Targeting the Warburg effect with a novel glucose transporter inhibitor to overcome gemcitabine resistance in pancreatic cancer cells

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Gemcitabine resistance remains a significant clinical challenge. Here, we used a novel glucose transporter (Glut) inhibitor, CG-5, as a proof-of-concept compound to investigate the therapeutic utility of targeting the Warburg effect to overcome gemcitabine resistance in pancreatic cancer. The effects of gemcitabine and/or CG-5 on viability, survival, glucose uptake and DNA damage were evaluated in gemcitabine-sensitive and gemcitabine-resistant pancreatic cancer cell lines. Mechanistic studies were conducted to determine the molecular basis of gemcitabine resistance and the mechanism of CG-5-induced sensitization to gemcitabine. The effects of CG-5 on gemcitabine sensitivity were investigated in a xenograft tumor model of gemcitabine-resistant pancreatic cancer. In contrast to gemcitabine-sensitive pancreatic cancer cells, the resistant Panc-1 and Panc-1GemR cells responded to gemcitabine by increasing the expression of ribonucleotide reductase M2 catalytic subunit (RRM2) through E2F1-mediated transcriptional activation. Acting as a pan-Glut inhibitor, CG-5 abrogated this gemcitabine-induced upregulation of RRM2 through decreased E2F1 expression and inhibition of cell survival. This CG-5-induced inhibition of E2F1 expression was mediated by the induction of a previously unreported E2F1-targeted miRNA, miR-520f. The addition of oral CG-5 to gemcitabine therapy caused greater suppression of Panc-1GemR xenograft tumor growth in vivo than either drug alone. Glut inhibition may be an effective strategy to enhance gemcitabine activity for the treatment of pancreatic cancer.

Introduction

Pancreatic cancer is the fourth leading cause of cancer death in the United States. Prognosis remains dismal, with a 5 year survival of <5% for all stages. Surgical resection followed by adjuvant therapy offers the only chance for cure; however, <15% of patients present with resectable disease. Cytotoxic chemotherapy with gemcitabine continues to be the standard of care and the backbone of experimental regimens in advanced pancreatic cancer for over a decade (1). Gemcitabine-based regimens will likely remain a mainstay of therapy for this disease in the foreseeable future, especially in light of the recent results of the Phase III MPACT trial, which showed that the addition of nab-paclitaxel to gemcitabine yielded clinically meaningful improvements in survival in metastatic pancreatic cancer (2). Additionally, adjuvant chemotherapy with gemcitabine has been shown to improve overall survival of patients with macroscopically resected pancreas cancer (3). However, a major issue associated with gemcitabine use remains the occurrence of cancer cell resistance (4). Given the wide applicability and use of gemcitabine in pancreatic cancer across all stages, development of novel strategies to reverse gemcitabine resistance continues to be an urgent need. However, minimal progress has been made to improve survival for patients with gemcitabine-based combination regimens, which is attributable the development of drug resistance (4). Evidence indicates that pancreatic cancer cells develop gemcitabine resistance by altering the transport or metabolism of gemcitabine (5–8) or through the upregulation of the PI3K/Akt survival signaling axis or DNA repair (9). In this study, our results indicate that gemcitabine-resistant pancreatic cancer cells exhibit an ability to sharply increase the expression of ribonucleotide reductase M2 catalytic subunit (RRM2) upon exposure to gemcitabine through E2F1-mediated transcriptional activation. This gemcitabine-induced RRM2 upregulation represents a DNA damage response that enhances the cellular DNA repair capability, thereby underlying the resistant phenotype of these cells. From a mechanistic perspective, targeting this gemcitabine-induced RRM2 upregulation represents a therapeutically relevant strategy to overcome drug resistance.

Cells undergoing malignant transformation often exhibit a shift in cellular metabolism from oxidative phosphorylation (OXPHOS) to glycolysis, known as the Warburg effect, to gain growth advantage (10,11). Evidence indicates that pancreatic carcinomas show increased glucose utilization, as indicated by 13C-fluorodeoxyglucose accumulation, as a result of overexpression of glucose transporters (Glut) (12). This glycolytic shift enables cancer cells to adapt to low-oxygen environments, to produce biosynthetic building blocks needed for cell proliferation, to acidify the local environment to facilitate tumor invasion, and to generate NADPH and glutathione through the pentose phosphate shunt to increase resistance to oxidative stress (10,11). As the Warburg effect is considered a fundamental property of neoplasia, targeting glycolysis represents a therapeutically relevant strategy for cancer treatment.

In the course of developing a novel class of Glut inhibitors (13,14), we discovered that these inhibitors, as represented by the proof-of-concept compound CG-5, effectively counteracted the ability of gemcitabine to activate E2F1-mediated RRM2 gene expression in resistant pancreatic cancer cells. As DNA repair capacity represents a determining factor in chemotherapeutic sensitivity, this unique mechanism sensitized resistant pancreatic cancer cells in vitro and in vivo by augmenting gemcitabine-induced DNA damage. Moreover, we identify a novel mechanism by which CG-5 downregulates E2F1 expression through posttranscriptional regulation by miR-520f, a relatively uncharacterized member of the miR-520 family of microRNAs (miRNAs), of which other members have been implicated as having tumor-suppressive functions in various cancers, including those of the pancreas, breast and liver (15–17).

Materials and methods

Cell culture and reagents

Non-malignant human primary pancreatic cells (NPC) were purchased from Applied Biological Materials (Richmond, British Columbia, Canada) and cultured in Prigrow 1 medium containing 10% fetal bovine serum. The human pancreatic cancer cell lines Panc-1, AsPC-1 and BxPC-3 were obtained from American Type Culture Collection (Manassas, VA), which

Abbreviations: 2-DG, 2-deoxyglucose; Glut, glucose transporter; miRNA, microRNA; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bro- mide; NPC, non-malignant human primary pancreatic cells; OXPHOS, oxida- tive phosphorylation; PBS, phosphate-buffered saline; PKM2, pyruvate kinase M2; RT–PCR, reverse transcription–polymerase chain reaction; UTR, untrans- lated regions.
authenticates human cells line in their collection using short tandem repeat analysis, and were maintained in RPMI 1640 medium (Invitrogen, Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum and penicillin/streptomycin. Gemiactin-resistant cells (Panc-1	extsuperscript{1,000k}, BxPC-3	extsuperscript{1,000k} and AsPC-1	extsuperscript{1,000k} cells) were generated from the respective cell lines by subcloning. Cells were expanded through 12 passages by selecting cells from concentrations of 0.1 to 1 µM, for 1–4 months. CG-5 was synthesized in our laboratory as described previously (18). Antibodies used and their sources are as follows: RRM1, RRM2, Sp1, NF-YA (Santa Cruz Biotechnology, Dallas, TX); E2F1, hENT1, TS (Cell Signaling Technology, Danvers, MA); MitoProfile® Total OXPHOS human WB antibody cocktail (Abcam, Cambridge, MA); β-actin (MP Biomedicals, Irvine, CA); goat anti-rabbit IgG-HRP conjugates, rabbit anti-mouse IgG-HRP conjugates (Jackson ImmunoResearch Laboratories, West Grove, PA).

Tissue collection

Primary pancreatic tumor and adjacent non-tumor tissues were collected from patients who had undergone resection for pancreatic ductal adenocarcinoma at the Ohio State University Comprehensive Cancer Center-James Cancer Hospital (Columbus, Ohio). Tissue samples were flash frozen immediately after resection. Use of these clinical specimens was reviewed and approved by the Ohio State University Institutional Review Board.

Cell viability and colony formation assay

Cell viability was determined by 3-(4,5-dimethylthiazol-2-y)-2,5-diphe- nylnitrazolium bromide (MTT) assays. Cells were seeded at 3 x 10⁴ cells per well in 96-well plates 24 h before treatment. For colony formation assays, cells were seeded at a density of 1 x 10³ cells per 6-cm dish. After 24 h, cells were exposed to different concentrations of gemcitabine for 1 day, with changes of drug-containing medium every 3 days thereafter. After 12 days of treatment, cells were fixed with 4% formaldehyde in phosphate-buffered saline (PBS) and stained with a 0.5% crystal violet solution in 25% methanol. Colonies of >50 cells were counted. IC₅₀ values of drug’s suppressive effects on cell viability and clonogenic survival were determined from the median-effect plots of the dose-response curves by using Compusyn software (3.0.1, CombinX, Paramus, NJ). Combining of CG-5 with gemcit- abine were evaluated in Panc-1	extsuperscript{1,000k} cells in colony formation assays using a non-constant ratio design. Data were analyzed for synergistic effects using the median-effect method (19), and combination indices were determined using Compusyn software.

Transient transfection and luciferase assay

Cells were transfected with various plasmids using Lipofectamine (Invitrogen), according to the manufacturer’s instructions. Cells were then seeded into six-well plates (5 x 10⁴ cells per well) and incubated in 10% fetal bovine serum-containing medium for 24 h before drug treatment. For shRNA experiments, cells were transfected with scrambled or E2F1 shRNA (Santa Cruz) and seeded into six-well plates (5 x 10⁴ cells per well) for drug treatments and subsequent analyses. For the luciferase assay, Panc-1 cells expressing RRM2 promoter-luciferase and herpes simplex virus thymidine kinase promoter- Renilla reni- forms luciferase were prepared as we described previously (20).

Reverse transcription–polymerase chain reaction

Total RNA was isolated from drug-treated cells with TRIzol (Invitrogen) and then reverse-transcribed to complementary DNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). For semiquantitative PCR, products were resolved by electrophoresis on 1% agarose gels and visualized by ethidium bromide staining. The sequences of the primers used were as follows: RRM2, forward 5'-GCT TGG TGT CGA CAA GGA GAAC-3' and reverse 5'-CCT CTG CTT CTT TAAT CAT C-3'; hENT1, forward 5'-TCT CTCT CAT AGG GCT GCC ATC TTCC-3' and reverse 5'-GCA GTA ACC ATC TGC A-3'; NF-YA, forward 5'-CCA AAG CTG TCT TGG GAG A-3' and reverse 5'-GAA CTG TGG GAT GAA G-3'; reverse 5'-CAT ATGG GAT GAA GGA-3' and reverse 5'-CAT ATGG GAT GAA GGA-3'; Sp1, forward 5'-GGCC AGC GCG AGC CCT ATG G-3' and reverse 5'-GAT GCG TCT CAT GAT GAT G-3'; NF-YA, forward 5'-AGA CTG TGG GAT GAA GGA-3' and reverse 5'-GAGG TCT CAG GCA CAG CAC TAC GCA-3'; reverse 5'-CTG CTA GAG CCA GCA A-3' and reverse 5'-GTA GAA GGA GGA GAA G-3'.

Glucose uptake assay and Glut isoform detection

For the glucose uptake assay, Panc-1 cells overexpressing specific Glut isoforms were seeded in six-well plates (3 x 10⁴ cells per well) for 24 h. Cells were washed twice with Krebs–Ringer phosphate buffer (126 mM NaCl, 2.5 mM KCl, 25 mM NaHCO₃, 1.2 mM Na₂HPO₄, 1.2 mM MgCl₂, 2.5 mM CaCl₂, pH 7.4) and were then treated with individual agents in Krebs–Ringer phosphate buffer. After 15 min, glucose uptake was initiated by adding 1 mM Krebs–Ringer buffer containing 100 mM 3-[3H]-deoxyglucose (2-DG) (PerkinElmer Life Science) and 100 mM non-radioactive 2-DG and was terminated by washing with cold PBS. The cells were lysed in 500 µl lysis buffer (10 mM Tris–HCl, pH 8.0, 0.1% sodium dodecyl sulfate), and aliquots were taken for measurement of radioactivity using a scintillation counter (Beckman LS6500). The Glut isoform expression profiles in pancreatic cancer cell lines were determined by real-time PCR using the Bio-Rad CFX96 Real-Time PCR Detection System. The sequences of the primers used were described previously (18).

Relative gene expression was normalized to 18s rRNA and calculated using the 2⁻ΔΔCt method (21).

Construction of the 3′-untranslated region–luciferase plasmid and reporter assays

The E2F1 3′-untranslated regions (UTR) target sites was amplified by PCR using the primers Fwd-5′-AGCTTTTGAATTCAACACCTCTGTTGACTGACG-3′ and Rev-5′-GAGACTATGCTTCCAGACACTGACGAGG-3′ and cloned downstream of the luciferase gene in the pMIR-REPORT luciferase vector (Ambion). This vector was sequenced and named WT-E2F1-3′-UTR. Site-directed mutagenesis of the miR-520f target site in the E2F1-3′-UTR was carried out using the Quick-change mutagenesis kit (Stratagene) and named Mut E2F1-3′-UTR, in which WT-E2F1-3′-UTR was used as a template. For reporter assays, the cells were transiently transfected with wild-type or mutant reporter plasmid using Lipofectamine 2000 (Invitrogen). Reporter assay was performed at 36 h posttransfection using the BriteLite plus reporter gene assay system (Perkin Elmer, Shelton, CT).

Quantitative reverse transcription–polymerase chain reaction assays for mature miRNA

The reverse transcription reactions of cell lines or human pancreatic non-tumor and tumor tissue cancer specimens were performed in a reaction containing 50 ng small RNA. Amplification and detection of specific products were performed with Bio-Rad CFX Manager 2.1 detection system with the cycle profile according to the miScript PCR starter kit (QIAGEN). The relative gene expression was calculated by comparing the cycle times for each target PCR. The target PCR Ct values were normalized by subtracting the internal control of U6 rRNA Ct value.

Stable cell generation

Panc-1 cells were transfected with pEP Null and pEP miR-520f vectors (Cell Biolabs, San Diego, CA) and selected with puromycin (0.5 µg/ml).

Immunofluorescent imaging

Treated cells were washed with cold PBS, fixed with 4% formaldehyde in PBS for 10 min at 37°C, permeabilized with 0.5% Triton X-100 in PBS for 5 min at room temperature, and then blocked with 5% bovine serum albumin in PBS for 1 h at room temperature. After washing with PBS, the cells were incubated with primary antibody against RRM2 or γ-H2AX in PBS containing 1% bovine serum albumin overnight at 4°C and then with secondary antibody conjugated to Alexa Fluor 488 for 1 h at room temperature. Nuclei were stained with 4′,6-diamidino-2-phenylindole contained in the Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Confocal images were obtained with an Olympus FV1000 confocal microscope (Olympus Corp., Japan).

In vivo study

Ectopic xenograft tumors were established in female athymic nude Foxn1nu mice (5–7 weeks of age; Harlan Laboratories, Indianapolis, IN) by subcutane- ous injection of 2 x 10⁶ Panc-1 cells in a total volume of 0.1 ml of PBS con- taining 50% Matrigel (BD Biosciences, San Jose, CA). To assess the activity of CG-5 on gemcitabine sensitivity, mice with established tumors (mean starting tumor volume, 53.7 ± 8.0 mm³) were randomized to four groups (n = 6–8) that received the following treatments: (i) CG-5 alone (200 mg/kg per os by gavage); (ii) gemcitabine alone (100 mg/kg, intraperitoneally [i.p.]); (iii) CG-5 (200 mg/kg, per os) plus gemcitabine (100 mg/kg, i.p.); and (iv) vehicles (0.5% methylcellulose/0.1% Tween 80/10% dimethyl sulfoxide in water, per os; and physiological saline, i.p.). CG-5 was administered once daily for the entire study. Gemcitabine was administered in two treatment cycles, each consisting of twice weekly treatments for 2 weeks, with the first and second cycles begin- ning at the 7 and 35 days, respectively, after the start of CG-5 treatment. Tumor volumes were calculated from weekly caliper measurements using a standard formula (volume = width² x length x 0.52). Body weights were measured weekly. At terminal sacrifice, tumors were harvested, snap-frozen in liquid
nitrogen, and stored at −80°C until used for biomarker analysis by western blotting. All experimental procedures using live animals were conducted in accordance with protocols approved by the Ohio State University Institutional Animal Care and Use Committee.

**Statistical analysis**

For all experiments performed in vitro, analysis of variance was used to test for significant differences between groups. For the in vivo murine solid tumor data, mixed-effect model, incorporating repeated measures for each subject, was used to model growth rates for the four groups and compare them. Multiplicity was adjusted by Holm’s method to control the family-wise error rate at 0.05 (23). Data were analyzed by the Center for Biostatistics using SAS 9.3 software (SAS, Cary, NC).

**Results**

Gemcitabine-resistant Panc-1 and Panc-1^{GemR} cells exhibit a higher extent of glycolytic phenotype and oxidative mitochondrial capacity relative to gemcitabine-sensitive AsPC-1 cells

Pancreatic cancer cells exhibit a wide range of sensitivity to gemcitabine. MTT and clonogenic survival assays indicate that Panc-1 and, to a greater extent, Panc-1^{GemR} cells (gemcitabine-resistant Panc-1 cells generated by chronic exposure to gemcitabine; see Materials and Methods) exhibited substantially greater resistance to gemcitabine, as manifested by IC_{50} values higher than two orders of magnitude compared with AsPC-1 and BxPC-3 cells (Figure 1A). These IC_{50} values, as estimated from median-effect plots using CompuSyn software, were as follows: MTT–BxPC-3, 8 nM; AsPC-1, 65 nM; Panc-1 and Panc-1^{GemR}, >10 µM (extrapolated to 20 and 32 µM, respectively); colony formation–BxPC-3, 11 nM; AsPC-1, 32 nM; Panc-1, 0.5 µM; Panc-1^{GemR}, 1 >1 µM (extrapolated to 10 µM).

In line with the reported link between therapeutic resistance and dysregulated cellular metabolism (24), these gemcitabine-resistant cells exhibit a strong glycolytic phenotype compared with the gemcitabine-sensitive AsPC-1 cells. As revealed by quantitative PCR analysis, Panc-1 and Panc-1^{GemR} cells, relative to AsPC-1 cells, expressed significantly higher mRNA levels of many glycolytic enzymes reported to contribute to aggressiveness and/or drug resistance in cancer cells, including hexokinase II (25), glucose phosphate isomerase (26), 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4 (27), aldolase (28), pyruvate kinase M2 (PKM2) (29), and pyruvate dehydrogenase kinase 3 (PDK3) (Figure 1B; all differences were significant at P < 0.05 with the exception of that between Panc-1 and AsPC-1 cells for PKM2 expression). It is noteworthy that the levels of expression of these glycolytic enzymes paralleled the extent of gemcitabine resistance among these cell lines.

Despite increased dependency on glycolysis, emerging evidence suggests that aggressive tumor cells might also exhibit increased mitochondrial oxidative capacity to make a more efficient use of nutrients or fuel molecules available from the tumor microenvironment (31). Thus, we examined the expression levels of key subunits of the OXPHOS enzyme system, including complex I, NADH dehydrogenase; complex II, succinate dehydrogenase complex; complex III, cytochrome c oxidase II; and complex V, mitochondrial ATP synthase (ATP5A). Consistent with the aforementioned hypothesis (31), western blot analysis indicated that these key OXPHOS components were upregulated in gemcitabine-resistant cells, especially Panc-1^{GemR} cells, relative to BxPC-3 and AsPC-1 cells (Figure 1C).

**Pancreatic cancer cells develop gemcitabine resistance by upregulating RRM2 expression**

To shed light onto the mechanism by which pancreatic cancer cells develop gemcitabine resistance, we correlated gemcitabine sensitivity with the expression of various drug resistance-associated biomarkers (4), including RRM1 (5), RRM2 (6), human equilibrative nucleoside transporter 1 (32), deoxyctydine kinase (33,34), p-Akt (9), and to a lesser extent, thymidine synthase (TS) (34), among the AsPC-1, BxPC-3, Panc-1, and Panc-1^{GemR} pancreatic cancer cell lines, which exhibited a range of gemcitabine sensitivities (Figure 1A). Western blot analysis revealed a positive correlation between the resistant phenotype of Panc-1 and Panc-1^{GemR} cells and high abundance of RRM2, TS and p-Akt in these two cell lines (Figure 1D, in the absence of gemcitabine), which, however, was not observed with the other markers of resistance examined. In addition, the unique ability of Panc-1 cells to upregulate RRM2 and TS expression in response to acute exposure to gemcitabine to a level comparable to that of Panc-1^{GemR} cells is noteworthy (Figure 1D). In contrast, this gemcitabine-induced upregulation of RRM2 and TS was not evident in gemcitabine-sensitive AsPC-1 and BxPC-3 cells. This coupregulation of RRM2 and TS suggested a shared mechanism by which gemcitabine stimulated their cellular accumulation. As TS is not commonly included among markers for gemcitabine resistance, we focused on interrogating the role of RRM2 upregulation in the development of gemcitabine resistance in this study. Moreover, an RRM1-immunoreactive band of lower molecular mass was noted in Panc-1 cells in response to acute or chronic exposure to gemcitabine (Figure 1D, right two lanes). Although the reason for the emergence of this second band in these two samples remains unclear, we speculate that it might arise from an effect of gemcitabine on the posttranslational modification and/or protein stability of RRM1, of the underlying mechanism warrants investigation.

Reverse transcription–polymerase chain reaction (RT–PCR) and luciferase reporter assays indicate that this gemcitabine-induced upregulation in RRM2 expression in Panc-1 cells was attributable to transcriptional activation of RRM2 gene expression (Figure 1E; P < 0.05). Pursuant to this finding, we examined the effect of shRNA-mediated knockdown of RRM2 on the cellular sensitivity of Panc-1^{GemR} cells to gemcitabine. As the expression of RRM2-silenced Panc-1^{GemR} cells to gemcitabine led to reexpression of RRM2, RRM2 knockdown caused only a fourfold increase in gemcitabine sensitivity (Figure 1F, left; IC_{50}, >10 µM [extrapolated to 35 µM] for control versus 8.7 µM for RRM2). Nevertheless, ectopic expression of RRM2 in gemcitabine-sensitive AsPC-1 cells shifted the cell viability dose–response curve to the right by more than a log unit relative to the pCMV control (IC_{50}, 70 nM for pCMV control versus 2.8 µM for pRRM2) (right).

**Gemcitabine activates RRM2 gene expression through E2F1 upregulation**

To understand the mechanism by which gemcitabine induced RRM2 gene expression in Panc-1 cells, we investigated the drug effects on the expression of transcription factors associated with the regulation of RRM2 expression, including E2F1, Sp1 and NF-YA (35–37). Exposure of Panc-1 cells to 1 µM gemcitabine for 24 h led to increased abundance of E2F1 mRNA and protein, in parallel with that of RRM2 (Figure 2A, left). In contrast, the expression of Sp1 and NF-YA was either attenuated or unaffected, at both mRNA and protein levels, thereby refuting their involvement in mediating gemcitabine-activated RRM2 expression.

This correlation between E2F1 and RRM2 expression was also evident in their relative abundances, at both protein and mRNA levels, in pancreatic cancer cells compared with those in normal primary pancreatic cells (NPC) (Figure 2B; P < 0.001). While cultured NPC lacked appreciable expression of E2F1 and RRM2, the pancreatic cancer cell lines AsPC-1, BxPC-3 and Panc-1 exhibited differential, but parallel, expression levels of E2F1 and RRM2. This expression pattern was also apparent in six paired pancreatic tumor and adjacent normal pancreas tissues resected from cancer patients (Figure 2C). The tumor tissues exhibited substantially higher expression levels of E2F1 and RRM2 than their normal counterparts, while no correlation was found between the expression levels of Sp1 and RRM2 in these clinical samples.

The mechanistic link between E2F1 and gemcitabine-induced RRM2 expression was confirmed by the ability of shRNA-mediated knockdown of E2F1, as confirmed by reduced expression of its target gene products CDK2 and cyclin A, to abrogate the stimulatory effect of the drug on RRM2 protein expression (Figure 2D, left) and RRM2 promoter-luciferase activity (right; P < 0.001) in Panc-1 cells. As TS has been reported to be an E2F1 target gene (38), this gemcitabine-induced
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E2F1 overexpression might also account for increased TS expression in gemcitabine-resistant cells. Again, this premise was supported by the enforced expression of E2F1 in AsPC-1 cells and the shRNA-mediated silencing of E2F1 in Panc-1 cells, which increased and reduced, respectively, the expression of TS (Figure 2E).

The Glut inhibitor CG-5 suppresses RRM2 expression through E2F1 downregulation

Previously, we developed a novel class of Glut inhibitors by pharmacologically exploiting the off-target effect of thiazolidinedione PPARγ agonists on glucose uptake through lead optimization of...
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ciglitazone (13,18). In the course of this development, complementary DNA microarray analysis revealed that RRM2 and E2F1 were among the genes most downregulated in cancer cells treated with the proof-of-concept inhibitor, CG-5 (data not shown). Based on this finding, along with those described above for the important role of E2F1 and RRM2 in the gemcitabine resistance of pancreatic cancer cells, we hypothesized that targeting the Warburg effect through Glut inhibition could overcome this gemcitabine-resistant phenotype.

In the present study, CG-5 (structure, Figure 3A, upper left) was used to address this hypothesis, CG-5 acted as a pan-Glut inhibitor as it inhibited a series of Glut isoforms examined, including Glut-1, -3, -4, -9 and -12, with similar potency (IC50, approximately 5 µM) (lower left). MTT assays indicated that CG-5 exhibited differential activities in suppressing the viability of pancreatic cancer cell lines, including AsPC-1, BxPC-3 and Panc-1. Right, quantitative PCR analysis of the relative mRNA abundance of E2F1 and RRM2 in NPC versus the aforementioned cancer cell lines after normalization to β-actin. Data are shown as means ±mSD (n = 3). (C) Western blot analysis of the expression of E2F1, RRM2 and Sp1 in paired pancreatic tumor tissues and adjacent normal tissues from pancreatic cancer patients. (D) Western blot and luciferase reporter assays of the effect of shRNA-mediated knockdown of E2F1 on the expression of RRM2, CDK2 and cyclin A (left) and RRM2 gene promoter activity (right) in gemcitabine-treated Panc-1 cells (1 µM, 24 h). Data are shown as means ±mSD (n = 3). (E) Evidence that TS is an E2F1-targeted gene. Western blot analysis of the effects of ectopic expression and shRNA-mediated knockdown of E2F1 on the expression of E2F1 and TS in AsPC-1 and Panc-1 cells, respectively.

A Novel Glut inhibitor overcomes gemcitabine resistance

Table 1: Paired tumor (T) and neighboring normal (N) pancreatic tissue

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Fig. 2. Evidence that gemcitabine activates RRM2 expression through E2F1 upregulation in pancreatic cancer cells. (A) RT–PCR (left) and western blot (right) analyses of the expression levels of RRM2 vis-à-vis E2F1, Sp1 and NF-YA in Panc-1 cells in the absence (-) or presence (+) of 1 µM gemcitabine (24 h exposure) versus those in Panc-1 GemR cells. (B) Left, western blot analysis of the expression of E2F1 and RRM2 in normal pancreatic epithelial cells (NPC) versus different pancreatic cancer cell lines, including AsPC-1, BxPC-3 and Panc-1. Right, quantitative PCR analysis of the relative mRNA abundance of E2F1 and RRM2 in NPC versus the aforementioned cancer cell lines after normalization to β-actin. Data are shown as means ±mSD (n = 3). (C) Western blot analysis of the expression of E2F1, RRM2 and Sp1 in paired pancreatic tumor tissues and adjacent normal tissues from pancreatic cancer patients. (D) Western blot and luciferase reporter assays of the effect of shRNA-mediated knockdown of E2F1 on the expression of RRM2, CDK2 and cyclin A (left) and RRM2 gene promoter activity (right) in gemcitabine-treated Panc-1 cells (1 µM, 24 h). Data are shown as means ±mSD (n = 3). (E) Evidence that TS is an E2F1-targeted gene. Western blot analysis of the effects of ectopic expression and shRNA-mediated knockdown of E2F1 on the expression of E2F1 and TS in AsPC-1 and Panc-1 cells, respectively.
Sp1 overexpression had no protective effect on RRM2 expression (Figure 3E), indicating that Sp1 does not mediate CG-5-induced reductions in RRM2 expression in Panc-1 cells. Evidence that this suppressive effect of CG-5 on RRM2 expression was mediated through glycolysis inhibition in Panc-1 cells included (a) the ability of the glycolysis inhibitor 2-DG and glucose starvation, like CG-5, to suppress E2F1 and RRM2 expression (Figure 3F, upper) and (b) the abrogation of CG-5-induced reduction of RRM2 by high glucose concentrations (lower) in Panc-1 cells. Cells were treated for 24h unless otherwise noted. (E) Western blot analysis of the dose-dependent suppressive effect of CG-5 on the expression of E2F1 and RRM2 in AsPC-1GemR cells after 24h of exposure.

Sp1 overexpression had no protective effect on RRM2 expression (Figure 3E), indicating that Sp1 does not mediate CG-5-induced reductions in RRM2 expression in Panc-1 cells. Evidence that this suppressive effect of CG-5 on RRM2 expression was mediated through glycolysis inhibition in Panc-1 cells included (a) the ability of the glycolysis inhibitor 2-DG and glucose starvation, like CG-5, to suppress E2F1 and RRM2 expression (Figure 3F, upper) and (b) the abrogation of CG-5-induced reduction of RRM2 by high glucose concentrations (lower). It is noteworthy that, in the absence of drug, the high glucose concentration increased the expression of E2F1 and RRM2 (10 versus 2% glucose).

Although the above findings demonstrated the mechanistic link between E2F1-mediated RRM2 upregulation and the intrinsic gemcitabine resistance of Panc-1 cells, it raised the question of whether increased E2F1/RRM2 expression also underlies the development of acquired gemcitabine resistance in sensitive cell lines, such as BxPC-3 and AsPC-1 cells. To address this issue, we isolated gemcitabine-resistant BxPC-3 and AsPC-1 cells (BxPC-3GemR and AsPC-1GemR, respectively) by subculturing through incrementally increasing gemcitabine concentrations, up to 1µM, for 1 month. The resistant phenotype was manifested by higher IC50 values relative to those of the parental cell lines (IC50: 2 nM for BxPC-3 versus 0.2 µM for BxPC-3GemR; 60 nM for AsPC-1 versus 1.3 µM for AsPC-1GemR) (Figure 3G, left). As it was for Panc-1 and Panc-1GemR cells, BxPC-3GemR and AsPC-1GemR cells exhibited substantially higher expression levels of E2F1 and RRM2 than the parental cell lines (right). Reminiscent of the effect on Panc-1 cells, AsPC-1GemR cells were susceptible to the suppressive effect of CG-5 on the expression of E2F1 and RRM2 in a concentration-dependent manner (Figure 3H).

CG-5 suppresses E2F1 expression through miR-520f upregulation
Considering the oncogenic role of E2F1 in mediating cancer progression and chemoresistance (40), the ability of CG-5 to suppress E2F1 expression was noteworthy. The transcription factors Sp1 (41) and c-Myc (42) have been shown to directly or indirectly regulate E2F1 expression. As both Sp1 (Figure 3B) and c-Myc (I.L.Lai and C.-S.Chen, unpublished experiments) were involved in the regulation of E2F1 expression, we examined the role of miR-520f, a known target of Sp1 (43) and c-Myc (44).}

Fig. 3. Evidence that the glucose inhibitor CG-5 suppresses RRM2 expression through E2F1 downregulation. (A) Left, structure of CG-5 (upper) and evidence that CG-5 is a pan-Glut inhibitor exhibiting comparable potencies for inhibition of glucose uptake in Panc-1 cells overexpressing various Glut isoforms (lower; data are shown as means ±mSD (n = 3)). Right, differential sensitivities of various pancreatic cancer cell lines and NPC to CG-5 by MTT assays. Points, means; error bars, SD (n = 6). (B) Western blot analysis of the expression of p-Akt, p-mTOR, p-AMPK, Skp2, cyclin D1, Sp1, RRM2 and E2F1 in CG-5-treated Panc-1 cells. (C) RT–PCR and luciferase reporter assays of the effect of CG-5 (24h exposure) on mRNA expression of RRM2 and E2F1 (upper) and RRM2 gene promoter activity (lower), respectively, in Panc-1 cells. Data are shown as means ±mSD (n = 3). (D) Western blot and luciferase reporter assays of the effect of ectopic overexpression of E2F1 on the CG-5-induced reductions in RRM2 expression (left) and RRM2 gene promoter activity (right), respectively, in Panc-1 cells. For the promoter-luciferase assays, cells were treated with 5 µM CG-5 for 24h. Data are shown as means ±mSD (n = 3). (E) Western blot analysis of the effect of ectopic overexpression of Sp1 on the CG-5-induced reduction in RRM2 expression in Panc-1 cells. (F) Western blot analyses of the effects of 2-DG and glucose (Glu) starvation on the abundance of E2F1 and RRM2 (upper) and the protective effect of high exogenous glucose levels (10 versus 2%) on CG-5-mediated downregulation of E2F1 and RRM2 expression (lower) in Panc-1 cells. Cells were treated for 24h unless otherwise noted. (G) Evidence that CG-5-mediated RRM2 upregulation underlies the acquisition of gemcitabine resistance in BxPC-3 and AsPC-1 cells. Left, MTT assays of the concentration-dependent effects of gemcitabine on the viability of BxPC-3GemR (Bre) and AsPC-1GemR (Ae) cells, vis-à-vis the parental BxPC-3 (B) and AsPC-1 (A) cells, after 24h of treatment. Points, means; bars, SD (n = 6). Right, western blot analysis of the expression levels of E2F1 and RRM2 in BxPC-3GemR and AsPC-1GemR cells relative to the respective parental cell lines. (H) Western blot analysis of the dose-dependent suppressive effect of CG-5 on the expression of E2F1 and RRM2 in AsPC-1GemR cells after 24h of exposure.
data) were targeted by CG-5, we investigated the role of these two transcription factors in the CG-5-induced reduction of E2F1 expression by examining the effects of ectopic expression. The enforced expression of c-Myc or Sp1 did not protect Panc-1 cells from the suppressive effect of CG-5 on E2F1 expression (data not shown), thereby refuting their involvement in this drug effect.

As a series of miRNAs, including miR-17, miR-20a, miR-34a, miR-106b, miR-149, miR-205 and miR-330, have been reported to regulate the expression of E2F1 (42–47), we analyzed preexisting microarray data generated from the analysis of the global effect of CG-5 (5 μM) on miRNA expression in LNCaP cells. These microarray results and subsequent confirmatory quantitative RT–PCR analyses, however, indicated that none of the above-mentioned E2F1-regulatory miRNAs was affected by CG-5 (data not shown). Consequently, we used the online software program TargetScanHuman (Release 6.2) (http://www.targetscan.org) to search for predicted targets for each of the most upregulated miRNAs in CG-5-treated cells, among which miR-520f was predicted to modulate E2F1 expression. Pursuant to this finding, we validated the ability of CG-5 to upregulate miR-520f expression in Panc-1 cells by quantitative RT–PCR, which showed a concentration-dependent increase (P < 0.0001) in miR-520f in response to CG-5 (Figure 4A, left). This miR-520f upregulation, however, was not noted after exposure to gemcitabine despite its ability to stimulate E2F1 expression, indicating that the increase in miR-520f was a CG-5-specific cellular response. The putative role of miR-520f in mediating CG-5-facilitated E2F1 downregulation was corroborated in two ways.Transient transfection with a miR-520f mimic dose dependently suppressed E2F1 and RRM2 expression in Panc-1 cells, and conversely, ectopically expressed anti-miR-520f-protected Panc-1 cells from the suppressive effect of CG-5 on E2F1 and RRM2 expression (Figure 4A, right).

Bioinformatic analysis using the RNAhybrid Webservice (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/submission.html) indicated that miR-520f has a seed region that is 100% complementary to a target sequence (nt 639–645) in the 3′ UTR of E2F1, which is highly conserved across the three species examined (Figure 4B, upper), with a calculated minimum free energy (∆G) of −23.7 kcal/mol (lower). The ability of miR-520f to regulate E2F1 expression by targeting the 3′ UTR was verified by several lines of evidence. First, we constructed two luciferase reporter plasmids, one that harbored the wild-type 3′ UTR of E2F1 at the 3′ position of the luciferase reporter gene (wt 3′ UTR) and another that contained a mutated 3′ UTR in which the conserved target sequence CCCUCUA (nt 639–645) for miR-520f binding was replaced by TATGACC as a negative control (mut 3′ UTR; Figure 4C, left). Cotransfection of Panc-1 cells with the wt-3′ UTR luciferase reporter plasmid and increasing amounts of

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**Fig. 4.** Evidence that CG-5 suppresses E2F1 expression through miR-520f upregulation. (A) Left, the expression of miR-520f in Panc-1 cells treated with CG-5 or gemcitabine for 24 h as determined by quantitative RT–PCR. Data are shown as means ± mSD (n = 3). Right, western blot analysis of E2F1 and RRM2 expression in Panc-1 cells expressing miR-520f mimic (upper) or anti-miR-520f in the presence or absence of CG-5 (lower, 5 μM 24 h). (B) Upper, the miR-520f seed region and its target sequences in the 3′ UTR of E2F1 are identified in bold-face type. Unpaired bases are listed above and below the duplex. (C) Diagram of wild-type (wt)- and mutant (mut)-E2F1-3′ UTR-containing luciferase reporter constructs (left; mutated target sequence is underscored). Luciferase assays of the effects of a miR-520f mimic on the activation of wt- or mut-E2F1-3′ UTR (center) and the effects of anti-miR-520f in the presence or absence of 5 μM CG-5 for 24 h on activation of wt-E2F1-3′ UTR (right) in Panc-1 cells. Data are shown as means ± mSD (n = 3). (D) Effects of the expression of miR-520f mimic or anti-miR-520f on the proliferation of Panc-1 cells. Points, means; bars, SD (n = 6). (E) Relative ectopic expression levels of miR-520f in a stably transfected Panc-1 clone (clone 2) versus that in control cells (WT) as determined by quantitative RT–PCR (left). Data are shown as means ± mSD (n = 3). Colony formation (center) and western blot analyses (right) of the effects of stable miR-520f expression on cell survival and the expression of E2F1 and RRM2, respectively, in gemcitabine-treated cells. Points, means; bars, SD (n = 6).
miR-520f mimic led to dose-dependent decreases in the luciferase activity relative to the vehicle control ($P < 0.001$) (center). This effect of miR-520f mimic on 3′ UTR-driven luciferase activity, however, was not noted with the mut-3′ UTR reporter construct (center), confirming the ability of miR-520f to interact with the 3′ UTR of E2F1 through the predicted target sequence. Moreover, consistent with the protective effect of anti-miR-520f against CG-5-induced reductions of E2F1 and RRM2 (Figure 4A, lower right), anti-miR-520f abolished the suppressive effect of CG-5 (5 µM) on the wt-3′ UTR-driven luciferase activity (Figure 4C, right; $P < 0.001$).

Consistent with the regulatory role of E2F1 in cell cycle progression, ectopic expression of miR-520f mimic and anti-miR-520f suppressed and increased, respectively, the proliferation of Panc-1 cells (Figure 4D; $P < 0.0001$). Furthermore, to examine the effect of miR-520f on gemcitabine sensitivity, we isolated a stable clone of Panc-1 cells overexpressing miR-520f mimic (clone #2) (Figure 4E, left) and evaluated responses to gemcitabine exposure. As shown, stable overexpression of miR-520f mimic enhanced cellular sensitivity to the antiproliferative activity of gemcitabine (center; IC$_{50}$: 0.5 µM for Panc-1 versus 0.1 µM for clone #2) by diminishing the ability of gemcitabine to upregulate E2F1 and RRM2 expression (right).

**CG-5 increases the sensitivity of Panc-1 and Panc-1$^{GemR}$ cells to gemcitabine**

Pursuant to the above findings, we investigated the ability of CG-5 to enhance the sensitivity of Panc-1 and Panc-1$^{GemR}$ cells to the genotoxic effect of gemcitabine. Clonogenic assays indicate that CG-5 at 2.5 and 5 µM sensitized Panc-1 and, to a greater extent, Panc-1$^{GemR}$ cells to gemcitabine (combination index < 1) (Figure 5A, left). For example, while gemcitabine alone at <100 nM was ineffective in suppressing Panc-1$^{GemR}$ cell proliferation, the combination with CG-5 at a submaximal inhibitory concentration, i.e. 2.5 µM, gave rise to multifold increases in growth inhibition, underscoring its chemosensitizing activity. This synergistic interaction correlated with the downregulation of E2F1 and RRM2 expression and Akt phosphorylation (center). Equally important, CG-5 augmented gemcitabine-induced DNA damage, as evidenced by increases in γ-H2AX formation (center) and the number of γ-H2AX foci-positive cells (right) compared with gemcitabine alone.

**Fig. 5.** Evidence that CG-5 increases the sensitivity of pancreatic cancer cells to gemcitabine. (A) Effects of CG-5 on gemcitabine sensitivity in Panc-1 and Panc-1$^{GemR}$ cells. Colony formation assay results were used for analysis of synergistic interactions between gemcitabine and CG-5 (left). Points, means; bars, SD (n = 6). The effects of CG-5 on gemcitabine-mediated changes in the expression levels of E2F1, RRM2, p-Akt and γ-H2AX were assessed by western blotting (center). Evaluation of the effect of CG-5 on gemcitabine-induced expression of γ-H2AX in Panc-1 cells as determined by immunofluorescent staining (10×) (right). CI, combination index. (B) Western blot (left) and immunofluorescent staining (right; 10×) analyses of γ-H2AX expression in Panc-1 cells treated for 24 h with CG-5. (C and D) Western blot analyses of the effects of (C) 2-DG and glucose starvation, and (D) the ribonucleotide reductase inhibitor Triapine on γ-H2AX expression in Panc-1 cells. (E) The protective effects of ectopic RRM2 expression on CG-5-induced γ-H2AX expression in Panc-1 cells. (F) Effects of CG-5 and gemcitabine, each alone and in combination, on the intracellular distribution of RRM2 in Panc-1 cells. Upper, immunofluorescent staining of RRM2 (green); nuclei stained with 4′,6-diamidino-2-phenylindole (blue); red lines indicate planes of cross-sectional analyses of fluorescence intensities, which are shown in two-dimensional histograms (blue, nuclei; green, RRM2). Magnification bar, 10 µm. Lower, western blot analysis of RRM2 expression in cytoplasmic and nuclear fractions of Panc-1 cells treated with CG-5 and gemcitabine, each alone and in combination. α-Tubulin and histone H3 were used as cytoplasmic and nuclear markers, respectively.
From a mechanistic perspective, the effect of CG-5-induced metabolic stress on DNA damage, as represented by increases in γ-H2AX abundance and foci (Figure 5B), was noteworthy, and was also observed in cells treated with 2-DG or glucose-depleted medium (Figure 5C). We obtained evidence that the ability of CG-5 to increase H2AX phosphorylation, which plays a key role in the response to DNA damage, was attributable to its suppressive effect on RRM2 expression. First, triamine (3-aminopyridine-2-carboxaldehyde thiosemicarbazone), a potent RRM2 inhibitor (48), exhibited a dose-dependent effect on H2AX phosphorylation, similar to that of CG-5, without altering RRM2 expression (Figure 5D). Second, ectopic expression of RRM2 protected cells from CG-5-induced γ-H2AX accumulation (Figure 5E).

It is also noteworthy that in addition to suppressing gemicitabine-induced RRM2 upregulation, CG-5 blocked the ability of gemicitabine to stimulate the translocation of RRM2 from cytoplasm to nucleus where it takes part in DNA repair. Immunocytochemical analysis indicated substantial increases in RRM2-specific immunofluorescence in both cytoplasmic and nuclear compartments in Panc-1 cells treated with 1 μM gemicitabine compared with the dimethyl sulfoxide-treated control, which, however, were attenuated by cotreatment with CG-5 (5 μM) (Figure 5F, upper panels). This CG-5-mediated inhibition of RRM2 nuclear localization was confirmed by western blot analysis of the cellular distribution of RRM2 in cytoplasmic versus nuclear fractions (lower).

The combination of CG-5 and gemicitabine suppresses Panc-1GemR xenograft tumor growth in nude mice

To investigate the effect of CG-5 on gemicitabine activity in vivo, athymic nude mice bearing established subcutaneous Panc-1GemR xenografts were treated with 2 week cycles, separated by 2 weeks, of twice weekly i.p. injections of gemicitabine (100 mg/kg), alone or in combination with CG-5 (200 mg/kg) administered once daily by oral gavage. Other groups of mice received either CG-5 or vehicles only. As shown in Figure 6A, treatment of mice with gemicitabine, CG-5 and the two drug combination reduced Panc-1GemR tumor growth by 42, 47 and 64% (corresponding to 2.9, 3.2 and 4.4 mm3/ day), respectively, compared with the vehicle-treated control (all P values < 0.0001). The two drug combination was more effective in reducing tumor growth than either drug alone (P values: gemicitabine, 0.015; CG-5: 0.04). The CG-5 and gemicitabine treatments were well tolerated. Mice receiving the drug combination exhibited transient weight loss during the gemicitabine treatment cycles during which they received moistened chow as precautionary supportive care. At the end of the study, mice exhibited no overt signs of toxicity, as indicated by the absence of gross lesions at necropsy and normal hematological and serum chemistry parameters (data not shown).

To correlate the in vivo tumor-suppressive response to mechanisms identified in vitro, the effects of CG-5 on biomarkers associated with gemicitabine resistance and DNA damage, including E2F1, RRM2 and γ-H2AX, were evaluated by immunoblotting in Panc-1GemR tumor homogenates collected after 63 days of treatment. As shown, the expression levels of E2F1 and RRM2 were significantly lower (P < 0.05; n = 3) in tumors from mice treated with the combination than in those treated with gemicitabine alone (Figure 6B). Equally important, as manifested by γ-H2AX expression levels, CG-5 augmented the effect of gemicitabine on DNA damage in vivo.

Discussion

Substantial evidence has demonstrated the mechanistic link between aberrant expression of many glycolytic enzymes, including hexokinase 2, PKM2 and pyruvate dehydrogenase kinase 3, and development of drug resistance in cancer cells through the upregulation of survival signaling. Accordingly, compared with AsPC-1 cells, Panc-1 and, to a greater extent, Panc-1GemR cells exhibit substantially higher mRNA expression levels of these glycolytic enzymes (Figure 1B), which bestow a glycolytic phenotype on these gemicitabine-resistant cells, i.e., the Warburg effect. The link between glycolytic phenotype and gemicitabine resistance provides a mechanistic rationale for our hypothesis that targeting the Warburg effect through Glut inhibition could overcome gemicitabine resistance in these cancer cells.

Ribonucleotide reductase catalyzes a key step in the synthesis of deoxyribonucleotide triphosphate, thus playing an essential role in DNA replication and damage repair. Although high endogenous RRM2 levels have been implicated as a major factor contributing gemicitabine resistance (49), our data indicate that the ability of Panc-1 pancreatic cells to increase RRM2 expression in response to gemicitabine underlies their resistant phenotype (Figure 1D). Although several transcription factors have been shown to transactivate the RRM2 gene, including E2F1, Sp1 and NF-YA, our data indicated that the gemicitabine-induced increase in RRM2 expression was mediated by E2F1 as gemicitabine selectively increased E2F1 expression and knockdown of E2F1 abolished the ability of gemicitabine to induce RRM2 gene activation in Panc-1 cells.

**Fig. 6.** Antitumor effects of CG-5 and gemicitabine, each alone and in combination, on Panc-1GemR xenograft tumors in athymic nude mice. (A) Effects of CG-5, gemicitabine and the combination on Panc-1GemR tumor growth. Arrows indicate days on which gemicitabine or vehicle was administered. Data are expressed as means ± SE. (B) Western blot analysis of the expression of E2F1, RRM2, γ-H2AX and H2AX in Panc-1GemR tumors from three mice in each treatment group. Left, immunoblot. Right, relative abundance of E2F1 and RRM2 in the tumors from drug-treated mice versus those from vehicle-treated mice after normalization to β-actin. (C) Schematic diagram depicting the mechanism by which CG-5 sensitizes pancreatic cancer cells to gemicitabine.
From a mechanistic perspective, this E2F1-mediated upregulation of RRM2 expression represents a gemcitabine-induced DNA damage response in these resistant cells to enhance DNA repair capacity, which we speculate could be mediated through a previously reported ataxia-telangiectasia and rad3-related (ATR)/ataxia-telangiectasia mutated (ATM)–checkpoint kinase 1 (Chk1) pathway (50) (Figure 6C).

However, it warrants attention that the role of E2F1 following DNA damage in regulating cellular fate, i.e. cell proliferation versus apoptosis, might be cell context-/cell type-specific as distinct forms of E2F1 with different posttranslational modifications might be organized in response to DNA damage (40,51). For example, it was previously reported that ectopic expression of E2F1 in combination with gemcitabine at 10 μM resulted in a strong induction of apoptosis through the E2F1-p73 pathway in AsPC-1 cells (52). In our study, exposure of AsPC-1 cells to gemcitabine did not increase the expression of E2F1 (not shown) or RRM2 (Figure 1D), in contrast to the response of Panc-1 cells, suggesting a different role of E2F1 in these two cell lines. Nevertheless, our data indicate that AsPC-1Gmut cells exhibited substantially higher expression levels of E2F1 and RRM2 relative to the parental cells (Figure 3H), suggesting that E2F1-induced RRM2 expression might also underlie the development of acquired gemcitabine resistance in these cells.

The Glut inhibitor CG-5 increased the sensitivity of Panc-1 and Panc-1Gmut cells to gemcitabine by countering gemcitabine-induced activation of E2F1-mediated RRM2 expression and nuclear recruitment (Figure 5). Evidence indicates that CG-5-mediated suppression of E2F1/RRM2 expression was attributable to its ability to block glycolysis. For example, this suppressive effect was shared by the glycolysis inhibitor 2-DG and glucose starvation and was abolished by high concentrations of exogenous glucose (Figure 3F). Moreover, higher glucose levels in the medium resulted in higher expression levels of E2F1 and RRM2 in Panc-1 cells (Figure 3F), which is consistent with the role of E2F1 as a regulator that links cell proliferation and glucose homeostasis (53). However, this finding raises a question of whether insulin resistance, which is prevalent among pancreatic cancer patients, might adversely affect the outcome of gemcitabine-based therapy (54).

To the best of our knowledge, we provide the first evidence that E2F1 is negatively regulated by miR-520f through binding to a specific target sequence (nt 639–645) within the 3′-UTR, and that this miR-520f-mediated repression of E2F1 occurs in response to Glut inhibition by CG-5. Overexpression of miR-520f in Panc-1 cells converted its resistant phenotype to a sensitive phenotype, to a level that is similar to that of AsPC-1 cells, by blocking the ability of gemcitabine to upregulate the E2F1-RRM2 pathway (Figure 4). Although information regarding the functions and targets of miR-520f and the miR-520 cluster in the literature is lacking or fragmentary, evidence suggests that the miR-520 family might be tumor suppressive. For example, miR-520h was reported to downregulate ABCG2 in pancreatic cancer cells, thereby inhibiting migration, invasion and side populations (15).

Investigation of the functional role of miR-520f in pancreatic cancer cells is currently underway. In conclusion, this study identifies a mechanism by which pancreatic cancer cells can acquire resistance to gemcitabine through the upregulation of RRM2 via an E2F1-dependent pathway. CG-5 restored the sensitivity of drug-resistant Panc-1 cells to gemcitabine by abrogating the effect of gemcitabine on E2F1-RRM2 expression by upregulating miR-520f expression. Together, these findings suggest that CG-5 or its structurally optimized derivatives have clinical value as part of therapeutic strategies for pancreatic cancer and warrants continued investigation in this regard.

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References
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44. Pickering,M.T. et al. (2009) miR-17 and miR-20a temper an E2F1-induced G1 checkpoint to regulate cell cycle progression. Oncogene, 28, 140–145.

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