Gemcitabine Sensitivity Can Be Induced in Pancreatic Cancer Cells through Modulation of miR-200 and miR-21 Expression by Curcumin or Its Analogue CDF

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Abstract
Curcumin induces cancer cell growth arrest and apoptosis in vitro, but its poor bioavailability in vivo limits its antitumor efficacy. We have previously evaluated the bioavailability of novel analogues of curcumin compared with curcumin, and we found that the analogue CDF exhibited greater systemic and pancreatic tissue bioavailability. In this study, we evaluated the effects of CDF or curcumin alone or in combination with gemcitabine on cell viability and apoptosis in gemcitabine-sensitive and gemcitabine-resistant pancreatic cancer (PC) cell lines. Mechanistic investigations revealed a significant reduction in cell viability in CDF-treated cells compared with curcumin-treated cells, which were also associated with the induction of apoptosis, and these results were consistent with the downregulation of Akt, cyclooxygenase-2, prostaglandin E2, vascular endothelial growth factor, and NF-κB DNA binding activity. We have also documented attenuated expression of miR-200 and increased expression of miR-21 (a signature of tumor aggressiveness) in gemcitabine-resistant cells relative to gemcitabine-sensitive cells. Interestingly, CDF treatment upregulated miR-200 expression and downregulated the expression of miR-21, and the downregulation of miR-21 resulted in the induction of PTEN. These results prompt further interest in CDF as a drug modality to improve treatment outcome of patients diagnosed with PC as a result of its greater bioavailability in pancreatic tissue. Cancer Res; 70(9); 3606-17. ©2010 AACR.

Introduction
Although significant progress has been made in systemic treatments, pancreatic cancer (PC) still remains the fourth leading cause of cancer-related deaths in the United States with an estimated 42,470 new cases and 35,240 deaths in 2009 (1). Many attempts in recent years aimed at improving the survival of patients diagnosed with PC have been disappointing, suggesting that newer treatment strategies must be developed.

Gemcitabine is considered the standard agent for the treatment of advanced disease and has offered some relief over the past two decades; however, the combination treatment using gemcitabine with other agents has not been successful in increasing the overall survival. These disappointing results call for novel combination therapies to improve the survival outcome of PC patients. Emerging evidence has shown combination therapies involving treatment with curcumin, an active component of turmeric, with gemcitabine in PC cell lines (2–4). Curcumin in combination with celecoxib, a cyclooxygenase-2 (COX-2) inhibitor, showed significant growth inhibition of PC cell lines (5) and, interestingly, in combination with α-3 fatty acids showed synergistic tumor inhibitory properties (6). These results suggest that curcumin could be useful in combination therapy, especially because curcumin is nontoxic to humans and showed multitargeted effects (7). Furthermore, curcumin alone can alter the expression of microRNAs (miRNA) in PC cells (8), which could be important in mediating its biological effects. Although curcumin could inhibit cell viability; induces apoptosis in pancreatic, breast, lung, prostate, and several other cancer cell lines (7, 9–11); and is also well tolerated, its limited absorbance across the gut and rapid metabolism in animal models and human clinical trials raised major concern regarding its target tissue bioavailability, limiting its therapeutic value (12, 13) especially for the treatment of patients with pancreatic tumor. Numerous analogues of curcumin have been created to overcome its low bioavailability and have attempted to increase its absorption without loss of activity (14–17); however, none has shown better target tissue bioavailability especially in the pancreas. We have previously shown the synthesis of a new analogue (CDF) with potent biological activity against PC cells and have also documented significantly...
greater pancreatic tissue bioavailability in mice compared with curcumin (18, 19), which led us to conduct the current study.

Studies have shown that the activation of phosphoinositide 3-kinase (PI3K) signaling pathway is due to the aberrant expression of PTEN in PC cell lines (20, 21). Phosphorylation and activation of PI3K/Akt can activate NF-κB, and the development and progression of PC are linked with the activation of NF-κB, a key transcriptional regulator of genes involved in cell survival, proliferation, and induction of apoptosis, thus suggesting that targeting inactivation of NF-κB could be therapeutically important (22, 23). Moreover, COX-2, a transcriptional downstream target of NF-κB, which mediates the production of prostaglandins [prostaglandin E2 (PGE2)] could also be a potential target for the treatment of PC (24). Interestingly, we have shown that curcumin and its analogue CDF could target both NF-κB and COX-2 (19), suggesting that CDF could be useful for the treatment of PC, especially because of its greater pancreatic tissue bioavailability.

Emerging evidence has shown that gene expression could be regulated by miRNAs, suggesting that miRNAs may play important functional role in a wide array of physiologic cellular processes, including differentiation, proliferation, and apoptosis (25). It has also been suggested that the processes of epithelial-to-mesenchymal transition (EMT) are regulated by the expression status of specific miRNAs during tumor development and progression (26, 27). The miRNA-200 family is one such example, which plays important role in regulation of EMT (28, 29), and is associated with cancer recurrence and overall survival. Low expression of miRNA-200 plays important roles in cancer metastasis in ovarian (30) and breast cancer (31). Hence reactivation of miR-200 by novel approaches could serve as inhibitors of EMT, which may either kill or reverse the EMT phenotype, thereby conventional agent, such as gemicitabine, could effectively kill those EMT-type cells. Another miRNA, miR-21, is overexpressed in solid tumors, including PC (32–34), breast cancer (35), and thyroid cancer (36), compared with paired benign and normal tissues. Induction of Stat-3 by interleukin-6 has been linked to miR-21 in multiple myeloma cells (37), and miR-21 is considered an oncogenic miRNA exhibiting ant apoptotic activity in various carcinomas cell lines (38, 39), suggesting that the inactivation of miR-21 could be therapeutically beneficial toward the treatment of PC.

Here we report for the first time that CDF, a novel analogue of curcumin, upregulated miRNA-200b and miRNA-200c and downregulated miR-21 in both gemicitabine-sensitive (BxPC-3) and gemicitabine-resistant (MIAPaCa-E and MIAPaCa-M) cell lines, which were associated with induction of apoptosis. We also showed that combination of CDF with gemicitabine was much more effective than either agent alone compared with curcumin, suggesting that CDF-mediated alterations in specific miRNAs, along with inactivation of NF-κB and its downstream genes, could be a novel approach for the treatment of patients diagnosed with PC.

**Materials and Methods**

**Cell culture, drugs, and reagents.** Human PC cell lines MIAPaCa-E, MIAPaCa-M, and BxPC-3 were chosen for this study based on their sensitivities to gemicitabine. MIAPaCa cells were exposed to gemicitabine every other week for 4 months to create a gemicitabine-resistant cell line. We named this paired cell line as MIAPaCa-E and MIAPaCa-M based on the changes in morphology from epithelial-like to mesenchymal-like phenotype. The cell lines have been tested and authenticated in core facility Applied Genomics Technology Center at Wayne State University on March 13, 2009. The method used for testing was short tandem repeat profiling using the PowerPlex 16 System from Promega. Gemicitabine and curcumin were purchased from Eli Lilly and Sigma-Aldrich, respectively. CDF was synthesized as described in our earlier publication (18, 19).

**Cell viability assay.** To test the viability of cells, 3,000 cells per well were plated in a 96-well plate for 24 hours. We initially tested a range of concentrations for CDF/curcumin (0.1–4 μmol/L) and gemicitabine (10–50 nmol/L). Based on the initial results for BxPC-3 cells, we chose the concentration of CDF/curcumin (1 μmol/L) and gemicitabine (10 nmol/L) for all subsequent assays. Next, we tested the effect of treatments on MIAPaCa-E (relatively resistant to gemicitabine) and MIAPaCa-M (highly resistant to gemicitabine) cell line with higher doses of CDF/curcumin (4 or 10 μmol/L), gemicitabine (10 nmol/L), and their combinations. Standard assay using MTT was performed after treatment for 72 hours, and experiments were repeated thrice. The color intensity was measured by TECAN’s microplate fluorometer (TECAN) at 595 nm.

**Clonogenic assay.** MIAPaCa-E cells were plated (30,000 per well) in a six-well plate. After 72 hours of exposure to 4 μmol/L of CDF/curcumin, 10 nmol/L of gemicitabine, and the combination, cells were trypsinized, and 1,000 viable cells were plated in 100-mm Petri dishes. The cells were then incubated for ∼10 to 12 days at 37°C in a 5% CO2/5% O2/90% N2 incubator. Colonies were stained with 2% crystal violet and quantitated.

**Quantification of apoptosis by enzyme-linked immunosorbent assay.** The cell death detection enzyme-linked immunosorbent assay (ELISA) kit (Roche Applied Science) was used to detect apoptosis in untreated and treated cells. Cells seeded in six-well plates were treated with CDF/curcumin (1, 4, or 10 μmol/L), gemicitabine (10 nmol/L), or their combination and trypsinized, and ∼10,000 cells were assayed as described earlier (40). TECAN’s microplate fluorometer (TECAN) was used to measure color intensity at 405 nm. The experiment was repeated thrice.

**Protein extraction and Western blot analysis.** BxPC-3, MIAPaCa-E, and MIAPaCa-M cells treated with CDF/curcumin (1 or 4 μmol/L), gemicitabine (10 nmol/L), or the combination for 72 hours were used to evaluate the effects on COX-2, E-cadherin, PTEN, pAkt, tropomyosin, and β-actin expression. Western blot analysis was performed as described previously (40), and the signal intensity was measured using chemiluminescent detection system (Pierce).

**PTEN cDNA transfection.** To determine the effect of PTEN cDNA transfection, MIAPaCa-E cells were plated in 100-mm
Petri dishes overnight and transfected with 15 μg of PTEN cDNA or the control empty vector by ExGen 500 (Fermentas) following the manufacturer’s protocol. Cells were treated with gemcitabine (10 nmol/L) or left untreated for 48 hours and assessed for the expression of PTEN, pAkt, NF-κB, and β-actin by Western blot analysis.

Antisense miR-21 oligonucleotide transfection. MIAPaCa-E cells were plated in 100-mm Petri dishes overnight. Cells were transfected with antisense miR-21 oligonucleotide or the nonspecific control using ExGen 500 (Fermentas) following the manufacture’s protocol. Treatment with 10 nmol/L of gemcitabine was done for 48 hours, and transfection efficiency of targeted proteins PTEN, pAkt, and NF-κB was determined by Western blot analysis.

Electrophoretic mobility shift assay for NF-κB activation. To evaluate the effect of CDF, curcumin, and gemcitabine on BxPC-3, MIAPaCa-E, and MIAPaCa-M cells, the cells were either untreated or treated with CDF/curcumin (1 or 4 μmol/L), gemcitabine (10 nmol/L), or the combination for 72 hours. The cells were lysed in 400 μL of lysis buffer, and the reaction was set up as described earlier (40). The gel was scanned using Odyssey Infrared Imaging System (LI-COR, Inc.). Equal protein loading was ensured by immunoblotting 10 μg of nuclear protein and probing with antiretinoblastoma antibody.

Determination of PGE2. To determine PGE2 levels, BxPC-3, MIAPaCa-E, and MIAPaCa-M cells were treated with CDF/curcumin (1–4 μmol/L), gemcitabine (10 nmol/L), or the combination for 24 hours in serum-free media. PGE2 secreted in the culture medium was analyzed using PGE2 immunoassay kit, as suggested by the manufacturer (R&D Systems).

Determination of vascular endothelial growth factor. All three human PC cells were seeded in six-well plates overnight followed by treatment with CDF/curcumin (1–4 μmol/L), gemcitabine (10 nmol/L), or their combination for 72 hours. The supernatant was assayed for vascular endothelial growth factor (VEGF) assay using AlphaLISA VEGF 500 data point kit from Perkin-Elmer.

TagMan miRNA real-time reverse transcription–PCR. To determine the expression of miRNAs (miRNA-200a, miR-200h, and miR-21) in all three PC cell lines, we used TagMan miRNA assay kit (Applied Biosystems) following manufacturer’s protocol. Cells were treated with CDF/curcumin (1–4 μmol/L), gemcitabine (10 nmol/L), or combination for 72 hours. Total RNA was extracted, and 5 ng from each sample were reverse transcribed as described earlier (29). Real-time PCR reactions were then carried out in a total volume of 25 μL reaction mixture as described earlier (29) using Smart Cycler II (Cepheid). Data were analyzed using C_{t} method and were normalized by RNU6B expression in each sample.

Statistical methods. Comparisons of treatment outcome were tested for statistical difference by the paired t test. Statistical significance was assumed at a P value of <0.05.

Results

The effects of CDF, curcumin, and gemcitabine on the viability of human PC cell lines, as assessed by MTT and clonogenic assays. BxPC-3 cells treated with different concentrations of CDF/curcumin (0.1–4 μmol/L) for 72 hours (Fig. 1A) were assessed for cell growth, as determined by MTT assay. Significant reduction in cell viability was seen in cells treated with CDF and curcumin. Based on our initial results, we selected the concentration of CDF/curcumin as (1 μmol/L) and gemcitabine (10 nmol/L) for BxPC-3 cells for conducting the combination experiments (Fig. 1B). Significant inhibition of cell viability was seen in combination treatments in BxPC-3 cells, MIAPaCa-E and MIAPaCa-M cells when treated with higher concentration of either 4 μmol/L or 10 μmol/L CDF/curcumin showed greater inhibition with CDF alone than curcumin alone; however, the growth inhibition was more superior in CDF combinations than curcumin combinations (Fig. 1B and C). The effect of treatment on cell growth was also assessed by clonogenic assay. The combination treatment resulted in a significant inhibition of colony formation in MIAPaCa-E cells when compared with single-agent treatment (Fig. 1D), and this was much more pronounced by CDF compared with curcumin combination. Similar results were observed with other cell lines (data not shown). Based on differential sensitivities of these cell lines to the above-mentioned agents, we chose different concentrations of CDF/curcumin for BxPC-3 (1 μmol/L), MIAPaCa-E (4 μmol/L), and MIAPaCa-M (4 μmol/L) for all subsequent experiments.

Light micrographic pictures. Figure 2A shows the morphologic differences in BxPC-3, MIAPaCa-E, and MIAPaCa-M cells. The MIAPaCa-E cells were exposed to gemcitabine every other week for a period of 4 months, which led to this mesenchymal phenotype (Fig. 2A). These cells (MIAPaCa-M) were subsequently used for our experiments.

Induction of apoptosis by CDF, curcumin, gemcitabine, and the combination. The underlying mechanism on the inhibition of cell viability was further studied by determining the apoptotic effects using the cell death detection ELISA. The combination of CDF and gemcitabine resulted in a significant induction of apoptosis in all three cell lines tested when compared with curcumin combination or single agents alone (Fig. 2B). We also wanted to test whether increasing the concentration of CDF and curcumin from 4 to 10 μmol/L in MIAPaCa-E and MIAPaCa-M would have any differential effects. We observed a substantial induction of apoptosis in cells treated with both combinations (Fig. 2C). These results are consistent with the cell viability assay. Subsequently, the mechanisms of such differences were further investigated, and the results are presented in the following sections.

Treatment effects on the expression of selective proteins. BxPC-3, MiaPaCa-E, and MiaPaCa-M cells were used to evaluate the effects of CDF, curcumin, and/or gemcitabine on the expression of COX-2, E-cadherin, PTEN, pAkt, and tropomyosin. Cells were treated with CDF/curcumin (1 or 4 μmol/L) and/or gemcitabine (10 nmol/L) for 72 hours. There was a complete loss of basal level of expression of COX-2 in MiaPaCa-M cells exposed to gemcitabine for a period of ~4 months. Expression of COX-2, pAkt, and tropomyosin proteins were significantly reduced in cells treated with CDF and its combination when compared with curcumin.
Figure 1. Growth inhibition of BxPC-3 cells treated with 0.1 to 4.0 μmol/L of CDF/curcumin (A); BxPC-3, MIAPaCa-E, and MIAPaCa-M cells treated with 1 to 4 μmol/L of CDF/curcumin, 10 nmol/L of gemcitabine, and the combination (B); and MIAPaCa-E and MIAPaCa-M cells treated with 10 μmol/L of CDF/curcumin, 10 nmol/L of gemcitabine, and the combination (C) were evaluated by the MTT assay. D, clonogenic assay of MIAPaCa-E cells were treated with 4 μmol/L of CDF/curcumin, 10 nmol/L of gemcitabine, and the combinations. The P values represent comparisons between cells treated by either of the drugs and their combinations by using the paired t test.
and its combination (Fig. 3A). Epithelial marker E-cadherin expression was significantly enhanced with CDF, curcumin, or its combination treatment only in BxPC-3 cells. No such effect was observed in MiaPaCa-E and MiaPaCa-M cells due to lack of basal level of expression of E-cadherin, which is consistent with the EMT phenotype. The expression of PTEN, a tumor suppressor gene, was found to be decreased in MiaPaCa-M cell line compared with BxPC-3 cells and was upregulated with...
treatment combinations. These results suggest that CDF is much more effective than curcumin. Because PTEN is a known target of miR-21, which has been reported to be upregulated in PC (32, 41, 42), we assessed the expression levels of miR-21 and assessed its interrelationship with the expression of PTEN in PC cells.

Modulation of miR-21 expression in BxPC-3, MIAPaCa-E, and MIAPaCa-M cells. We determined the expression levels

![Figure 3.](image-url)

A, the expression of COX-2, E-cadherin, PTEN, pAkt, tropomyosin, and β-actin in BxPC-3, MIAPaCa-E, and MIAPaCa-M cell lines treated with 1 to 4 μmol/L CDF/curcumin, 10 nmol/L gemcitabine, or the combination for 72 h. B, comparative expression analysis of miR-21 in BxPC-3, MIAPaCa-E, and MIAPaCa-M cells by real-time miRNA RT-PCR. Cells were treated with 1 to 4 μmol/L CDF/curcumin, 10 nmol/L gemcitabine, or their combinations for 72 h. The expression of PTEN, pAkt, and NF-κB in MIAPaCa-E cells after transfection with miR-21 antisense oligo (C) and transfection with PTEN cDNA (D), followed by gemcitabine treatment for 48 h.
of miR-21, which is an oncogenic miRNA and showed to have antiapoptotic activity in various carcinomas cell lines (38, 39), between BxPC-3 (gemcitabine-sensitive), MIAPaCa-E (relatively resistant to gemcitabine), and MIAPaCa-M (highly resistant to gemcitabine) cells by real-time reverse transcription–PCR (RT-PCR). Overexpression of miR-21 was observed in both MIAPaCa-E and MIAPaCa-M cells compared with BxPC-3 cells. In addition, we found a significant reduction in the expression of miR-21 in cells treated with either CDF alone or in combination with gemcitabine (Fig. 3B). To further validate whether miR-21 indeed could target the expression levels of a tumor suppressor PTEN, we investigated the effect of transfection of PTEN cDNA and miR-21 antisense oligonucleotide in MIAPaCa-E cells.

**Effects of PTEN cDNA transfection and miR-21 antisense oligonucleotide transfection on selective proteins.** MIAPaCa-E cells showed increased expression of miR-21, and thus, this cell line was used to evaluate the effect of both PTEN cDNA and miR-21 antisense oligonucleotide transfection on the expression of PTEN, pAkt, and NF-κB. The cells were transfected with either PTEN cDNA or miR-21 antisense oligonucleotide for 48 hours followed by gemcitabine treatment for 48 hours. The expression of PTEN was enhanced by both transfection studies compared with either untreated cells or cells treated with control vector or oligos, respectively. On the other hand, the expression of pAkt and NF-κB was further reduced with both the transfection (Fig. 3C and D). These findings clearly suggest that CDF and curcumin are capable of reactivating the expression of PTEN, which is normally lost in malignant tumors, and this is mediated by downregulating the expression of miR-21. To gain mechanistic insight on how CDF and curcumin could sensitize PC cell lines to gemcitabine-induced inhibition of cell viability and induction of apoptosis, we investigated the DNA binding activity of NF-κB and assessed the effects of treatments on NF-κB downstream gene, such as COX-2.

**CDF and curcumin inhibits NF-κB DNA binding activity.** The DNA binding activity of NF-κB, a nuclear transcription factor, was assessed in all three PC cell lines treated with CDF, curcumin, gemcitabine, and the combination treatment. Gemcitabine-treated cells caused activation of NF-κB in all three cell lines, whereas CDF-treated cells caused significant inhibition in the DNA binding activity of NF-κB in all three cell lines. Interestingly, gemcitabine-induced activation of NF-κB was attenuated by CDF treatment (Fig. 4). However, the curcumin-treated cells or its combination with gemcitabine showed much lower effects on NF-κB DNA binding activity compared with CDF-treated cells (Fig. 4). These results suggest that the combination of CDF and gemcitabine causes greater inhibition of cell growth, induction of apoptosis, inhibition of COX-2 protein, and all of which could in part be due to inactivation of NF-κB in both gemcitabine-resistant and gemcitabine-sensitive cell lines. Because we found that CDF could inhibit COX-2, we assessed the COX-2 enzymatic product PGE2 in the conditioned medium under our experimental conditions.

**Inhibition of PGE2 synthesis.** Because it is known that the inhibition of COX-2 will reduce the synthesis of PGE2, we have tested the effects of treatments on the production of PGE2. We measured the levels of PGE2 in the conditioned medium collected from BxPC-3, MIAPaCa-E, and MIAPaCa-M cells after treatments. It is important to note that we found a higher level of PGE2 secretion by BxPC-3 cells (Fig. 5A), whereas MIAPaCa-E and MIAPaCa-M cells showed very low basal levels of secreted PGE2, which are consistent with low constitutive expression of COX-2 protein (43). There was a substantial increase in the PGE2 level in gemcitabine-treated BxPC-3 cells compared with untreated cells, suggesting that gemcitabine-induced NF-κB could be responsible for the induction of COX-2 and thereby caused induction in the secretion of PGE2. A significant reduction in PGE2 level was observed in cells treated with CDF, curcumin, or their combinations with gemcitabine; however, the effect was much more pronounced in cells treated with CDF than curcumin. Collectively, these results suggest that the production of PGE2 is mediated through NF-κB and COX-2 pathway and that CDF could downregulate both NF-κB and COX-2 and thereby reduces the synthesis and secretion of PGE2.

**Inhibition of VEGF secretion.** We have shown that CDF and curcumin attenuated gemcitabine-induced activation of the DNA binding activity of NF-κB in the previous section; thus, we investigated the effect of CDF, curcumin, gemcitabine, or their combination on another downstream transcriptional target of NF-κB, i.e., VEGF secretion by BxPC-3, MIAPaCa-E, and MIAPaCa-M cells. Conditioned media from cells treated with CDF, curcumin, gemcitabine, or their combination were analyzed with AlphaLISA VEGF 500 data point kit from Perkin-Elmer. Compared with untreated cells and cells treated with curcumin, both MIAPaCa-E and MIAPaCa-M cells treated with CDF showed a significant inhibition in the secreted levels of VEGF (Fig. 5B). Because previous studies have shown that the loss of miR-200 family is associated with drug-resistant phenotype especially in those cells having mesenchymal phenotype (29), we assessed the level of expression of miR-200 in PC cells under our experimental conditions to gain further mechanistic insight as to the biological effects of CDF.

**Modulation of miR-200b and miR-200c expression in BxPC-3, MIAPaCa-E, and MIAPaCa-M cells.** We determined the comparative level of expression of miRNA-200 family among gemcitabine-sensitive (BxPC-3 cells) and gemcitabine-resistant MIAPaCa-E (relatively resistant to gemcitabine) and MIAPaCa-M (highly resistant to gemcitabine) by real-time RT-PCR. The miR-200b and miR-200c, which are known regulators of EMT, were significantly decreased in both MIAPaCa-E and MIAPaCa-M compared with BxPC-3 cells, which are also consistent with their differential sensitivities to gemcitabine (Fig. 6A). BxPC-3, MIAPaCa-E, and MIAPaCa-M cells treated with CDF, curcumin, or gemcitabine and their combination for 72 hours showed increased expression of miR-200b and miR-200c, but the effect was much more pronounced in CDF-treated cells (Fig. 6B and C), suggesting the superiority of CDF compared with curcumin.

These findings suggest that the phenotypic characteristics of both MIAPaCa-E and MIAPaCa-M cells that are resistant
to gemcitabine and having the EMT characteristics could be reversed by CDF treatment and, as such, will render these cells to gemcitabine chemosensitivity, which of course needs further in-depth investigation.

**Discussion**

Here, we have shown that a synthetic analogue of curcumin, CDF, is significantly more effective in the killing of gemcitabine-resistant cells, which could in part be due to better cellular uptake and retention of CDF by PC cells, which is consistent with our published findings on pharmacokinetic data in mice (18, 19). Mechanistically, this could be due to inactivation of NF-κB and COX-2 as predicted previously (19). Because the expression and activation of COX-2 and NF-κB pathways is common in PC cells and contributes to the observed resistance of PC cells to chemotherapeutic agents (44), a rational approach for the successful inhibition of cell growth would be to combine natural agents that are nontoxic but could be highly effective in the inhibition of NF-κB and COX-2. To that end, curcumin, a natural dietary chemopreventive agent, has shown multitargeted effects, including the inhibition of NF-κB and COX-2, and thus, curcumin when combined with gemcitabine could be very effective in vitro, but its limited absorbance across the gut in humans limits its therapeutic utilities. In our study, we have found that our novel synthetic analogue (CDF) of curcumin is much more effective in inhibiting NF-κB, COX-2, PGE2, and VEGF either alone or when combined with gemcitabine. The COX-2 generated PGE2 plays important roles during pancreatic tumorigenesis, and our data clearly suggest that CDF alone or in combination with gemcitabine could inhibit the production of PGE2. These results are also due to CDF-mediated inactivation of NF-κB, which in turn inactivates the transcription of COX-2 and thereby leads to the inhibition in the production of PGE2. These results suggest that the combinatorial approach led to the discovery of our novel agent.
CDF, which could be useful for the prevention of tumor progression and/or treatment of PC.

Here, we also report for the first time that both CDF and curcumin are effective in reducing specific miRNAs. miRNAs are moderately stable compared with large molecules, such as proteins, and can be efficiently extracted because they are well preserved in both formalin-fixed and paraffin-embedded tissues (45). miRNAs can also normalize multiple coding genes associated with tumor growth, and thus, assessment of specific miRNA expression could be useful for predicting disease outcome. It is well known that the development of cancer involves alterations in the expression of multiple genes regulated by transcriptional, posttranscriptional, translational, and posttranslational modification, and thus, a single gene or protein expression cannot accurately reflect the status of the disease. miR-21 is overexpressed in many solid tumors and has been shown to be associated with tumor progression, poor survival, and reduced therapeutic effects (25, 38, 46). In fact, we have reviewed the role of miRNAs in cancer (41), which is consistent with another review article recently published (42). Here, we found that miR-21 expression is upregulated in gemcitabine-resistant gemcitabine-resistant

Figure 5. PGE2 ELISA (A) and VEGF ELISA (B) were performed using conditioned medium collected from BxPC-3, MIAPaCa-E, and MIAPaCa-M cells. Cells were treated with 1 to 4 μmol/L CDF/curcumin, 10 nmol/L gemcitabine, or their combinations for 24 h. Please note that the basal level of PGE2 in BxPC-3 cells was 10 times higher than MIAPaCa-E and MIAPaCa-M cells.
cell lines (MIAPaCa-E and MIAPaCa-M) compared with gemcitabine-sensitive BxPC-3 cells and that the expression of miR-21 could be significantly downregulated by CDF and its combination with gemcitabine. There are several genes that are regulated by miR-21, and the increased expression of miR-21 could downregulate specific genes among such genes. PTEN, a well-known tumor suppressor gene, has been reported to be lost in tumors and is regulated by miR-21 (32, 41, 42), which is consistent with our findings showing the reactivation of PTEN in PC cells treated with CDF or curcumin. Consistent with our results, it was shown that inactivation of miR-21 by treatment of cells with antisense oligonucleotide of miR-21 increased PTEN protein expression and induced cell cycle arrest in human PC cells (47). The implication or the effect of our data would be enormous because natural nontoxic agents or their synthetic analogues could be useful for the upregulation of PTEN mediated via inactivation of miR-21, and our data further suggest that such strategy could in fact be useful for sensitization of drug-resistant PC cells to conventional cytotoxic agents that are not very effective by themselves, such as gemcitabine. The activation of PTEN by CDF or curcumin would decrease Akt phosphorylation as shown in Fig. 3, which would likely contribute to the inhibition of cell growth and induction of apoptosis, and such effects could be exploited in a preclinical animal model in future studies.

The data presented on miR-21 inactivation is in sharp contrast to the data on miR-200b and miR-200c, whose expression was drastically reduced in gemcitabine-resistant PC cells, which is consistent with previous findings showing that the expression of these miRNAs was either lost or substantially reduced in various tumors, including pancreas (29). Interestingly, we found that the expression of miR-200b and miR-200c could be upregulated by CDF and curcumin, suggesting that the mesenchymal phenotype of gemcitabine-resistant PC cells could be reversed by simply treating the cells with either CDF or curcumin. We found that both MIAPaCa-E and MIAPaCa-M cells showed lower expression of both miR-200b and miR-200c and loss of E-cadherin

Figure 6. A, comparative expression of miR-200b and miR-200c in BxPC-3, MIAPaCa-E, and MIAPaCa-M cells as assessed by real-time miRNA RT-PCR. Cells were treated with 1 to 4 μmol/L CDF/curcumin, 10 nmol/L gemcitabine, or their combinations. Expression of miR-200b (B) and expression of miR-200c (C) are shown.
expression, consistent with mesenchymal-like morphology (Fig. 2A) compared with BxPC-3 cells having epithelial morphology and higher expression of both miR-200b and miR-200c and E-cadherin. Our results also showed that the treatment of MIAPaCa-E and MIAPaCa-M cells with CDF alone or in combination with gemcitabine significantly upregulated miR-200 expression, although the expression of E-cadherin was not upregulated, suggesting that only partial reversal of the EMT could be achieved, which will lead to sensitization of gemcitabine-resistant cells to gemcitabine-induced killing.

In conclusion, our results suggest that CDF could sensitize PC cells to gemcitabine by inactivation of NF-κB, COX-2, and their downstream target molecules, which is in part due to inactivation of miR-21 and reactivation of miR-200b and miR-200c. The inactivation of miR-21 led to the reactivation of PTEN, resulting in the inactivation of phosphorylated Akt. In addition, CDF was able to cause reactivation of miR-200b and miR-200c, which may in turn result in the reversal of EMT phenotype and thus, sensitizing gemcitabine-resistant PC cells to gemcitabine again, could be exploited for designing novel strategies for the prevention of tumor progression and/or treatment of pancreatic tumors using the combination of CDF and gemcitabine in the immediate future.

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

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References
Correction: Online Publication Dates for Cancer Research April 15, 2010 Articles

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Dudka AA, Sweet SMM, Heath JK. Signal transducers and activators of transcription-3 binding to the fibroblast growth factor receptor is activated by receptor amplification. Cancer Res 2010;70:3391–401. Published OnlineFirst April 13, 2010. doi:10.1158/0008-5472.CAN-09-3033.

Gemcitabine Sensitivity Can Be Induced in Pancreatic Cancer Cells through Modulation of miR-200 and miR-21 Expression by Curcumin or Its Analogue CDF


Cancer Res 2010;70:3606-3617. Published OnlineFirst April 13, 2010.