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p8 Is a New Target of Gemcitabine in Pancreatic Cancer Cells

Valentin Giroux, Cédric Malicet, Marc Barthet, Meritxell Gironella, Cendrine Archange, Jean-Charles Dagorn, Sophie Vasseur, and Juan L. Iovanna

Abstract Gemcitabine is the only available chemotherapeutic treatment of pancreatic cancers. It is, however, moderately effective, showing a tumor response rate of only 12%. The aim of this work was to identify new pathways involved in the resistance of pancreatic cancer cells to gemcitabine, in the hope of developing new adjuvant strategies to enhance its therapeutic efficacy. Comparison of gene expression patterns of five human pancreatic cancer cell lines showing different degrees of resistance to gemcitabine revealed specific overexpression of several genes in the most resistant. One of them encoded the antiapoptotic p8 protein. We found that (a) knocking down p8 expression in gemcitabine-resistant cells promoted cell death and increased caspase-3 activity; (b) forced overexpression of p8 in gemcitabine-sensitive cells increased their resistance to gemcitabine-induced apoptosis; and (c) gemcitabine down-regulated p8 mRNA expression. These results suggest that, in pancreatic cancer cells, a large part of gemcitabine-induced apoptosis results from the inhibition of the constitutive antiapoptotic activity of p8. Hence, targeting the p8-associated pathway could be a new adjuvant therapy improving the response of patients with pancreatic cancer to gemcitabine treatment.

Pancreatic cancer is the fifth most common cause of death by cancer in the Western world (1). The anatomic localization of the pancreas and the lack of specificity of the symptoms result in a complex and delayed diagnosis. Therefore, at the time of detection, 85% of patients show metastatic infiltration in proximal lymph nodes, liver, or lungs. The current 5-year survival rate is ~3% and only surgical resection (possible in 15% of the cases) can increase survival rate to 20%. Chemotherapy and radiation may only allow for marginal increase in survival (2). Recently, gemcitabine (2',2'-difluorodeoxycytidine), a novel nucleoside analogue, showed some efficacy in the treatment of pancreatic cancers (reviewed in ref. 3). This molecule inhibits growth of human pancreatic cells *in vitro* and *in vivo*, these cells being otherwise insensitive to classic anticancer drugs such as 5-fluorouracil, doxorubicin, or cisplatin (4). In fact, gemcitabine has become the standard first-line treatment for patients with pancreatic cancer. When used in monotherapy, gemcitabine results in a tumor response rate of 12% (5) and offers a median survival time of 5 months

(6). Several adjuvant chemotherapies are presently under evaluation in an effort to increase tumor response to gemcitabine and improve the median survival duration.

The aim of our work was to identify the intracellular pathways involved in the resistance to gemcitabine of pancreatic cancer cells. This should help establishing the bases of new adjuvant strategies that would enhance the efficacy of the drug. To this end, we used the Affymetrix microarray approach to compare the gene expression profiles of 5 pancreatic cancer-derived cells that exhibit different sensitivities to gemcitabine. Expression of a few genes was apparently associated with increased resistance to the drug. Among them the p8 gene, which encodes a stress-associated protein with antiapoptotic properties, was strongly expressed in the two most resistant cell lines. We observed that knocking down p8 expression in gemcitabine-resistant cells made them sensitive to the drug and, conversely, that overexpression of p8 in gemcitabine-sensitive cells increased their resistance. Therefore, inhibiting the p8-dependent pathway could improve pancreatic cancer treatment with gemcitabine.

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Materials and Methods

Cell lines and cell culture conditions. The human pancreatic cancer cell lines Panc-1 and Mia-PaCa2 were grown in DMEM supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, 100 IU/mL penicillin G, and 100 µg/mL streptomycin. The Capan-1, Capan-2, and BxPC3 cells were cultivated in RPMI 1640 in the presence of 2 mmol/L L-glutamine, 4.5 g/L glucose, 10 mmol/L HEPES, 1 mmol/L sodium pyruvate, 10% fetal bovine serum, and 100 IU/mL penicillin G and 100 µg/mL streptomycin. All cell lines were routinely cultivated in humidified 5% CO₂ atmosphere.

Dose-response curves for gemcitabine. Cells (10⁴ per well) were seeded on 96-well plates in 100 µL media. The next day, gemcitabine (purchased from Eli Lilly, Suresnes, France) was added in 100 µL of media to the desired final concentration (from 0 to 500 µmol/L). After

72 hours, 20 μ L of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS; Promega, Charbonnieres, France) reagent were added, the plates were incubated at 37°C for 30 minutes, and the absorbance read at 490 nm. The percentage of viable cells was defined as A_{490} of gemcitabine divided by the A_{490} of cells receiving no treatment, multiplied by 100%.

Microarray. Total RNA from pancreatic cancer cells from three independent experiments was isolated by Trizol (Life Technologies, Inc., Cergy-Pontoise, France). Twenty micrograms of total RNA were converted to cDNA with SuperScript reverse transcriptase (Life Technologies) using T7-oligo-d(T)₂₄ as a primer. Second-strand synthesis was done using T4 DNA polymerase and *E. coli* DNA ligase followed by blunt ending by T4 polynucleotide kinase. cDNA was transcribed *in vitro* using the T7 BioArray High Yield RNA Transcript Labeling Kit (Enzo Biochem, New York, NY) to produce biotinylated cRNA. Purified cRNA was fragmented to 200- to 300-mer cRNA using a fragmentation buffer (100 mmol/L potassium acetate, 30 mmol/L magnesium acetate, 40 mmol/L Tris-acetate, pH 8.1) for 35 minutes at 94°C. The cRNA probes were hybridized to an U133 2.0 Plus GeneChip (Affymetrix, Santa Clara, CA). Fifteen micrograms of fragmented cRNA were hybridized for 16 hours at 45°C with constant rotation (60 rpm). Microarrays were processed in an Affymetrix GeneChip Fluidic Station 400. Staining was made with streptavidin-conjugated phycoerythrin followed by amplification with a biotinylated antistreptavidin antibody and a second round of streptavidin-conjugated phycoerythrin, and then scanned using an Agilent GeneArray Scanner (Agilent Technologies, Ontario, Canada). The signal intensities for the β -actin and GAPDH genes were used as internal quality controls. The ratio of fluorescent intensities for the 5' and 3' ends of these housekeeping genes was <2. Scanned images were analyzed with the Microarray Suite 5.0 software (Affymetrix).

Regulation of p8 mRNA expression by gemcitabine. Mia-PaCa2 cells (2×10^5) were seeded in 60-mm culture dishes. Twenty-four hours later, cells were treated with 150 μ M/L gemcitabine for different times. Total RNA was isolated by Trizol and p8 mRNA expression was measured by semiquantitative and quantitative reverse transcription-PCR (RT-PCR).

siRNA design. The four p8 siRNAs employed in this work were designed by Qiagen (Courtboeuf, France) in accordance with the Basic siRNA Design Tool algorithm. The sequences are p8-1 siRNA (sense 5'-CCCUAGCAAAGAGCAAGAd(TT)-3'), p8-2 siRNA (sense 5'-AGGUCGCACCAAGAGAAAd(TT)-3'), p8-3 siRNA (sense 5'-CUGGU-GACCAAGCUGCAGAd(TT)-3'), and p8-4 siRNA (sense 5'-GGAGACC-CAGGACAGGAUd(TT)-3'). Control siRNA (Qiagen) cannot recognize any eukaryote sequence (sense 5'-UUUCUCCGACGUGUCACGUd(TT)-3').

Transfection. The day before siRNA transfection, cells were plated in six-well plates to eventually give 30% to 50% confluence. After removal of the medium, cells were washed once with serum-free medium and transfection was done in serum-free medium by addition of a mix composed of 10 μ L Oligofectamine Reagent (Invitrogen, Cergy-Pontoise, France) and 200 pmol siRNA diluted in 240 μ L serum-free medium. Plasmids were transfected by using Lipofectamine 2000 (Invitrogen). Cells were plated the day before transfection so that they reached 80% to 90% confluence at the time of experiment. After a wash with serum-free medium, transfection was done in serum-free medium by addition of a mix composed of 10 μ L Lipofectamine 2000 and 4 μ g DNA diluted in 500 μ L serum-free medium. After an incubation period of 4 hours at 37°C, the transfection medium containing siRNA or DNA was replaced by fresh medium.

Semiquantitative RT-PCR analysis. RNA (1 μ g) were analyzed by RT-PCR with the Promega One-Step RT-PCR system. RT-PCR was done using various numbers of cycles to verify that the conditions chosen were within the linear range. The mRNA encoding p8 was specifically amplified with sense (5'-TAGAGACGGACTGCG-3') and antisense (5'-GCGTGTCTATTTATTGTTGC-3') primers. As a control, the transcript coding for the TATA-binding protein (TBP) was specifically amplified with sense (5'-TGCACAGGACCAAGAGTAA-3') and antisense (5'-CACATCACAGCTCCCCACCA-3') primers. Reverse transcription was carried out for 45 minutes at 45°C followed by 24 to 32 cycles of

PCR, each cycle consisting in a denaturing step for 30 seconds at 95°C, an annealing step for 1 minute at 57°C, and a polymerization step for 1 minute at 72°C. PCR products were separated on a 2.0% agarose gel containing ethidium bromide and photographed under UV light.

Quantitative RT-PCR analysis. First-strand cDNA was synthesized in 20 μ L reaction with 1 μ g total RNA using Expand Reverse Transcriptase (Roche, Meylan, France) following the instructions of the manufacturer. Quantitative PCR was done with the Light Cycler system (Roche) and Takara (Berkeley, CA) reagents. Five microliters of 10-fold diluted cDNA were mixed with 10 μ L SYBR Premix Ex Taq (including Taq polymerase, reaction buffer, MgCl₂, SYBR green I dye, and deoxynucleotide triphosphate mix) and 4 nmol forward and reverse primers (TBP primers are used as a control) in a volume of 20 μ L. After an initial Taq activation for 10 seconds at 95°C, Light Cycler PCR was done using 45 to 55 cycles with the following cycling conditions: 95°C for 5 seconds, 58°C for 6 seconds, and 72°C for 12 seconds. Each sample was analyzed in duplicate and the experiment was repeated two or thrice. Results were analyzed using RelQuant (Roche) and expressed as percent of control values.

Preparation of p8^{-/-} and p8^{-/-} hump8 transformed fibroblasts. Primary embryo fibroblasts were isolated from 14.5-day-old p8^{-/-} mouse embryos and transformed with pBabe-ras^{V12}/E1A retrovirus as previously described (7). To restore p8 expression in transformed p8^{-/-} fibroblasts, we infected these cells with a retrovirus expressing human p8. The retroviral vector was constructed as follows: human p8 cDNA was subcloned into *Hind*III and *Xho*I restriction sites of the pLPC plasmid (obtained from S. Lowe, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). Ecotropic human p8-expressing retrovirus was then generated by transient transfection using Bosc 23 ecotropic packaging cells. Virus supernatant was used to infect transformed p8^{-/-} fibroblasts and the population of p8-expressing transformed p8^{-/-} fibroblasts (p8^{-/-} hump8 cells) was isolated by selection in the presence of puromycin (0.7 μ g/mL). As control, transformed p8^{-/-} fibroblasts were infected with pLPC empty vector.

Cell death analysis. Mia-PaCa2 were seeded in 60-mm culture dishes the day before siRNA transfection. One day after p8-4 siRNA transfection, cells were treated with 150 μ M/L gemcitabine and harvested at 48 hours. BxPC3 were seeded in 60-mm culture dishes to reach 80% to 90% confluence before plasmid transfection. One day after transfection, cells were treated with 10 μ M/L gemcitabine and harvested at 48 hours. p8^{-/-} empty and p8^{-/-} hump8 fibroblasts were seeded in 60-mm culture dishes 24 hours before treatment with 10 μ M/L gemcitabine for 8 hours.

DNA analysis by flow cytometry. Cells were washed with PBS and fixed in cold-ethanol 70% for 30 minutes at 4°C. After a wash with phosphate-citrate buffer, cells were treated with 50 μ L RNase A (100 μ g/ μ L), labeled with 200 μ L propidium iodide (50 μ g/ μ L), and immediately analyzed by flow cytometry (FACSCalibur, Becton Dickinson, Le Pont-De-Claix, France). Cell death analysis was done on 30,000 cells, evaluating the sub-G₁ ratio (ModFit LT). The experiments were repeated at least thrice and each sample was assayed in triplicate.

Cell viability after gemcitabine treatment. The number of viable cells after treatment with gemcitabine was determined by the trypan blue dye exclusion test. The percentage of dead cells was calculated by the following formula: percent death = $(N - N_T) / (N - N_0) \times 100\%$, where N is the number of untreated cells cultured still alive after a given time of culture, N_T is the number of treated cells alive at the same time, and N_0 is the cell number at the beginning of the experiment.

Caspase-3 activity. Caspase-3 activity was measured using the Apo-ONE Homogeneous Caspase-3 Assay Fluorometric Kit (Promega). Mia-PaCa2 cells were initially seeded at 5×10^4 per well on 48-well plates. Twenty-four hours later, these cells were transfected with p8-4 or control siRNAs. BxPC3 were seeded at 10^5 per well on 48-well plates and transfected with pcDNA3p8 (8) or the empty vector. Twenty-four hours later, BxPC3 were treated with 10 μ M/L gemcitabine for 48 hours and caspase-3 activity was measured by the cleavage of the fluorometric substrate Z-DEVD-R110 according to the instructions of the manufacturer (Promega).

Statistical analysis. Results shown represent means \pm SD. Statistical analysis was done by ANOVA with a post hoc analysis by the Student-Neuman-Keuls test.

Results

Resistance of pancreatic cancer-derived cell lines to gemcitabine correlates with p8 mRNA expression. As a first step to identify genes associated to gemcitabine resistance, we examined the relative sensitivity of five pancreatic cancer-derived cell lines (Panc-1, Mia-PaCa2, Capan-1, Capan-2, and BxPC3) to the drug. Whereas LD₅₀ was 25 and 7 μ mol/L for Panc-1 and Mia-PaCa2, respectively, LD₅₀ was 0.04, 0.01, and 0.02 μ mol/L for Capan-1, Capan-2, and BxPC3, respectively (Fig. 1). Therefore, we considered Panc-1 and Mia-PaCa2 as resistant cells and Capan-1, Capan-2, and BxPC3 as sensitive cells. The second step was to establish the mRNA expression profiles of the cells to select the candidate genes involved in gemcitabine resistance. To this end, we did an Affymetrix microarray analysis of the five pancreatic cells on more than 22,000 genes. About 300 genes seem to be overexpressed in Panc-1 and Mia-PaCa2 compared with Capan-1, Capan-2, and BxPC3. Among them, we found the gene encoding the stress protein p8, which is strongly involved in the resistance of cells to injuries and tumor development (9–11). Strong expression of p8 mRNA in Panc-1 and Mia-PaCa2 was confirmed by semiquantitative and quantitative RT-PCR (Fig. 2).

p8 mRNA is down-regulated by gemcitabine. p8 is known to be induced by several stresses, including minimal stresses such as routine change of the culture medium in the absence of any added substance, and by several drugs (7, 12, 13). We investigated the effect of gemcitabine at a dose of 150 μ mol/L

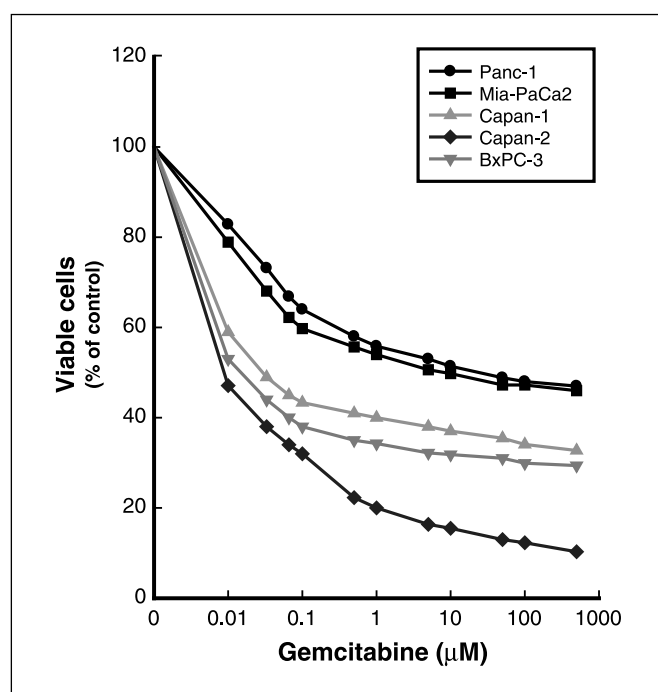


Fig. 1. Effects of gemcitabine on pancreatic cancer cell viability. Human pancreatic cancer cell lines Panc-1 (●), Mia-PaCa2 (■), Capan-1 (▲), Capan-2 (◆), and BxPC3 (▼) were treated with increasing concentrations of gemcitabine (0–500 μ mol/L) for 72 hours. The viabilities indicated on the y axis were determined by MTS assays and normalized to control as described in Materials and Methods.

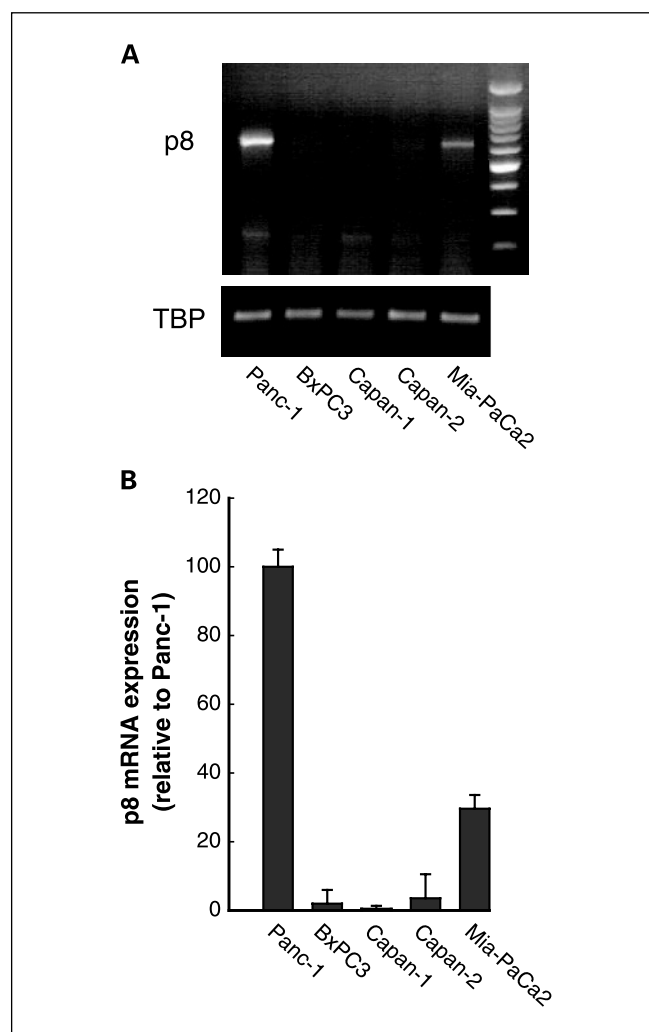


Fig. 2. Expression of the p8 mRNA in pancreatic cancer-derived cells. Total RNA was extracted from five human pancreatic cancer cells (Panc-1, Mia-PaCa2, Capan-1, Capan-2, and BxPC3) and p8 mRNA expression was measured by semiquantitative (A) and quantitative RT-PCR (B). TBP coding for the TATA-binding protein was used as a control.

on p8 mRNA expression in Mia-PaCa2 cells. Unexpectedly, p8 mRNA expression was down-regulated by gemcitabine as shown by semiquantitative and quantitative RT-PCR. As shown in Fig. 3, p8 mRNA expression was decreased by 85% after 8 hours of treatment.

Silencing p8 induces cell death and increases the sensitivity of Mia-PaCa2 cells to gemcitabine. We selected Mia-PaCa2 among our five cell lines to study the role of p8 in gemcitabine resistance of the pancreatic cancer cells because it expresses high levels of p8 and is resistant to gemcitabine treatment. We intended to knock down p8 in these cells by using specific siRNAs. We tested four sequences and established that although all four worked, p8-4 siRNA was the most efficient 48 hours after transfection (Fig. 4). It was therefore used in functional studies. After transfecting the p8-4 siRNA or control siRNA, Mia-PaCa2 cells were treated with 150 μ mol/L gemcitabine. After 48 hours, cell death was monitored by measuring by flow cytometry the sub-G₁ ratio of the population (Table 1). Cell death was significantly higher in p8-silenced cells than in control cells (65% versus 50%). Then, viability was determined

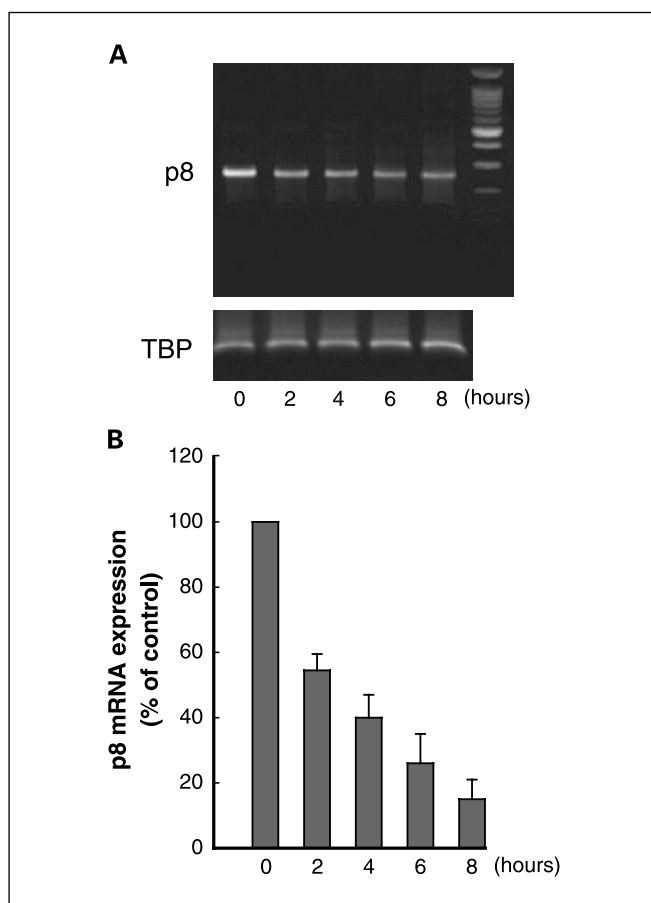


Fig. 3. Effects of gemcitabine on p8 mRNA expression in Mia-PaCa2 cells. Mia-PaCa2 cells were treated with 150 $\mu\text{mol/L}$ gemcitabine for 0, 2, 4, 6, and 8 hours. Purified RNA was then analyzed by semiquantitative (A) and quantitative RT-PCR (B) to monitor p8 mRNA expression. TBP coding for the TATA-binding protein was used as a control.

by direct counting of cells excluding trypan blue dye (Fig. 5A). By this method, we observed an even stronger decrease of viability in p8-4 siRNA-transfected cells, compared with control siRNA-transfected cells, than by flow cytometry analysis. We ruled out that such decrease was due to a toxic effect of the reagent because no cell death was observed after transfecting the same siRNA to HeLa or NIH 3T3 cells (data not shown). These data strongly suggest that p8 expression is necessary to the survival of gemcitabine-treated Mia-PaCa2 cells. A similar conclusion was reached for Panc-1 cells (data not shown).

Increased caspase-3 activation in gemcitabine-treated Mia-PaCa2 cells after p8 silencing. Gemcitabine induces cytochrome *c* release from mitochondria, which binds to Apaf-1 to promote the activation of procaspase 9 (14). Then, caspase 9 activates procaspase 3. Because caspase-3 activity seems to be required for cell death induction by gemcitabine (14), we evaluated the role of p8 expression in caspase-3 activation after gemcitabine treatment of Mia-PaCa2 cells. Cells were transfected with p8 siRNA or control siRNA and treated 24 hours later with 150 $\mu\text{mol/L}$ gemcitabine. Caspase-3 activity was measured after 48 hours. Results normalized to cell viability are shown in Fig. 5B. Interestingly, the mere fact of silencing p8 increased significantly caspase-3 activity in Mia-PaCa2 cells,

confirming that p8 is antiapoptotic in the pancreatic cell line. As expected, treating Mia-PaCa2 cells with gemcitabine strongly increased caspase-3 activity. Finally, when p8 was silenced in these cells before gemcitabine treatment, a further increase in caspase-3 activity was observed.

Overexpressing p8 prevents cell death and increases resistance to gemcitabine in BxPC3 cells. Knowing that p8 mRNA inhibition increases the sensitivity of Mia-PaCa2 cells to gemcitabine, we wished to check the opposite situation in which p8 would be overexpressed in BxPC3, a cell line with very low constitutive expression of p8 and very sensitive to gemcitabine. BxPC3 cells were transiently transfected with the pcDNA3p8 or an empty vector and 24 hours later cells were treated, or not, with 10 $\mu\text{mol/L}$ gemcitabine. More than 85% of the cells were efficiently transfected as judged by immunocytochemistry (data not shown). The first observation was that, in the absence of other treatment, p8 overexpression decreased the spontaneous death rate of BxPC3 cells (10% versus 15%)

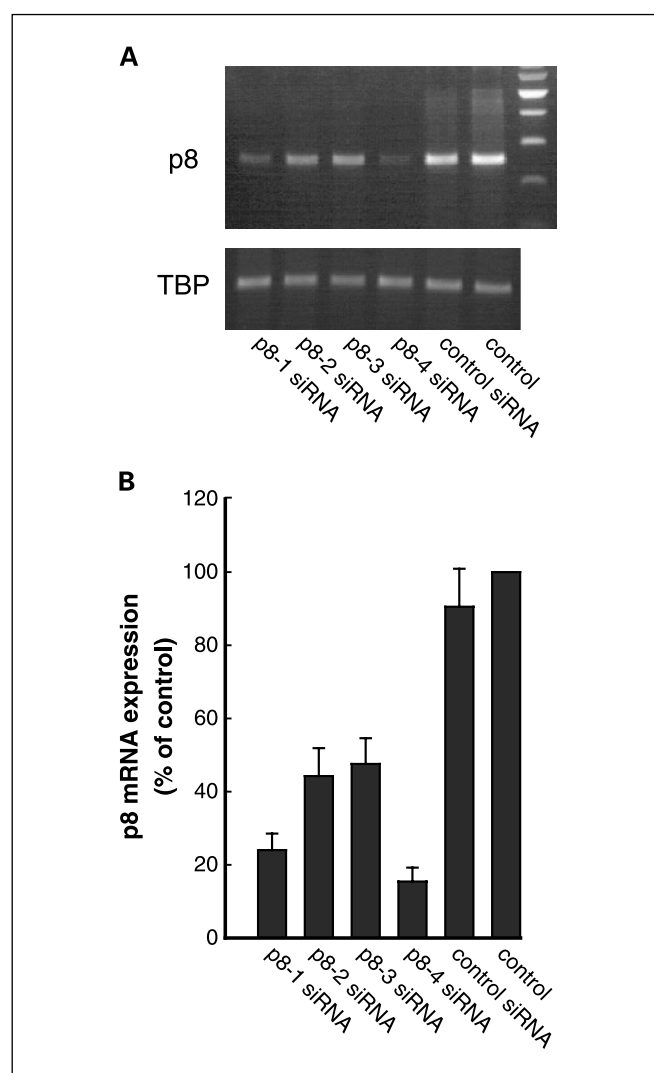


Fig. 4. Silencing the p8 mRNA in Mia-PaCa2 cells. Mia-PaCa2 cells were transfected with the four p8-siRNAs or control siRNA. Forty-eight hours after transfection, total RNA was extracted and analyzed by semiquantitative (A) and quantitative RT-PCR (B) to monitor the inhibition by the p8-siRNAs of p8 mRNA expression.

Table 1. Effect of p8-4 siRNA in Mia-PaCa2 cells treated or not with gemcitabine (sub-G₁ ratio)

Control cells		Gemcitabine 150 μ mol/L	
Control siRNA (n = 9)	p8-4 siRNA (n = 9)	Control siRNA (n = 9)	p8-4 siRNA (n = 9)
13.1 \pm 3.3	37.3 \pm 6.1	50.2 \pm 2.2	65.8 \pm 8.8
<i>P</i> < 0.05		<i>P</i> < 0.05	

estimated by sub-G₁ ratio measurement (Table 2). In addition, after 48 hours of gemcitabine treatment, less apoptosis was observed in p8-overexpressing cells than in control cells (44% versus 59%). As shown in Fig. 6A, these results correlated with those obtained by trypan blue exclusion.

Overexpression of p8 inhibits caspase-3 activation by gemcitabine in BxPC3 pancreatic cells. The involvement of caspase 3 in the antiapoptotic effect of p8 overexpression was evaluated in BxPC3 cells. We used the same experimental setup as above and measured caspase-3 activity in cells in normal conditions and after exposure to gemcitabine. Results normalized to cell viability are shown in Fig. 6B. Forced expression of p8 was accompanied by a significant decrease in caspase-3 activity in basal conditions as well as after gemcitabine treatment. Altogether, these results indicate that p8 expression in pancreatic cancer-derived cells antagonizes the cell death program triggered by gemcitabine.

p8^{-/-} hump8 fibroblasts are more resistant to gemcitabine than p8^{-/-} fibroblasts. To check whether the protective role of p8 against gemcitabine-induced cell death was specific to cell lines of pancreatic origin, we generated ras^{V12}/E1A-transformed p8^{-/-} fibroblast. They were infected either with an empty pLPC retrovirus or with a pLPC retrovirus expressing human p8 (p8^{-/-} hump8) to reestablish p8 expression. Cells were treated

Table 2. Effect of p8 overexpression in BxPC3 cells treated or not with gemcitabine (sub-G₁ ratio)

Control cells		Gemcitabine 10 μ mol/L	
Empty vector (n = 9)	p8-vector (n = 9)	Empty vector (n = 9)	p8-vector (n = 9)
15.6 \pm 1.9	10.1 \pm 1.6	59.6 \pm 4.6	44.2 \pm 3.2
<i>P</i> < 0.05		<i>P</i> < 0.05	

with gemcitabine (10 μ mol/L) for 8 hours and cell death was monitored by flow cytometry (Table 3) and by trypan blue exclusion (Fig. 7A). Both approaches showed that p8^{-/-} hump8 were more resistant to gemcitabine and that caspase-3 activity was decreased in p8^{-/-} hump8 cells compared with p8^{-/-} cells (Fig. 7B). In conclusion, these results suggest that the mere expression of p8 in a cell counteracts gemcitabine-induced cell death.

Discussion

Although much progress has been made during the last decade in the treatment of human solid tumors, the prognosis of pancreatic cancer remains poor, with a mean survival rate of 4 to 6 months (15, 16). One of the reasons for this dismal prognosis is the resistance of pancreatic cancer cells to anticancer treatments such as radiotherapy, immunotherapy, antihormonal therapy, and chemotherapy (15, 17, 18). Gemcitabine, a novel nucleoside analogue, is presently a standard first-line treatment for patients with pancreatic cancer, but it only exhibits a 12% tumor response rate (5). A number of mechanisms may contribute to drug resistance, including inadequate intracellular drug concentration, rapid inactivation of the drug, and increased rate of DNA repair in cancer cells

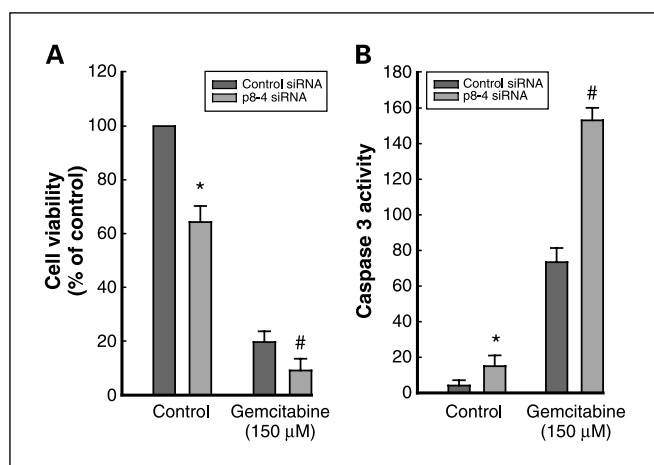


Fig. 5. Influence of p8 expression on cell death and caspase-3 activity in Mia-PaCa2 cells on treatment with gemcitabine. Mia-PaCa2 cells were transfected with control or p8-4 siRNA and, 24 hours later, treated with 150 μ mol/L gemcitabine for 48 hours. No gemcitabine was added to control cell medium. **A**, cell viability, relative to control cells, was determined by the trypan blue exclusion test. **B**, caspase-3 activity was determined by monitoring the cleavage of the fluorometric Z-DEVD-R110 substrate and normalized to cell viability. *, *P* < 0.05, versus untreated control; #, *P* < 0.05, versus treated control.

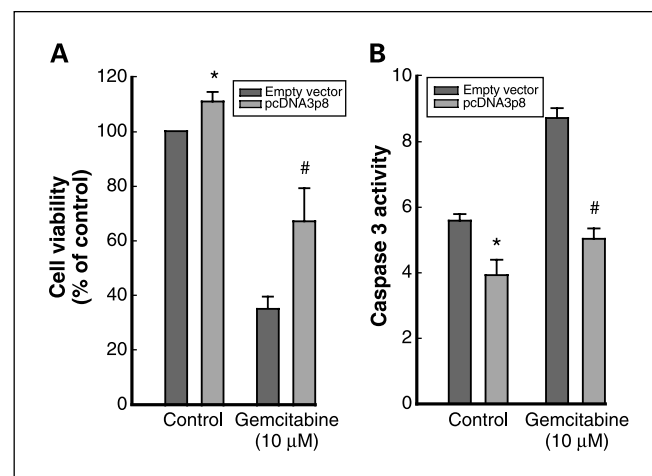


Fig. 6. Influence of p8 expression on cell death and caspase-3 activity in BxPC3 cells on treatment with gemcitabine. BxPC3 cells were transfected with a p8 expression vector or an empty vector as control. After 24 hours, they were treated with 10 μ mol/L gemcitabine for 48 hours. No gemcitabine was added to control cell medium. **A**, cell viability, relative to control cells, was determined by the trypan blue exclusion test. **B**, caspase-3 activity was determined by monitoring the cleavage of the fluorometric Z-DEVD-R110 substrate and normalized to cell viability. *, *P* < 0.05, versus untreated control; #, *P* < 0.05, versus treated control.

Table 3. Effect of p8 overexpression in ras^{V12}/E1A-transformed p8^{-/-} fibroblasts treated or not with gemcitabine (sub-G₁ ratio)

Control cells		Gemcitabine 10 μmol/L	
Empty vector (n = 9)	hump8-vector (n = 9)	Empty vector (n = 9)	hump8-vector (n = 9)
7.8 ± 2.6	2.1 ± 0.4	38.6 ± 4.7	14.3 ± 1.9
<i>P</i> < 0.05		<i>P</i> < 0.05	

(19, 20). The precise mechanisms of resistance to chemotherapy in pancreatic cancer are not well understood. Recently, evidence has emerged that chemotherapeutic agents exert their toxicity through activation of apoptosis and that promotion of apoptosis can increase the chemosensitivity of cancer cells. Therefore, specific silencing of genes that inhibit apoptosis might improve the sensitivity of cancer cells to chemotherapeutic agents.

Five human pancreatic cell lines were used in the present study. All of them responded to gemcitabine with increased apoptosis but the LD₅₀ spanned over more than 4 orders of magnitude, Mia-PaCa2 and Panc-1 being more resistant (Fig. 1). A panel of cells showing quite different responses to gemcitabine seemed to be an interesting tool to look for genes that control or interfere with gemcitabine-induced apoptosis. A systematic analysis using Affymetrix microarray technology revealed that several genes were specifically overexpressed in cells most resistant to gemcitabine, suggesting their possible implication in the mechanisms that antagonize the effects of the drug. Among them, p8 was found especially interesting because it encodes a stress protein overexpressed in several types of cancer (21–23) and because p8 expression in pancreas is inversely correlated with the extent of apoptosis (24). To get insight into the mechanisms by which p8 interferes with the gemcitabine-induced apoptosis pathway, we inhibited endogenous p8 expression in the gemcitabine-resistant Mia-PaCa2 cell line and observed increased apoptosis on exposure to the drug with concomitant increase in caspase-3 activity. Conversely, overexpression of p8 in the gemcitabine-sensitive BxPC3 cell line, on transfection with an appropriate vector, resulted in increased resistance to the drug, evidenced by a decreased rate of apoptosis. Similar results were obtained when using p8^{-/-} fibroblasts instead of BxPC3 cells. We concluded that p8 inhibited gemcitabine-induced apoptosis. On that basis and because p8 expression is known to be strongly induced by many inducers of apoptosis such as doxorubicin, staurosporine, and ceramide (7, 12, 13), we thought that the moderate efficacy of gemcitabine on pancreatic cancer was due to a concomitant overexpression of p8. In fact, as shown in Fig. 3, gemcitabine strongly down-regulated p8 mRNA expression, contrary to other inducers of apoptosis. These results suggest that gemcitabine treatment increases the rate of apoptosis because it inhibits the p8

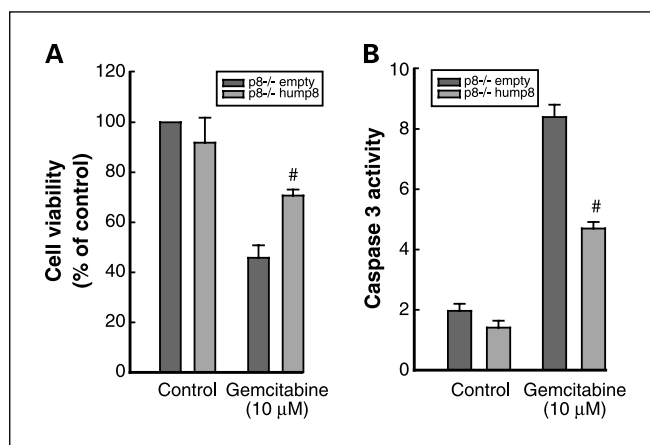


Fig. 7. Influence of p8 expression on cell death and caspase-3 activity in ras^{V12}/E1A-transformed p8^{-/-} fibroblasts on treatment with gemcitabine. p8^{-/-} empty or p8^{-/-} hump8 transduced fibroblasts were treated with 10 μmol/L gemcitabine for 8 hours. No gemcitabine was added to control cell medium. **A**, cell viability, relative to control, was determined by the trypan blue exclusion test. **B**, caspase-3 activity was determined by monitoring the cleavage of the fluorometric Z-DEVD-R110 substrate and normalized to cell viability. #, *P* < 0.05, versus treated control.

antiapoptotic pathway, in addition to mechanisms already described. Hence, targeting the p8-dependent pathway might be an interesting adjuvant to gemcitabine in the treatment of pancreatic cancer patients.

Several molecules are known to be involved in gemcitabine resistance, including those of the phosphatidylinositol 3-kinase/Akt survival pathway (25), the focal adhesion kinase (26), the integrin-linked kinase (27), p38 mitogen-activated protein kinase (28), nuclear factor κB (29), c-Src tyrosine kinase (30), Bcl-2 (31), Bcl-X_L (32), the M2 subunit of ribonucleotide reductase (33), the deoxycytidine kinase (34), and other molecules involved in gemcitabine transport through membranes or in its metabolism (reviewed in ref. 35). It is interesting to note that all these molecules, except for deoxycytidine kinase and molecules controlling gemcitabine transport and metabolism, are involved in classic antiapoptotic pathways. Yet, there is to our knowledge no report that the expression of any of them is down-regulated by gemcitabine, suggesting that their role in gemcitabine resistance is indirect. The p8 gene is, on the contrary, a direct target of gemcitabine because its expression is rapidly down-regulated by the drug. These observations support the above-mentioned hypothesis that down-regulation of p8 expression by gemcitabine is an important mechanism of induction by the drug of pancreatic cancer cell death. Further studies should determine whether p8 is also implicated in the resistance of pancreatic cancer to other chemotherapies because of its antiapoptotic capacities and whether gemcitabine is more efficient than other drugs simply because it can inhibit p8 expression. Nevertheless, it can already be said that any drug capable of inhibiting p8 expression, given as adjuvant to gemcitabine treatment, should improve the response of patients with pancreatic cancer.

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