v/v FBS. Cells were then either harvested for Western blot analysis 48 hours later, or trypsinized and replated for clonogenic assay. BLOCK-it Fluorescent Oligo treated samples were fixed with 4% formaldehyde 6 hours after transfection and transfection efficiency determined by fluorescence microscopy.
dCK overexpression

Full-length dCK was cloned into pcDNA3.1 (Invitrogen) from cDNA generated from RT112 cells using the following primers:

dCK F-5’-AAAGTCAAAACCGACACCC-3’; dCK-R 5’-TTGGCTGCTTGATCCTAC-3’.

Ten micrograms of DNA was transfected into subconfluent cells with 30 µL Fugene HD (Roche) and expression verified by Western blot analysis 24 to 48 hours posttransfection.

Flow cytometry

Cells were scraped in PBS 24 hours after treatment with gemcitabine or dFdU and spun at 1,000 rpm for 5 minutes. Cell pellets were resuspended in PBS containing 40 µg/mL propidium iodide (Sigma) and 100 µg/mL RNase A, incubated at 37°C for 1 hour and then analyzed on a FACScan flow cytometer (Beckton Dickinson). Cell-cycle phases were modeled using ModFit LT (Verity Software House).

qRT-PCR

Relative quantitation of dCK in cell lines was measured using the ΔΔCt method with dCK TaqMan probe Hs01176127_m1 (catalog number 763630, Applied Biosystems) and normalized to ACTB, using TaqMan probe Hs00176127_m1 (catalog number 785259, Applied Biosystems). Samples were run on an Applied Biosystems 7500 Fast Real-Time PCR system.

Determination of deoxynucleotides by HPLC

Cells (2 × 10^5–1 × 10^6) were treated with 20 nmol/L gemcitabine for 24 hours before trypsinization, counting, and extraction of deoxynucleotides with 100 µL cold 6% trichloroacetic acid. Samples were then processed for high performance liquid chromatography (HPLC) as outlined in Supplementary Methods.

Xenograft model

Cells (2 × 10^6 cells/ml) were prepared in phenol red-free Matrigel (BD Biosciences) and 50 µL injected into the flank of 6-week-old female athymic nude mice (Harlan Laboratories). Tumors were measured daily with callipers, using the formula a × b × c × π/6 and were allowed to reach 100 mm³ before a single intraperitoneal injection of 100 mg/kg gemcitabine, followed by daily doses of 2 Gy IR for 5 consecutive days, as appropriate. All work was done in accordance with UK Home Office Guidelines and under project license PPL 30/2922 at the University of Oxford (Oxford, United Kingdom).

IHC

A formalin-fixed paraffin-embedded human bladder tumor sample was purchased from ProteoGenex, Inc. Untreated CAL29 and CALgem xenografts (n = 2 each) were also processed; the CAL29 xenografts were less than 100 mm³. IHC staining was performed on 4-µm thick formalin-fixed paraffin-embedded tissue sections using the Novolink Polymer Detection System (Novocastra Laboratories) and analyzed as outlined in the Supplementary Methods. Rabbit polyclonal anti-dCK (Abcam, ab96599) was used at 1:100 dilution, rabbit polyclonal anti-CD31 (Abcam, ab28364) at 1:100 dilution, all incubated for 1 hour at room temperature.

Statistical analysis

All statistical analyses were two sided and the statistical analysis was performed using GraphPad Prism (version 4.0b; GraphPad Software Inc). Statistical significance was defined as P < 0.05. Student t test was used to compare the mean values between two groups and two-way ANOVA used to compare drug and radiation interactions. Data are presented as mean values and SEM.

Gemcitabine was determined to have a supra additive effect on all cell lines at doses of IR of 4 Gy and above, using the isobologram technique previously described (17).

Results

Gemcitabine cytotoxicity and levels of key proteins in its metabolism differ between bladder cancer lines

All four bladder cancer cell lines were sensitive to gemcitabine in the nanomolar range, with IC_{50}s of 14 nmol/L for RT112 (95% confidence interval (CI) 12.6–14.5 nmol/L), 10 nmol/L for T24 (9.5–11.1 nmol/L), 38 nmol/L for CAL29 (34–41 nmol/L), and 40 nmol/L for VMCUB1 (35–46 nmol/L), respectively, as determined by clonogenic assay (Fig. 1A). There was no consistent pattern of changes in protein levels in five proteins described as key regulators of gemcitabine cellular uptake and metabolism across the cell lines in response to gemcitabine, IR, and combination gemcitabine/IR by Western blot analysis (Fig. 1B and Supplementary Fig. S2).

Radiosensitization of each cell line was tested after 24-hour treatment with 10 and 20 nmol/L gemcitabine or equivalent concentrations of its metabolite dFdU (Fig. 1C). dFdU caused no radiosensitization (all P = NS) but gemcitabine radiosensitized all four cell lines effectively, with sensitizer enhancement ratios (SER) ranging from 1.07 to 1.3 for 10 nmol/L gemcitabine and 1.42 to 1.63 for 20 nmol/L gemcitabine [Fig. 1D; all P < 0.01 except VMCUB1 untreated (UT) vs. 10 nmol/L gem P = 0.315, see Fig. 1D legend for exact P values]. Interestingly, dCK protein levels correlated with SER by linear regression (r² = 0.804; Fig. 1E). Gemcitabine treatment was associated with a large accumulation of cells in S phase, while dFdU had no impact on cell cycle (Fig. 1F). The extent of S phase accumulation in the four cell lines was inversely correlated with gemcitabine IC_{50} (Supplementary Fig. S3). These data suggest that gemcitabine radiosensitizes bladder cancer cells through induction of stalled replication forks. In support of this, γH2AX induction was detectable by Western blot analysis following treatment of cells with gemcitabine and gemcitabine/IR, more so than for IR alone (Fig. 1B).

siRNA knockdown of dCK reduces sensitivity to gemcitabine

We hypothesized that altering expression levels of proteins involved in gemcitabine uptake and metabolism by
siRNA knockdown would elucidate their relative importance in gemcitabine cytotoxicity and radiosensitization. In the RT112 cell line, which had intermediate radiosensitization by gemcitabine, we transiently knocked down RRM1, hENT1, CDA, and dCK and determined effects on clonogenic cell survival (Fig. 2A and B and Supplementary Fig. S2). We assessed siRNA transfection efficiency by microscopy 6 hours after transfection of a control, fluorescent siRNA and found that all cells analyzed had taken up siRNA (Supplementary Fig. S1).

RRM1 of the RNR complex encodes the regulatory subunit, required by both RRM2 and RRM2B (a p53 inducible subunit; ref. 18). Seventy percent knockdown of RRM1 had a large impact on cell viability (Fig. 2C) but did not result in intrinsic radiosensitization (P = 0.937; Fig. 2E). Gemcitabine was still able to induce radiosensitization in RRM1 siRNA knockdown cells (P = 0.0003), indicating that the mechanism for radiosensitization does not require RNR inhibition. Further evidence supporting lack of RNR inhibition in radiosensitization of bladder cells was that deoxyribo- nucleotide levels in RT112 cells treated with 20 nmol/L gemcitabine for 24 hours did not show a significant decrease in deoxyribo- nucleotide pools (Fig. 2D). In contrast, the most striking change in deoxyribo- nucleotide pools was a 3.6-fold increase in dTTP levels.

Knockdown of CDA to 30% of control levels had no effect on gemcitabine cytotoxicity (P = 0.72; Fig. 2B), but reduction in CDA radiosensitized RT112 cells, even in the absence of gemcitabine (CDA siRNA vs. NSC, P = 0.038; Fig. 2E). Reduction in hENT1 protein by 50% resulted in a slight increase in resistance to gemcitabine, with IC50 increasing from 14 nmol/L to 18 nmol/L (P = 0.024, 95% CI, 16.5–18.5 nmol/L; Fig. 2B), but there was no significant change in radiosensitization compared with nonsilencing control (NSC), with 20 nmol/L gemcitabine radiosensitizing both cell lines (P < 0.0001 for both hENT1 siRNA and NSC; Fig. 2E). However, a 70% reduction in dCK protein induced resistance to gemcitabine, with an increase in IC50 from 14 nmol/L gemcitabine to 47.5 nmol/L (P < 0.0001, 95% CI, 31–56 nmol/L; Fig. 2B). Knockdown of dCK also significantly reduced the radiosensitizing effect of gemcitabine (P = 0.023; Fig. 2E). Complete knockdown of dCK was not achieved (Fig. 2A) and this is likely to account for some of the radiosensitization seen with 20 nmol/L gemcitabine.

Resistance to gemcitabine is achieved by reduction of dCK

We generated RT112 and CAL29 cell lines resistant to gemcitabine and to dFdU by gradually increasing the dose of each nucleoside over several passages. Having found no radiosensitization by dFdU, we reasoned that changes in expression of proteins seen in the gemcitabine-resistant cells that did not occur in the dFdU-resistant cells would be of greatest interest. In both gemcitabine-resistant cell lines (RTgem and CALgem), a marked reduction in dCK was observed, consistent with this enzyme being the rate-limiting factor for gemcitabine metabolism to active antimetabolite (Fig. 3A). Reduction in dCK protein level was commensurate with a significant reduction at the mRNA level, as measured by qRT-PCR (RT112 vs. RTgem, P < 0.0001, CAL29 vs. CALgem P < 0.0001; Fig. 3B). The reduction in dCK levels in both gemcitabine-resistant cell lines rendered them refractive to the radiosensitizing effects of 20 nmol/L gemcitabine (RTgem vs. RTgem + 20 nmol/L gem, P = 0.45, CALgem vs. CALgem + 20 nmol/L gem, P = 0.31; Fig. 4A). Reexpression of dCK in the CALgem cell line (Fig. 4B) was sufficient to resensitize these cells to gemcitabine, and was also sufficient to reestablish gemcitabine radiosensitivity, highlighting the importance of this enzyme in the response to gemcitabine (Figs. 4C and D).

Gemcitabine-resistant xenografts still respond to gemcitabine with IR

CAL29 and CALgem cells were implanted into the flanks of mice. In line with a previously published study (19), we found CAL29 xenografts to be poorly tumorigenic and an insufficient number of xenografts reached the size required for treatment. CALgem xenografts were allowed to grow to a size of 100 mm³ before being treated with daily IR, gemcitabine single dose, or IR/gemcitabine at a gemcitabine dose of 100 mg/kg. Unexpectedly, xenografts treated with combination IR/gemcitabine had a significant growth delay compared with the xenograft treated with IR alone (29.9 days to triple volume for combination IR/gemcitabine vs. 16.6 days for IR alone (95% CIs, 27.3–32.4 days vs. 15.2–18 days, P = 0.002; Fig. 5A). After mouse sacrifice, tumor cells were grown out in vitro as explants in 10 µmol/L gemcitabine, and clonogenic survival assay demonstrated no radiosensitization with 20 nmol/L gemcitabine (Fig. 4A). Western blot

Figure 1. Low-dose gemcitabine is a radiosensitizer in bladder cancer cells. A, clonogenic assay to determine gemcitabine cytotoxicity in a panel of bladder cancer cell lines. Subconfluent cells were treated with 20 nmol/L gemcitabine before plating 600 to 2,000 cells per dish, depending on cell line, and colony formation was measured 2 weeks later. B, Western blot analyses for proteins involved in the uptake and metabolism of gemcitabine (n = 3). RT112, CAL29, T24, and VMUB21 bladder cancer cell lines were treated with 20 nmol/L gemcitabine for 24 hours and with 5 Gy IR where appropriate, collected 2 hours after treatment, and immunoblotted for RRM1 and hENT1, CDA, and dCK. Equal loading was confirmed by blotting for total H2AX, and DNA damage was confirmed by blotting for γH2AX. C, cells were treated as in A and subsequently treated with IR immediately after plating. Colonies were counted 2 weeks after plating. D, SEERs at 10% survival. P values for comparisons were calculated using two-way ANOVA and were as follows: RT112: UT versus 10 nmol/L gem P = 0.308; UT versus 20 nmol/L gem P < 0.0001; CAL29; UT versus 10 nmol/L gem P = 0.004, UT versus 20 nmol/L P < 0.0001; T24; UT versus 10 nmol/L gem P = 0.002, UT versus 20 nmol/L gem P < 0.0001; VMUB21: UT versus 10 nmol/L gem P = 0.315, UT versus 20 nmol/L gem P = 0.0013. E, correlation between dCK protein levels and SER. dCK protein levels were determined by densitometry of Western blot analyses from four independent experiments. These values were plotted against SEERs obtained from clonogenic assays performed in triplicate. F, cell-cycle analysis of gemcitabine- and dFdU-treated bladder cancer cell lines. Cells in exponential growth phase were incubated with gemcitabine or dFdU for 24 hours before fixing and staining with propidium iodide.
analysis confirmed that protein levels of dCK remained undetectable in these explants (Fig. 4B). This intriguing result led us to study dCK expression in the tumor vasculature, in parallel with CD31, an endothelial marker used to assess tissue angiogenesis. We found strong positive nuclear staining for dCK in tumor stroma and the vasculature in both CALgem mouse xenografts and a human muscle-invasive bladder tumor (Fig. 5B); this coincided with CD31 positivity in the vessel linings. Quantitative analysis of three independently stained slides from one CAL29 xenograft was strongly positive for dCK in 70% of nuclei within the tumor and in 66% of nuclei in the stroma and vasculature, whereas in three replicates of a CALgem xenograft, only 18% of tumor cell nuclei were dCK positive with 61% positive nuclei in the stroma and vasculature (Fig. 5C and Supplementary Table S1).
Gemcitabine radiosensitizes mouse fibroblasts and human vascular endothelial cells

Having established that the CALgem explants had remained dCK negative and were refractive to gemcitabine, we tested the ability of gemcitabine to radiosensitize 3T3 mouse fibroblasts and HUVECs, as in vitro models for xenograft stroma and vasculature, respectively. Gemcitabine radiosensitized 3T3 cells, with an SER of 1.29 for 20 nmol/L gemcitabine. We found 20 nmol/L gemcitabine to be almost completely inhibitory for colony formation in HUVECs (Supplementary Fig. S4), but found that a dose as low as 1 nmol/L gemcitabine was sufficient to radiosensitize these cells with an SER of 1.39 and SER increased to 1.98 with 5 nmol/L gemcitabine (Fig. 6A). Western blot analysis demonstrated stronger expression of dCK in HUVECs than in 3T3 cells, consistent with our finding that dCK levels correlate with SER (Fig. 6B). We then sought to establish if stromal response to gemcitabine could influence the sensitivity of CALgem cells by performing a coculture assay. We found that in this assay, 3T3 cells were still radiosensitized by gemcitabine, but that colony formation of CALgem cells was completely unaffected (Fig. 6C). Following this, we plated a monolayer of 3T3 cells that were treated with and without 20 nmol/L gemcitabine, upon which we seeded CALgem. Similarly, there was no significant difference in colony formation of CALgem cells using this method (Fig. 6D).

Discussion

Gemcitabine is an effective radiosensitizer in muscle-invasive bladder cancer but also carries side effects, including diarrhea, which can be dose limiting (16). An understanding of the mechanisms of action of gemcitabine as a radiosensitizer and the enzymes involved in its metabolism might help drive the search for appropriate biomarkers of patient response, allowing optimum selection of patients for this combined treatment. We have shown that gemcitabine radiosensitizes bladder cancer cells at low nanomolar concentrations and the primary mode of radiosensitization seems to be via the activity of dCK.

hENT1 knockdown to 50% of control levels only resulted in a small increase in gemcitabine resistance and did not significantly alter its radiosensitizing effects. However, in vitro expression of hENT1 does increase sensitivity to gemcitabine (9, 20), and a recent study has found that high hENT1 expression in bladder tumor cells is a prognostic biomarker for prolonged survival after gemcitabine treatment (21). In pancreatic cancer, high hENT1 has been identified as a predictive marker for response to gemcitabine (22). Although siRNA knockdown of hENT1 had a limited effect on gemcitabine cytotoxicity or radiosensitization, it should be noted that knockdown in our study was not particularly effective (approximately 50%); this may be sufficient to allow entry of low concentration gemcitabine.

RNR inhibition had a significant impact on cell viability (Fig. 2C) but 70% reduction of RRM1 by siRNA did not significantly alter the radiosensitizing effects of gemcitabine. The decreased plating efficiency of RRM1 knockdown cells might argue that gemcitabine does not inhibit RNR at low concentration and that, although RNR is important for cell viability, it is not a major contributor to the radiosensitization caused by gemcitabine. Moreover, RRM1 knockdown itself does not lead to radiosensitization of these cells, whereas gemcitabine still radiosensitizes, so RRM1 inhibition does not seem to be contributing to this radiosensitization.
RRM2 inhibition with the HDM-2 inhibitor or knockdown with siRNA synergistically enhances gemcitabine cytotoxicity, which also suggests that gemcitabine effects on RNR are not major (23, 24). Although we did not observe dramatic reduction in deoxynucleotide pools, we did observe a 3.6-fold increase in dTTP levels. This may be due to upregulation of thymidine kinase 1 (TK1), which has been shown to be upregulated during S phase (25) and after genotoxic stress (26). Interestingly, it has been observed that nucleotide pool perturbations cause genomic instability (27, 28) and in particular elevated dTTP levels are sufficient to cause instability (29).

Knockdown of CDA to 30% of control levels was sufficient to radiosensitize cells, even in the absence of gemcitabine, while addition of gemcitabine further radiosensitized cells. This implies that CDA is important in maintaining normal nucleotide pool balance, but given that depletion of CDA did not increase the SER of gemcitabine, we hypothesize that deamination of gemcitabine is not a major negative regulator of gemcitabine activity at low dose.

Knockdown of dCK did not fully abolish radiosensitization by gemcitabine, but this is likely due to only partial knockdown, calculated to be a 70% reduction compared with control cells (Fig. 2A and Supplementary Fig. S2). However, potent reduction in dCK levels in gemcitabine-resistant cells was observed at both the level of protein and mRNA expression and these cells were not radiosensitized by gemcitabine (Fig. 3A). Plasmid-based overexpression of dCK in resistant cells was sufficient to restore gemcitabine sensitivity in RTgem and CALgem bladder cancer cell lines. A, radiation clonogenic survival curves. RTgem (left), CALgem (middle) (resistant to 10 μmol/L gemcitabine), and CALgem explant (right) cell lines were treated with 20 nmol/L gemcitabine for 24 hours before replating and irradiation, B, Western blot analysis for expression of dCK in CAL29, CALgem, and CALgem explant cell lines and β-tubulin serves as a marker for equal loading (n = 4). C, gemcitabine cytotoxicity clonogenic assay. D, cells treated as for Fig. 1C. Colonies were counted 2 weeks after plating.
sensitivity, indicating that dCK is of key importance in the response to gemcitabine, as observed by others (30–33).

Surprisingly, in a xenograft model, gemcitabine-resistant tumors responded better to combination gemcitabine and irradiation than either treatment alone. This result was unexpected as the gemcitabine-resistant cells (CALgem) had been shown not to be radiosensitized by gemcitabine in vitro (Fig. 4A). This pointed toward the possibility that the vasculature/stroma formed within the tumor remained dCK positive and that perhaps radiosensitization of the vasculature by gemcitabine is sufficient to cause tumor kill. This hypothesis is strengthened by our IHC evidence of dCK colocalization with CD31 staining. Our cell line experiments investigating stromal and vascular sensitivity to gemcitabine also favor this model. Furthermore, our coculture experiments suggest that radiosensitization of gemcitabine-resistant tumors is not a direct effect caused by a diffusible stromal factor but is an indirect effect mediated by elevated stromal dCK and thus radiosensitization of the stroma, ultimately leading to tumor cell killing.

This finding warrants further investigation, as it implies that patients may respond to gemcitabine/IR even when their

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**Figure 5.** Tumor growth delay after gemcitabine and IR. A, mice bearing subcutaneous gemcitabine-resistant CALgem xenografts were randomly assigned to vehicle or treatment (gemcitabine, 5 consecutive days of 2 Gy IR, or gemcitabine and 5 × 2 Gy IR) groups when tumor volumes were approximately 100 mm³, and tumors were measured every 1 to 3 days. Mice were sacrificed when their tumor volume reached 400 mm³. (* denotes significance <0.001). B, IHC analysis of xenograft tumor vasculature. Thin walled vessel stained using CD31 (left), dCK (middle), and hematoxylin and eosin (right) in CALgem xenograft (top) and a larger vessel in human stage T2 muscle invasive bladder tumor (bottom). C, IHC analysis for dCK (left and middle) and hematoxylin and eosin (right) of xenografts from CAL29 cells (top) and CALgem (bottom). Middle panels represent dCK IHC scoring of nuclei using the Aperio nuclear V9 algorithm, performed for three independently stained replicates of the same tumors.

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tumor is gemcitabine resistant. We are now measuring gemcitabine and metabolite levels in blood and urine from patients receiving gemcitabine/IR (http://www.clinicaltrials.gov/ct2/show/NCT01343121) to look for prognostic biomarkers, and will study dCK expression by IHC in their pretreatment tumor specimens. It will be of particular interest to study dCK expression in the tumor vasculature and/or stroma as a potential biomarker of treatment outcome.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: M. Kerr, A.E. Kiltie
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Figure 6. Gemcitabine radiosensitizes mouse fibroblasts and human vascular endothelial cells. A, radiation clonogenic survival curves. 3T3 cells (left) were treated with 20 nmol/L gemcitabine for 24 hours before replating and irradiation. HUVECs (right) were treated with 1 and 5 nmol/L gemcitabine for 24 hours before replating and irradiation. B, Western blot analysis for expression of dCK in 3T3 and HUVEC cell lines and β-actin serves as a marker for equal loading (n = 2). C, coculture radiation clonogenic survival curves. 3T3 and CALgem cells were treated with 20 nmol/L gemcitabine separately for 24 hours, before being replated together and irradiated. D, coculture clonogenic survival. 3T3 cells were grown as a monolayer, before treatment with 20 nmol/L gemcitabine for 24 hours. CALgem cells were subsequently seeded on top of 3T3 cells and irradiated.
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