Mechanisms of Overcoming Intrinsic Resistance to Gemcitabine in Pancreatic Ductal Adenocarcinoma through the Redox Modulation
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Abstract
Pancreatic ductal adenocarcinoma (PDAC) frequently develops therapeutic resistances, which can be divided into extrinsic and intrinsic resistance. The extrinsic resistance that arises from the surrounding dense tumor stroma is much better understood. However, the mechanisms of intrinsic resistance are not well understood. Here, we report that reactive oxygen species (ROS) induced by gemcitabine treatment, a newly discovered cytotoxic activity, served as a probe in our study to reveal the mechanisms of the intrinsic therapeutic resistance. Our results showed that gemcitabine-induced ROS is generated by NOX and through the increase of p22phox expression via NF-kB activation. As a feedback mechanism, nuclear translocation of Nrf2 stimulated the transcription of cytoprotective antioxidant genes, especially genes encoding enzymes that catalyze glutathione (GSH) production to reduce elevated ROS as an intrinsic resistance countermeasure. RNAi-mediated depletion of Nrf2 or addition of β-phenylethyl isothiocyanate inhibited the ROS detoxification process by reducing GSH levels, which, in turn, increased the efficacy of gemcitabine in vivo and in vitro. Thus, our study suggests that a redox-mediated pathway contributes to the intrinsic resistance of PDAC to gemcitabine and provides a basis for developing strategies to preferentially kill PDAC cells through ROS-mediated mechanism. The combination of gemcitabine and PeITC has a selective cytotoxic effect against pancreatic cancer cells in vivo and could thus prove valuable as a cancer treatment. Mol Cancer Ther; 14(3); 788–798. ©2014 AACR.

Introduction
Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer-related deaths in the United States (1). At diagnosis, approximately 80% of patients with pancreatic cancer have therapy-resistant locally advanced or metastatic disease with a median survival of less than 6 months (2, 3). The 5-year survival rate has remained around 1% to 3% for the past 25 years and is the lowest of any cancer, and the therapy regimens have been largely ineffective (4). Thus, pancreatic cancer poses one of the greatest challenges in cancer research. Numerous efforts have been made to improve treatment for patients with PDAC (1, 5). However, the treatments are still largely ineffective (4). The failure is attributable to many factors, including extrinsic (6) or intrinsic (7) resistance to the conventional therapeutic approaches of chemotherapy. The chemotherapeutic agent gemcitabine is usually the recommended first-line drug for patients with PDAC and is given alone or in combination with other agents (8). Unfortunately, a large number of patients are resistant to these therapies mainly because the dense tumor stroma functions as a barrier and extrinsic resistance (7). The mechanisms of intrinsic resistance to gemcitabine still remain as an active area of ongoing investigation.

Like certain other anticancer agents, gemcitabine induces reactive oxygen species (ROS), which serves as an additional anticancer mechanism (9, 10). Excessive production of ROS can cause cellular damage that ultimately leads to cell death. Therefore, cells have to develop a highly regulated antioxidant defense system to prevent oxidative damage (11, 12). The cellular defense systems against ROS consist of various antioxidant enzymes, especially glutathione (GSH) generating enzymes, including glutamate cysteine ligase (GCL), glutathione reductase (GSR), and glutathione S-transferase (GST). Most of these enzymes are under the control of the transcription factor NF-E2–related factor-2 (Nrf2; refs. 11–13). Our observation is consistent with the previous reports that gemcitabine stimulates ROS generation in PDAC cells (9, 10). However, the source of ROS remains unclear. Furthermore, the role of antioxidants in cellular defense against gemcitabine-induced ROS still needs to be investigated. Thus, elucidating the mechanism of intrinsic resistance to gemcitabine in the context of ROS generation and ROS-dependent selectivity is important for the design of drug combinations to overcome the resistance.
β-Phenylethyl isothiocyanate (PEITC), a natural compound found in cruciferous vegetables, depletes GSH and selectively kills cancer cells while sparing healthy cells (14–17). Given the fact that stimulation of ROS is generated by gemcitabine in PDAC cells, we hypothesized that the cells increase cellular GSH to prevent oxidative damage and that PEITC enhances the effect of gemcitabine-induced ROS through the redox modulation. In this study, we demonstrated the mechanism by which gemcitabine induces ROS generation in PDAC cells, identified the key molecules involved in ROS-induced cytoprotective mechanisms, and determined the combinational approach of the key molecules involved in ROS-induced cytoprotective mechanisms, and determined the combinational approach of gemcitabine and PEITC that induced a synergistic cytotoxic effect against PDAC cells. Thus, our study provides important information for understanding the mechanism of intrinsic resistance to gemcitabine.

**Materials and Methods**

**Cell lines and cell culture**

Human pancreatic cancer cell lines AsPc-1 and MIAPaCa-2 were purchased from the ATCC. Panc-28 was obtained at The University of Texas MD Anderson Cancer Center (Houston, TX). All cells were cultured at 37°C with 5% CO₂ in DMEM medium containing glucose, glutamine, and nonessential amino acids with 10% FBS. The cell line MIAPaCa-2 was exposed to 200 nmol/L of gemcitabine every other week for about 6 months to create gemcitabine-resistant cell lines MIAPaCa-2/GR (18). The MIAPaCa-2/GR cell line was maintained in gemcitabine (200 nmol/L) every five passages to maintain resistance. All cell lines were authenticated by short tandem repeat fingerprinting before using at the Characterized Cell Line Core of The University of Texas MD Anderson Cancer Center.

**ROS detection**

Hydroethidine (HEt) and CM-H₂DCF-DA were used to measure cellular ROS by flow cytometry, respectively. MIAPaCa-2, AsPc-1, and Panc-28 cells were seeded in 6-well plates at 2 × 10⁵ cells per well overnight. After incubation with HEt (100 ng/mL) or CM-H₂DCF-DA (3 μmol/L) at 37°C for 1 hour, samples were washed with PBS and analyzed with a FACSCalibur flow cytometer (BD Biosciences). Data were analyzed using FlowJo software.

**NOX activity assay**

NAD(P)H oxidase (NOX) activity was measured by a lucigenin-derived chemiluminescence assay as previously described (19). Briefly, 5 mg of homogenized protein was incubated with its substrate 100 mmol/L NADPH in a phosphate buffer (50 mmol/L, pH 7.0) containing 150 mmol/L NaCl and 1 mmol/L EGTA for 15 minutes, followed by an addition of 5 mmol/L lucigenin for 15 minutes in the dark. The chemiluminescent signal (photon emission) was measured using a Turner 20/20 luminometer (Turner Designs).

**Animal study**

Two weeks after being injected with the cells, AsPc-1 orthotopic tumor-bearing NOD/SCID mice were randomly assigned (n = 5/group) to receive the following on a weekly schedule: group 1 served as a control and received no treatment; group 2

![Figure 1](image-url)

**Figure 1.**

Significant increase in ROS generation compensated with increased cellular antioxidant activity in MIAPaCa-2/GR cells compared with parental cells. A, representative histograms of significant increase of ROS levels in MIAPaCa-2/GR and MIAPaCa-2 cells as detected by the fluorescent probes HEt and CM-H₂DCF-DA, respectively (log scale). B, MIAPaCa-2, AsPc-1, and Panc-28 cells were treated with 5 μmol/L gemcitabine for 24 hours, then the cells were labeled with HEt and CM-H₂DCF-DA for 1 hour followed by flow cytometric analysis to detect ROS levels. C, comparison of mRNA expression of antioxidant genes in MIAPaCa-2 and MIAPaCa-2/GR cells by real-time PCR analysis. GCLC, GCLM, GST-P, GSR, and SOD1. D and E, the expression of the indicated antioxidant proteins (D) and cellular GSH level (E) were analyzed in MIAPaCa-2 and MIAPaCa-2/GR cells. β-Actin was used as loading control. Data in B, C, and E, mean ± SD (n = 5); *, P < 0.05; and **, P < 0.01 versus control (no treatment) or MIAPaCa-2 cells.
received 50 mg/kg intraperitoneal gemcitabine once a week; group 3 was treated with 25 mg/kg intraperitoneal PEITC 3 consecutive days per week; group 4 was treated with 50 mg/kg intraperitoneal gemcitabine once a week followed by 25 mg/kg PEITC for the next 3 days. Treatments were continued for 4 weeks. All mice were weighed every 5 days and observed for tumor growth. Bioluminescence imaging of tumors was conducted using a cryogenically cooled IVIS 100 imaging system as previously described (20). The day after the end of treatment, the mice were euthanized by carbon dioxide inhalation and the tumors were excised and weighed. The tissue was then fixed in formalin and embedded in paraffin. The expression of p-NF-κB/p65 and p22phox was analyzed by Western blotting using the protein extracted from three representative mice tumor tissue from each treatment group. The cellular GSH level was also determined. These animal experiments were repeated once.

Statistical analysis

All data are presented as mean ± SD. For comparison of the statistical differences of more than two groups, one-way ANOVA and Newman-Keuls multiple comparison tests were used. All other statistical analyses were evaluated using the Student's unpaired t test (Prism GraphPad). A P value of <0.05 was considered statistically significant. The cytotoxic effect of PEITC combined with gemcitabine was calculated using CalcuSyn (Biosoft).

Figure 2.

Gemcitabine induced NOX-derived ROS generation through the increase of p22phox expression via NF-κB activation. A, NOX activity in MIAPaCa-2 and MIAPaCa-2/GR cells that were untreated or treated with DPI for 24 or 48 hours. B, NOX activity in PDAC cells treated with gemcitabine, DPI, or both for 48 hours. C, comparisons of mRNA expression of NOX components in MIAPaCa-2 (MIA) and MIAPaCa-2/GR (GR) cells. D, comparisons of mRNA expression of p22phox in MIAPaCa-2 and MIAPaCa-2/GR cells (left) and PDAC cells treated with gemcitabine (right). E, quantitative analysis of ROS levels in indicated PDAC cells treated with gemcitabine, siRNA (targeting p22phox), or both (Gem + siRNA). F, the expression of p-NF-κB/p65, NF-κB, and p22phox in indicated PDAC cell lines that were treated with gemcitabine were analyzed by Western blot analysis. G, immunofluorescence staining of NF-κB/p65 (green) in MIAPaCa-2 and AsPc-1 cells treated with gemcitabine (5 μmol/L) for 24 hours. DAPI (blue) was used to indicate the nuclei. Scale bar, 10 μm. H, the expression of p-NF-κB/p65, NF-κB, and p22phox in indicated PDAC cell lines after siRNA depletion of NF-κB. β-Actin was used as loading control. Data in A, B, D, and E, mean ± SD (n = 3); **, P < 0.01 versus the corresponding control groups.
Redox Modulation for Overcoming Gemcitabine Resistance

Results
Significant increase in ROS generation compensated with increased cellular antioxidant activity in MIAPaCa-2/GR cells

To study the redox mechanisms underlying cellular resistance to gemcitabine (18), we compared the ROS levels between MIAPaCa-2 and MIAPaCa-2/GR cells (Supplementary Fig. S1). We found that MIAPaCa-2/GR cells exhibited significantly higher levels of ROS as quantified by flow cytometry (Fig. 1A). We observed that gemcitabine induced significant accumulation of cellular ROS in not only MIAPaCa-2 cells, but also AsPc-1 and Panc-28 cells after incubation with 0.05, 0.5, and 5 µmol/L gemcitabine for 24 hours (Fig. 1B and Supplementary Fig. S1B), confirming previous reports of ROS induction by gemcitabine (9, 10). As expected, gene expression of GSH-related enzymes [glutamate-cysteine ligase, catalytic subunit (GCLC), glutamate-cysteine ligase, modifier subunit (GCLM), glutathione S-transferase pi (GST-pi), and GSR] was significantly upregulated in MIAPaCa-2/GR cells compared with the parental cells (Fig. 1C; Supplementary Table S1). Superoxide dismutase-1 (SOD1; an enzyme responsible for eliminating O$_2^-$), catalase (an enzyme responsible for eliminating H$_2$O$_2$), and Nrf2, which regulates GSH synthesis (12), were significantly upregulated as well in these cells (Fig. 1C, D, and E). Taken together, these results suggested that gemcitabine treatment increases ROS generation with a compensatory increase in cellular antioxidant activity. The significant upregulation of these antioxidants may protect cells from ROS injury caused by gemcitabine.

Gemcitabine-induced NOX-derived ROS generation though increase of the expression of p22$^{phox}$ via NF-κB activation

Because it is known that NOX is a major source of ROS generation (11, 12, 14), we compared the activity of NOX in these cells. MIAPaCa-2/GR cells exhibited an almost 4-fold higher NOX activity compared with parental cells (P < 0.01; Fig. 2A), and the activity of NOX was inhibited specifically by DPI, a compound known as a flavoprotein inhibitor of NOX (19), by 90% after 48 hours of treatment in both cell lines (Fig. 2A). Consistently, the increase of NOX activity induced by gemcitabine treatment in PDAC cells was completely blocked by additional incubation with DPI (Fig. 2B). To investigate how NOX activity was induced after gemcitabine treatment, we examined the expression of different components of the NOX complex in these cells (Fig. 2C, Supplementary Table S1). Consistent with reported studies (21), NOX3 and NOX4 were expressed in both cell lines. In addition, the expression of p22$^{phox}$, a catalytic subunit of the NOX complex was significantly higher in MIAPaCa-2/GR cells than in MIAPaCa-2 cells.

Figure 3.
Gemcitabine promoted ROS detoxification by inducing GSH synthesis through Nrf2 activation. A and B, the upregulation of mRNA expression of Nrf2 (A) or Nrf2 target genes (B) before and after treatment was compared by real-time analysis. C, gemcitabine induced translocation of Nrf2 from cytosol to the nucleus in PDAC cells treated with 5 µmol/L gemcitabine for 48 hours. Lamin A/C was used as the nucleus loading control. β-Actin was used as the whole-cell loading control. D, increase of cellular GSH level after gemcitabine treatment in PDAC cells. E, decrease of cellular GSH level after siRNA depletion of Nrf2 in MIAPaCa-2 cells detected by flow cytometric analysis. G, effect of siRNA depletion of Nrf2 on sensitivity of three PDAC cell lines to gemcitabine detected by MTS assay. PDAC cells were transfected with 10 nmol/L Nrf2-targeting siRNA, or with RNAi control for 24 hours, which was followed by exposure to gemcitabine for 72 hours, at the indicated concentrations. Data, mean ± SD (n = 3); **, P < 0.01 versus the corresponding control groups.
Similar results were observed in AsPC-1 and Panc-28 cells after 48 hours treatment of gemcitabine (Fig. 2D). The increased cellular ROS after gemcitabine treatment in PDAC cells was significantly decreased by siRNA depletion of p22phox (Fig. 2E). However, DPI showed opposite results, which may be due to its nonspecificity (data not shown). The increase of NOX activity and ROS generation was correlated with elevated p22phox expression, suggesting that p22phox might be the limiting factor in the NOX complexes. These results demonstrated that NOX is involved in gemcitabine-induced ROS generation through the increase of p22phox expression.

We then investigated the molecular mechanism involved in p22phox regulation by gemcitabine in PDAC cells. Gemcitabine has been reported to modulate the activity of the transcription factor NF-kB (10, 20). Interestingly, NF-kB has also been reported to target the p22phox promoter, and to upregulate p22phox expression and NOX activity in human aortic smooth muscle cells (22). To determine whether NF-kB mediates gemcitabine induced upregulation of p22phox expression in PDAC cells, we examined the phosphorylated (p)-NF-kB/p65 (i.e., activated NF-kB/p65) and p22phox expression levels in PDAC cells following gemcitabine treatment. We found that gemcitabine treatment increased the level of p-NF-kB/p65 and
the nuclear localization of NF-κB/p65 in tested PDAC cells (Fig. 2F and G). Also, the expression of p22^phox^ was substantially increased following NF-κB activation in the PDAC cells treated with gemcitabine (Fig. 2F). siRNA depletion of NF-κB decreased the expression level of p22^phox^, notably in all three cell lines (Fig. 2H). Further studies also confirmed the increased p-NF-κB/p65 and p22^phox^ expression in MIAPaCa-2/GR cells compared with the parental cells (Supplementary Fig. S2A). siRNA depletion of NF-κB/p65 decreased the elevated ROS level in MIAPaCa-2/GR cells and MIAPaCa-2 cells treated with gemcitabine (Supplementary Fig. S2B and S2C). These data suggest that NF-κB also positively regulates p22^phox^ expression in PDAC cells. Taken together, these results indicated that gemcitabine induces NOX-derived ROS generation though the increase of NF-κB activation and p22^phox^ expression.

**Figure 5.** Synergistic activity between gemcitabine and PEITC in various PDAC cell lines. A, cellular GSH level in MIAPaCa-2 cells treated with 5 μmol/L gemcitabine (Gem), 2.5 μmol/L PEITC, or both (G + P). Values are normalized to the levels in untreated (control) cells. B, quantitative analysis of ROS accumulation induced by 5 μmol/L gemcitabine, 2.5 μmol/L PEITC, or both (G + P) in MIAPaCa-2 and AsPc-1 cell lines. C, MIAPaCa-2 cells were incubated with various concentrations of gemcitabine (0.1–10 μmol/L) with or without PEITC (3 μmol/L) for 72 hours. The effect on cell viability was determined by MTS assay. And the CI of gemcitabine and PEITC treatment in MIAPaCa-2 cells was analyzed by a median dose-effect method using CalcuSyn software (Biosoft). CI = 1, additive effect; CI < 1, synergistic effect; CI > 1, antagonist effect. D, inhibition of colony formation in MIAPaCa-2, Panc-28, and AsPc-1 cells by gemcitabine (20, 10, and 5 nmol/L, respectively) with 2 μmol/L PEITC. PDAC cells were incubated with gemcitabine, PEITC, or both for 2 weeks and the cell colonies were fixed in formalin, stained with crystal violet, and counted. Data, mean ± SD (n = 3); **, P < 0.01 compared with the corresponding control value.
Figure 6.
Antitumor activity of PEITC plus gemcitabine in vivo in an AsPC-1 pancreatic tumor orthotopic xenograft mouse model. A, a digital grayscale image of each mouse in each group was acquired on treatment day 28, which was followed by the acquisition and overlay of a pseudocolor image representing the spatial distribution of detected photons emerging from active luciferase within the mouse. B, the luciferase quantitative value for tumor variations in each group. C and D, mice were killed by carbon dioxide inhalation, tumors were excised (C) and weighed (D) on day 28 after drug treatment. (Continued on the following page.)
Gemcitabine induced GSH synthesis by activation of Nrf2 to promote ROS detoxification, and depletion of Nrf2 enhanced the sensitivity of PDAC cells to gemcitabine

The transcription factor Nrf2 plays a vital role in regulating the expression of genes that encode antioxidants (23). To investigate the effect of gemcitabine on expression of major antioxidants, the three PDAC cell lines were exposed to 5 μmol/L gemcitabine for 48 hours, and the gene expression levels were analyzed by real-time PCR. Treatment significantly upregulated Nrf2 in all three cell lines (Fig. 3A). The levels of target genes responsible for GSH synthesis (GCLM and GCLC), GSH regeneration (GSR), and GSH in detoxification (GST-pi) were all higher in treated cells than those in control cells (Fig. 3B). In addition, Western blot analysis demonstrated that similar treatment caused a remarkable increase in the nuclear level of Nrf2 and the expression of its representative target gene GCLC (Fig. 3C). Accordingly, the cellular GSH level was significantly higher in these three PDAC cells treated with gemcitabine (Fig. 3D). These observations indicated that gemcitabine treatment produces oxidative stress and consequently induces antioxidant/prosurvival/cytoprotective response in PDAC cells.

We then explored the role of Nrf2 in regulating the redox balance in MIAPaCa-2 cells treated with gemcitabine. We posited that due to the intrinsic increase of ROS caused by gemcitabine treatment, the cells are highly dependent on GSH to maintain redox homeostasis, and that the interruption of antioxidant systems results in excessive accumulation of ROS to a threshold that triggers cell death. To test this possibility, we first investigated the effect of Nrf2 depletion in PDAC cells by using siRNA. The increased GSH level and cellular ROS that had been observed in PDAC cells after gemcitabine treatment were significantly decreased by siRNA depletion of Nrf2 (Fig. 3E and F). Accordingly, siRNA depletion of Nrf2 caused a mild decrease in the viability of PDAC cells and elicited increased sensitivity to gemcitabine in MIAPaCa-2, AsPC-1, and Panc-28 cells as determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenol)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) assay (Fig. 3G). These results suggested that Nrf2 plays an important role in determining the sensitivity of PDAC cells to gemcitabine. Therefore, increased gemcitabine efficacy may be achieved by strategically targeting the Nrf2-regulated antioxidant pathway with pharmacologic agents.

Preferential induction of ROS accumulation and cell death by PEITC in gemcitabine resistance cells

On the basis of important role of GSH as a major cellular antioxidant, we postulated that PEITC, a novel compound conjugates with GSH to export it from cells (14, 24), circumvents cellular resistance to gemcitabine. To test this possibility, we first examined the effect of PEITC on GSH content in PDAC cells. Incubation of PDAC cells with 5 μmol/L PEITC for 3 hours significantly depletes cellular GSH (80%), whereas, incubation with 5 μmol/L PEITC for 24 hours significantly increase the cellular ROS level (Fig. 4A). Annexin V/propidium iodide (PI) and apoptotic assays showed that treatment with 5 μmol/L PEITC for 48 hours resulted in an apoptosis level of approximately 60% in all three PDAC cell lines (Fig. 4B). Treatment with N-acetylcysteine (NAC, a GSH precursor) before PEITC resulted in an apoptosis level of approximately 15% in these same cells, demonstrating that NAC suppresses the cytotoxicity of PEITC (Fig. 4B). In contrast, PEITC demonstrated minimal toxicity to an immortalized/nontumorigenic pancreatic ductal epithelial cell line HPDE according to MTS and Annexin V/PI assays (Supplementary Fig. S3A and S3B), suggesting that PEITC may selectively cause death of PDAC cells.

Consistent with the treatment of PEITC, similar results (decreased GSH content, increased ROS level, and more apoptosis cells) were also observed in PDAC cells treated with the glutamylcysteine synthetase inhibitor BSO (Supplementary Fig. S4A and S4B). These results suggested that PEITC induces ROS accumulation in addition to cell death by depleting cellular GSH in PDAC cells. To further test whether PEITC can preferentially induce cell death by interrupting the redox balance in gemcitabine-resistant cells, we treated both MIAPaCa-2 and MIAPaCa-2/GR cells with 5 μmol/L PEITC for 3 or 6 hours and examined the effect on GSH content and ROS level. For both cell lines, 5 μmol/L PEITC depleted cellular GSH by 80% at 3 hours and almost completely at 6 hours (Fig. 4C). Treatment of MIAPaCa-2/GR cells with 5 μmol/L PEITC increased the amount of ROS in a time-dependent manner and reached a 4-fold increase at 6 hours. In contrast, the parental MIAPaCa-2 cells demonstrated a moderate H2O2 elevation, indicating that the parental cell line was less sensitive to PEITC (Fig. 4C). These findings suggested that the parental cells are better able to survive PEITC-induced accumulation, probably because they tend to have a low basal ROS output. Consistently, Annexin V/PI and MTS and colony formation assays demonstrated that PEITC effectively induced cell death and suppressed cell growth in both MIAPaCa-2 and MIAPaCa-2/GR cells in a dose-dependent manner, whereas MIAPaCa-2 cells were less sensitive to PEITC (Fig. 4D–F).

Furthermore, pretreatment with glutathione monoester (GME), a cell permeable derivative of GSH, showed partial inhibition of PEITC-induced cell apoptosis and more resistant to gemcitabine in MIAPaCa-2 cells as demonstrated by Annexin V/PI and colony formation assay, respectively (Supplementary Fig. S4C and S4D). Taken together, these results suggested that gemcitabine-resistant cells are “additive” to high GSH for survival, and thus are more sensitive to GSH depletion by PEITC.

PEITC enhanced gemcitabine cytotoxicity in PDAC cell lines and in orthotopic xenograft mouse models by modulating cellular redox status

To test our hypothesis that the pharmacologic depletion of cellular GSH modulates the chemoresistance of pancreatic cancer, we examined in vitro antitumor capability of PEITC with the combination of gemcitabine. First, we used a relatively low concentration of PEITC (2 μmol/L) in combination with 5 μmol/L gemcitabine to treat MIAPaCa-2 cells. Results showed that this combination significantly depleted cellular GSH in MIAPaCa-2 cells versus treatment with gemcitabine only (Fig. 5A). Flow cytometric analysis demonstrated that, compared with gemcitabine treatment only, this combination resulted in significantly
higher accumulation of ROS in MiaPaCa-2 cells and in AsPC-1 cells (Fig. 5B). Moreover, combination of gemcitabine and PEITC decreased cell viability (Fig. 5C, left). These results suggested that disruption of an antioxidant-mediated cytoprotective pathway by PEITC significantly enhances the antipancreatic cancer activity of gemcitabine in PDAC cells. The median-effect analysis software CalcuSyn was used to calculate the drug combination index (CI). The results showed a synergistic interaction between gemcitabine and PEITC, as demonstrated by a consistent CI value less than 1.0 (Fig. 5C, right). Colony formation assay was then used to test the effect of the combination on PDAC cell growth. Different concentrations of gemcitabine were used in combination with 2 μmol/L PEITC to treat MiaPaCa-2, Panc-28, and AsPC-1 cells. Either agent alone caused some delay in cell growth in all three cell lines, but together gemcitabine and PEITC showed synergistic effect of colony formation inhibition compared with the calculated/predicted additive value (Fig. 5C).

Finally, we determined whether PEITC plus gemcitabine at clinically relevant doses can inhibit the growth of AsPC-1 cells in an orthotopic xenograft nude mouse model. Twenty mice were orthotopically injected with AsPC-1 cells and randomly assigned to four groups (n = 5/group). Treatment with gemcitabine, PEITC, both agents, and vehicle control began 2 weeks after injection and lasted for 4 weeks. No significant weight loss (Supplementary Fig. S3C) or other signs of acute or delayed toxicity were observed in any mouse during treatment. Compared with the control group after 4 weeks of treatment, gemcitabine alone and PEITC alone had little effect (Fig. 6A and B). However, the mice treated with both agents experienced a large reduction in tumor burden (mean tumor weight: vehicle control, 1.23 ± 0.16 g; gemcitabine, 1.26 ± 0.12 g; PEITC, 1.01 ± 0.18 g; gemcitabine plus PEITC, 0.49 ± 0.13 g; Fig. 6C and D). Western blot analyses were carried out to determine the expression of p-NF-κB/p65 and p22phox in these tumors. Consistent with our in vitro results, the expression of these two proteins was increased in representative tumors tissue extracted from mice treated with gemcitabine compared with those left untreated (control; Fig. 6E). Also, tumor tissues from mice treated with PEITC either alone or with gemcitabine demonstrated significant depletion of cellular GSH compared with control (Fig. 6F). The combination of gemcitabine and PEITC resulted in the greatest amount of apoptosis compared with gemcitabine or PEITC alone detected by TUNEL assay (Fig. 6G). These in vitro results indicated that PDAC cells treated with gemcitabine may rely on the GSH antioxidant system against ROS stress for cell survival, and that depletion of GSH may cause further oxidative stress to achieve significant cell killing not only in vitro but also in vivo.

Discussion

In this study, we investigated the molecular mechanisms of ROS generation and the role of antioxidants in gemcitabine resistance. When ROS accumulates past the detoxification capacity of the cell, ROS cytotoxicity damages mitochondria and cell membranes causing cell death (11, 12). The NOX family is the major source of ROS that are implicated in cancer (25–27). Our data showed that gemcitabine increased NOX activity, which could be reversed by the NOX inhibitor DPI. Interestingly, gemcitabine induced p22phox upregulation and activation of NF-κB, whereas silencing NF-κB by RNAi reduced gemcitabine-induced p22phox expression. This finding was consistent with a previous report that NF-κB upregulated p22phox expression in human aortic smooth muscle cells (22). Taken together, these results showed that the gemcitabine treatment induces NF-κB activation and NOX-derived ROS generation through p22phox expression in PDAC cells. In addition, CXCR4 signaling regulated by NF-κB has also been reported serving as a counter defense mechanism against gemcitabine in PDAC (10), which is most like another parallel pathway regulated by NF-κB. Our previous studies showed that PDAC cells with silenced TAK1 had lower NF-κB activity and were more sensitive to gemcitabine, suggesting that activation of NF-κB played an important role in chemoresistance (20).

Responding to oxidative stress, Nrf2 induces transcription of antioxidant response element-bearing genes, in addition to antioxidant-encoding genes (e.g., GSH-synthesizing enzymes), and phase II detoxifying enzymes and transporters (13, 28). Nrf2 induces expression of many cytoprotective proteins responsible for maintaining cellular redox stability. Recent study also showed that nuclear Nrf2 expression play an important role in ROS detoxification during pancreatic carcinogenesis (29), and is related to a poor survival in patients with pancreatic adenocarcinoma (30). Western blot analysis showed that the expression of Nrf2-targeting antioxidants such as SOD1, GPX, GST-Pi, GCLC, and GCLM, was increased following gemcitabine treatment. It is well established that Nrf2 is activated by oxidative stress (28). Keap1 regulates antioxidant responses by repressing Nrf2 activation (13, 28). Taking together, we conclude that gemcitabine-induced ROS increases Nrf2-mediated antioxidants, thus resetting a toxic threshold for oxidative stress by upregulation of antioxidant agents. So, adaptive countermeasure against gemcitabine enables PDAC cells to endure ROS accumulation.

Excessive levels of ROS stress can exhaust the antioxidant capacity of cells and lead to apoptosis by pushing the ROS stress level beyond a threshold (12, 27). Therefore, manipulating ROS levels by redox modulation is a way to selectively kill cancer cells without causing significant toxicity to normal cells (31). Using genetic manipulation, we demonstrated that additional Nrf2 depletion induced a decrease of in GSH level and an increase in ROS generation following gemcitabine treatment in PDAC cells. Furthermore, knockdown of Nrf2 with Nrf2-siRNA revealed an increased sensitivity to gemcitabine in different PDAC cell lines. This result is consistent with a recent report that inhibition of Nrf2 by PIK-75 augments sensitivity of pancreatic cancer cells to gemcitabine (32). We reason that modulating gemcitabine-induced redox regulatory mechanisms by reducing GSH pool may affect cell survival and drug sensitivity. PEITC, a novel compound that has been reported to deplete GSH and subsequently elevate oxidative stress (15, 17), may circumvent such protection and enhance the antipancreatic cancer activity of gemcitabine.

To validate the cellular antioxidant system as a potential therapeutic target for treatment of patients with pancreatic cancer, we then investigated the antitumor activity of PEITC in pancreatic cancer cells. Our results showed that PEITC effectively disabled the GSH antioxidant system and selectively killed cancer cells with increased ROS generation. Consistently, our results showed that disruption of the antioxidant-mediated cytoprotective pathway by PEITC significantly enhanced the antipancreatic cancer activity of gemcitabine in three PDAC cell lines. Moreover, the intratumoral NF-κB activity was significantly elevated in gemcitabine-treated mice than in control mice, consistent with a recent report
(33). Our findings provide the combination strategy to modulate cellular redox status and overcome resistance to gemcitabine, resulting in a synergistic and selective cytotoxic effect in vivo. Although recent reports showed that NF-xB and Nrf2 were important for pancreatic cancer development and chemoresistance (30, 34, 35), we showed that gemcitabine activates NOX-induced ROS via NF-xB activation and increased ROS results in activation of Nrf2 and the increase of cellular GSH, contributing to the intrinsic resistance of PDAC. A combination of gemcitabine, which stimulates endogenous ROS generation, and PEITC, which inhibits ROS elimination, would be a logical strategy to induce pancreatic cell death, in that it would compose severe oxidative stress (Fig. 6H).

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

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Conception and design: H.-Q. Ju, T. Gocho, P. Huang, P.J. Chiao
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