

Enhancing apoptosis and overcoming resistance of gemcitabine in pancreatic cancer with bortezomib: a role of death-associated protein kinase-related apoptosis-inducing protein kinase 1

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ABSTRACT

Aims and background. To investigate the role of the apoptosis gene, DAP (death-associated protein) kinase-related apoptosis-inducing protein kinase 1 (DRAK1), which is involved in enhancing cell sensitivity and overcoming cell resistance to gemcitabine in pancreatic cancer cells by the proteasome inhibitor bortezomib.

Methods. Cultured human pancreatic cancer gemcitabine-sensitive cell lines (bxc-3) and gemcitabine-resistant (panc-1) cell lines were divided into four groups: control, treatment with bortezomib, treatment with gemcitabine, and the two-drug combination. Expression of DRAK1 genes in each group was detected by using reverse transcription-polymerase chain reaction and western blot. Apoptosis in the pancreatic cancer cell lines was measured by flow cytometry.

Results. We found that the effects of growth inhibition and apoptosis of gemcitabine on both pancreatic cancer cell lines were enhanced by bortezomib. Treatment of panc-1 and bxc-3 cells with bortezomib (100 nM) and gemcitabine (50 µg/ml and 0.05 µg/ml, respectively) induced an increase in the levels of DRAK1 mRNA compared with the control and single-agent treatment. Furthermore, immunoblotting analysis in panc-1 but not bxc-3 cells showed similar changes in the expression of DRAK1 protein produced by combination therapy.

Conclusions. Our results demonstrated that bortezomib enhanced cell sensitivity and overcame cell resistance to gemcitabine in pancreatic cancer cells, which may be attributed to DRAK1 induced by bortezomib and the combination with gemcitabine.

Introduction

Pancreatic cancer is one of the most devastating cancers. It is responsible for 5 to 6% of all cancer-related deaths. It remains a disease with a poor prognosis, with a 5-year survival rate of <4%, which has remained unchanged in the last decades¹. Systemic chemotherapy may increase survival and improve quality of life for patients with resected and locally advanced pancreatic cancer. Currently, gemcitabine (2',2'-difluorodeoxycytidine), a novel pyrimidine nucleoside analogue, has become the standard chemotherapeutic drug for pancreatic cancer. However, gemcitabine treatment alone is not very effective and is associated with multiple adverse events and drug resistance^{2,3}. In view of the problem of gemcitabine drug resistance, it is important to develop new agents and innovative approaches to treat pancreatic cancer.

In eukaryotic cells, the ubiquitin-proteasome is essential for the disposal of damaged or unwanted proteins. As a large multicatalytic protease complex, the proteasome is responsible for most non-lysosomal intracellular protein degradation⁴. The proteasome inhibitor bortezomib (also known as Velcade [Mellinnum Pharmaceuticals] or PS-341) represents a new type of chemotherapeutic drug that has been

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shown to induce apoptosis independently or synergistically with conventional cancer therapy in both solid and hematological tumors. Bortezomib is a dipeptidyl boronic acid derivative that specifically inhibits the function of the 26 S proteasome. It is the first proteasome inhibitor to be evaluated in human studies⁵⁻⁹. Bortezomib by injection has been approved for the treatment of multiple myeloma in selected patients¹⁰. Several studies on the anticancer and chemosensitization effects of bortezomib in pancreatic cancer have shown that it has significant activity *in vitro* and *in vivo*. However, the molecular mechanisms underlying proteasome inhibitor-mediated antitumor effects on pancreatic cancer are poorly understood, although possible explanations include inhibition of the NF- κ B pathway¹¹.

DAP (death-associated protein) kinase also participates in apoptosis, since its reduced expression by antisense mRNA protected HeLa cells from apoptosis induced by interferon- γ ¹². In addition, overexpression of DAP kinase induced apoptosis and kinase-negative mutant-blocked apoptosis triggered by interferon- γ ¹³. DAP kinase-related apoptosis-inducing protein kinase 1 (DRAK1), which belongs to the DAP kinase subfamily of serine/threonine kinases, can induce the morphological changes of apoptosis in NIH 3T3 cells¹⁴. In our previous study¹⁵, oligonucleotide microarray analysis showed that DRAK1 responded to bortezomib in panc-1 and bxp-3 cell lines. In the present study, we confirmed that bortezomib could potentiate the antitumor effects of gemcitabine against pancreatic cancer cells growing *in vitro* by up-regulating DRAK1.

Materials and methods

Drugs and chemicals

The 26S-proteasome inhibitor bortezomib was kindly provided by Dr. Jiangping Wu (Laboratory of Immunology, Research Center, CHUM-Notre Dame Hospital, Montreal, Canada). The agent was dissolved in dimethyl sulfoxide (DMSO) to make a 0.2 mmol/L stock solution and stored at -80 °C. Bortezomib was diluted in culture medium immediately before use. In all experiments, the final concentration of DMSO did not exceed 0.1%. Gemcitabine was purchased from Eli Lilly and dissolved in sterile 0.9% sodium chloride to make a 50 g/L stock solution.

Cell lines and culture

panc-1 and bxp-3 human pancreatic cancer cell lines were obtained from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, sodium pyruvate, nonessential amino acids and L-glutamine. Cells were maintained in a humidified atmosphere of

5% carbon dioxide at 37 °C and subcultured upon reaching 80% confluence.

Growth inhibition assay

Cells were plated in 96-well sterile plastic plates (100 μ l/well at a density of 5×10^4 cells/ml) and allowed to attach for 24 h. Cells were then placed in media containing specified treatment regimens. At the end of the specified treatment period, cells were pulsed with 20 μ l of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO, USA) in each well and incubated at 37 °C in 5% CO₂ for 4 h. The medium was aspirated, and the formazan crystals were dissolved in 150 μ l of DMSO. Absorbance was measured at 570 nm using a spectrophotometer (Bio-Tek ELx800, Winooski, VT, USA). Growth inhibition was defined in relation to that of the untreated cells by the following equation: [growth inhibition (% control) = $100 \times (\text{absorbance untreated sample} - \text{absorbance treated sample}) / (\text{absorbance untreated sample})$]. For each concentration and combination, experiments were repeated three times, and each experimental condition was repeated in quadruplex wells in each experiment.

Cell cycle analysis

Cell cycle analysis and quantification of apoptosis analysis was carried out using propidium iodide (PI) staining and fluorescence-activated cell sorting (FACS). In brief, panc-1 and bxp-3 cells cultured for 24 h in gemcitabine (50 μ g/ml and 0.05 μ g/ml, respectively), bortezomib (100 nM), and combination and control media were collected by gentle trypsinization, washed in PBS, and pelleted by centrifugation. Cells were fixed in 70% ethanol and stored at -20 °C until staining. Immediately prior to staining, cells were washed twice in PBS and then resuspended in PBS containing RNase A (20 μ g/ml). Cells were then stained with PI (0.1 mg/ml) at room temperature in the dark for 30 min. DNA histograms were obtained by FACS using FACSCalibur with CellQuest 3.1f software (Becton Dickinson, San Jose, CA, USA). Cell cycle analysis was performed using Modfit 3.0 Program. Cell cycle progression was measured with corresponding absorbance for G₀-G₁, S, and G₂-M phases. The relative percentage of cells in the sub-G₀ phase was then quantitated and used as an estimate of cells undergoing apoptosis. All the experiments were performed in triplicate.

Flow cytometric assessment of apoptosis

The measurement of phosphatidylserine redistribution in a plasma membrane was conducted according to the protocol outlined by the manufacturer of the Annexin V-FITC/PI apoptosis detection kit (Abcam, Cambridge, MA, USA). After a 24-h treatment, harvested 1×10^5 cells were suspended in 500 μ l of $1 \times$ Annexin V bind-

ing buffer. Five μl of Annexin V-FITC and 5 μl of PI were added and the mixture incubated for 15 min in the dark. Binding buffer (1 \times) (400 μl) was added to each sample. The stained cells were analyzed directly by flow cytometry using the Cell Quest program (Becton Dickinson).

Reverse transcription-polymerase chain reaction (RT-PCR) analysis and quantitative real-time RT-PCR

Total RNA was isolated with the reagent TRIzol (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. RNA concentrations were determined by spectrophotometry. cDNAs were synthesized by moloney murine leukemia virus reverse transcriptase with oligo(dT)16 primer. Each reverse transcript was amplified with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. RNA templates were used to generate cDNAs for DRAK1 (sense, 5'-TGA-CAAGTGAATCTCCATTGGGTG-3'; antisense, 5'-TGAAATCAACAGCCGACTCAGAC-3') and GAPDH (sense, 5'-AACGGATTGGTTCGTATTGGG-3'; antisense, 5'-TCGCTCCTGGAAGATGGTGAT-3') by PCR. RT-PCR was performed by a DNA thermal cycler (BIO-RAD, Hercules, CA, USA). The PCR products were visualized on 2% agarose gels with ethidium bromide staining under UV transillumination.

Fluorescent PCR analysis was performed using the BIO-RAD iCycler 5 (BIO-RAD). RNA was amplified by qPCR in 20 μl reactions using SYBR[®] Premix Ex Taq[™] (TAKARA Biotechnology, Dalian, China) with 10 μmol of each appropriate primer (sense, 5'-TGAACCTGTCAAAGCACCTG-3'; antisense, 5'-ACCTGGTTGTCTGAAGTGCC-3'). Assays were performed in triplicate and analyzed using I cycler version 3.1.7050 (BIO-RAD). The gene expression levels obtained were normalized by mRNA expression of GAPDH. The relative mRNA expression was then presented in relation to the control.

Western blotting

Cells were grown at 1×10^6 cells and incubated with special agents for the indicated times. Following treatment, cells were washed with PBS, and total cells were prepared by scraping in 200 μl of lysis buffer (50 mM Tris-HCl, pH 8.0, 1.0 mM ethylenediaminetetraacetate, 150 mM NaCl, 0.1% SDS, 1% NP40, 1% sodium deoxycholate, 1 mM phenylmethanesulfonylfluoride). Fifty micrograms of proteins were determined by using BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). For protein analysis, total cellular lysates were separated on a 10% (w/v) SDS-polyacrylamide gel and transferred to polyvinylidene fluoride. The filters were blocked in tris-buffered saline with 5% skim milk and incubated overnight with primary antibodies specific for DRAK1, procaspase-3, poly (ADP-ribose) polymerase (PARP) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as a primary antibody. The filters were then incubated with secondary antibody conjugated with

horseradish peroxidase (Santa Cruz Biotechnology). Immunoreactive bands were detected by the enhanced chemiluminescence kit for western blotting detection with hyper-ECL film. Equal loading was confirmed by probing the blots with the anti- β -actin antibody (Santa Cruz Biotechnology).

Statistical analysis

Data were expressed as mean values \pm SE and analyzed by a two-tailed *t*-test with $P < 0.05$ considered as significant. Analyses were performed using SPSS 11.5 statistical software package (SPSS Inc., Chicago, IL, USA).

Results

Effects of bortezomib and gemcitabine on growth of pancreatic cancer cells

To test the effects of bortezomib on pancreatic cancer cell growth, cells were treated with increasing concentrations of bortezomib (0-160 nM) for 24, 48 and 72 h. As shown in Figure 1A, panc-1 cell growth was inhibited by bortezomib treatment in a dose- and time-dependent manner. Low doses of bortezomib (20 and 40 nM) did not increase bxp-3 cell growth inhibition for 24, 48 and 72 h. We subsequently evaluated the effect of gemcitabine on cell growth *in vitro* and found that gemcitabine was effective in inhibiting cell growth in both cell lines (Figure 1B). The results revealed that bxp-3 was most and panc-1 least sensitive to gemcitabine-induced growth inhibition.

Bortezomib potentiates growth inhibition induced by gemcitabine in pancreatic cancer cells

Next, the effect of a combination of bortezomib and gemcitabine on cell viability was investigated by MTT assay. For these studies, cells were treated with bortezomib (100 nM), gemcitabine (panc-1, 50 $\mu\text{g}/\text{mL}$; bxp-3, 0.05 $\mu\text{g}/\text{mL}$) or the two-drug combination for 24 h. Viable cells were evaluated by MTT assay. We found that treatment with bortezomib plus gemcitabine for 24 h resulted in more growth inhibition than either bortezomib or gemcitabine alone in pancreatic cancer cells (Figure 1C).

Effects of bortezomib and gemcitabine on apoptosis of pancreatic cancer cells and cells in the sub-G₀ phase

bxp-3 and panc-1 cells were treated with bortezomib (100 nM) or gemcitabine (0.05 $\mu\text{g}/\text{mL}$ and 50 $\mu\text{g}/\text{mL}$, respectively) alone, or the combination of the two drugs for 24 h, and cell cycle analysis was performed as described. As shown in Figure 2A, combined treatment resulted in a significant increase in the number of cells in the sub-G₀ phase, compared with bortezomib and gemcitabine alone.

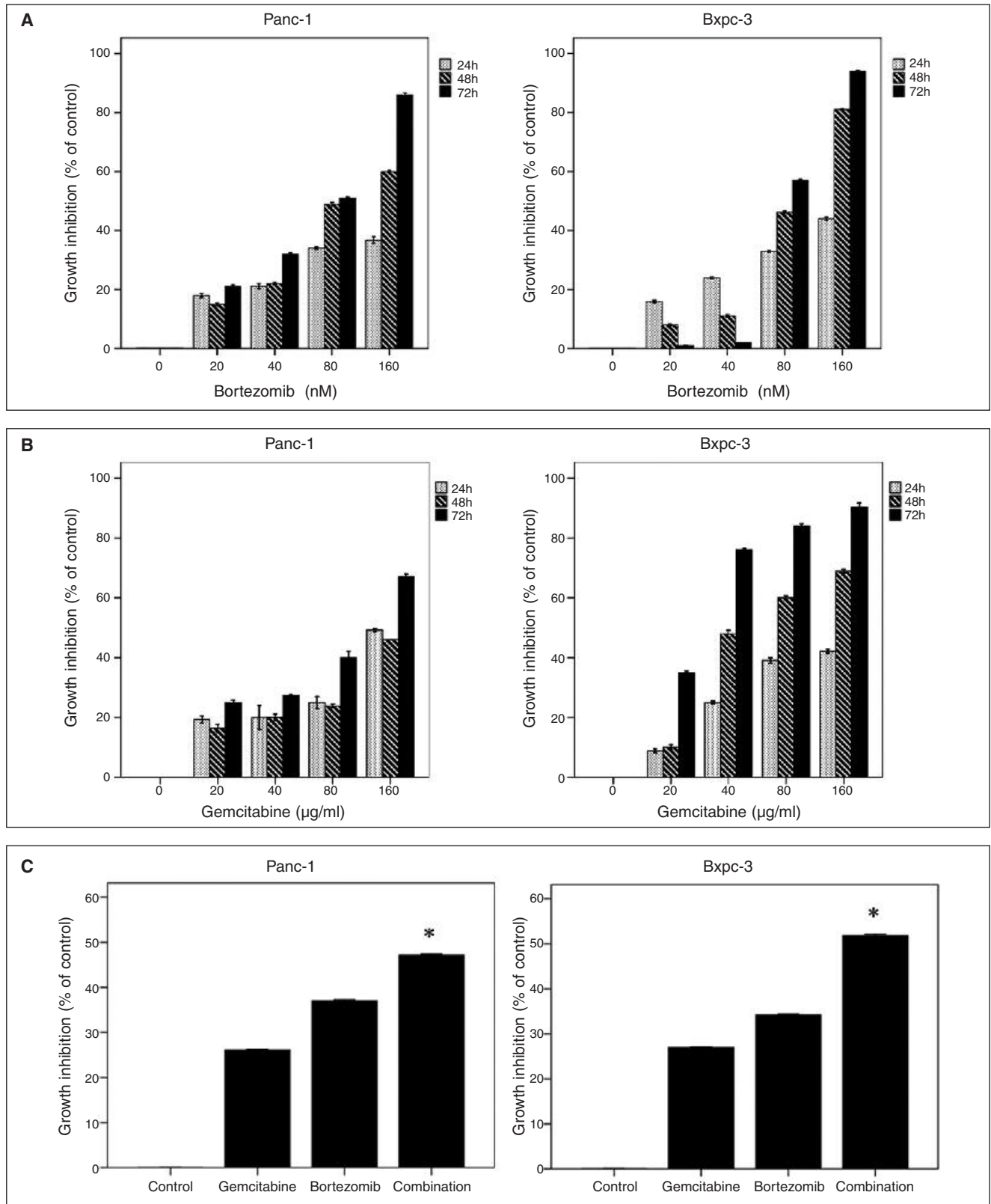


Figure 1 - Cell growth inhibition by bortezomib and gemcitabine. Cells were either untreated or treated with increasing concentration of bortezomib (A) or gemcitabine (B), for 24, 48 and 72 h and analyzed for viable cells by MTT assay as described in *Materials and methods*. Data are presented as mean \pm SE of nine replicates from three independent experiments. C) Pancreatic cancer cells treated with bortezomib (100 nM), gemcitabine (panc-1 (50 μ g/ml), or bxp-3 (0.05 μ g/ml), or the two-drug combination for 24 h. Columns, mean of three experiments; bars, SE. * $P < 0.05$, combination treatment group compared with control group, single treatment with gemcitabine or bortezomib group.

Whether bortezomib enhances the induction of apoptosis by gemcitabine was examined. We observed the induction of apoptosis in pancreatic cancer cells treated with bortezomib (100 nM) or gemcitabine (0.05 µg/ml and 50 µg/ml) alone or with the combination for 24 h. At these concentrations, relative to single agents, the combined treatment with gemcitabine and bortezomib for 24 h induced much more apoptosis in both the cell lines as shown by Annexin V/PI flow cytometry. Treatment with gemcitabine alone showed an apoptosis induction with 5.15% and 9.06% apoptosis in bxp-3 and panc-1 cells, respectively. However, treatment with bortezomib alone demonstrated 11.45% apoptosis in panc-1 and 6.83% in bxp-3 cells. Furthermore, the combination of gemcitabine and bortezomib resulted in a strong apoptotic effect. Panc-1 cells showed 15.42% apoptosis and bxp-3 cells 11.11% (Figure 2B), suggesting that bortezomib is able to enhance the extent of gemcitabine-induced apoptosis.

To further examine the ability of the combination therapy to induce apoptosis, we evaluated procaspase-3 and PARP activation in both the tested cell lines. The combination of bortezomib and gemcitabine caused an additional increase in PARP cleavage and a decrease in procaspase-3 compared with either agent alone (Figure 2C).

Bortezomib upregulates DRAK1 in panc-1 and bxp-3 cells

To investigate the mechanisms by which bortezomib induced apoptosis, we evaluated the expression of the apoptotic gene DRAK1 in pancreatic cancer cells after drug exposure. We first examined the mRNA levels of the gene in panc-1 and bxp-3 cells using PCR. As shown in Figures 3A and 3B, treatment of panc-1 and bxp-3 cells with bortezomib (100 nM) and gemcitabine (50 µg/ml and 0.05 µg/ml) induced an increase in the levels of DRAK1 mRNA compared with control and single treatment. Furthermore, immunoblotting analysis in panc-1 but not in bxp-3 cells showed similar changes in the expression of DRAK1 protein produced by the combination, suggesting that bortezomib enhanced cytotoxicity of gemcitabine by up-regulating the DRAK1 gene (Figure 3C).

Discussion

Pancreatic cancer is characterized clinically by a wide resistance to all current chemotherapy treatments. Previous studies have reported that human pancreatic cancer cell lines display marked resistance to gemcitabine¹⁶. Therefore, representative gemcitabine-sensitive (bxp-3) and gemcitabine-resistant (panc-1) cell lines¹⁷ were selected to evaluate the effects of bortezomib combined with gemcitabine on pancreatic

cancer cell growth *in vitro*. Cell growth inhibition analysis revealed that panc-1 cells responded weakly and bxp-3 cells showed high sensitivity to gemcitabine, whereas both responded to bortezomib. Next, we found that the combination of gemcitabine and bortezomib treatment markedly enhanced tumor cell growth inhibition compared with either agent alone.

Bortezomib is a potent and reversible proteasome inhibitor that functions to degrade intracellular polyubiquitinated proteins. It is an approved drug for the treatment of patients with relapsed multiple myeloma. Bortezomib induces apoptosis and has shown broad antitumor activity with selectivity for transformed cells¹⁸⁻²⁰. Currently, many preclinical trials that researched the treatment of bortezomib in combination with gemcitabine in pancreatic cancer have shown various degrees of efficacy²¹⁻²³, whereas the exact underlying mechanisms have not been fully delineated. Using oligonucleotide microarrays, we identified a large number of genes differentially expressed, some of which are potentially involved in bortezomib's antitumor effects in pancreatic cancer and have not been previously reported¹⁵.

In our study, analysis of apoptosis demonstrated differential responses of individual cell lines to treatment with bortezomib, whereas the combination led to greater apoptosis in both gemcitabine-sensitive (bxp-3) and gemcitabine-resistant (panc-1) cell lines. However, no difference was observed when the two (gemcitabine and bortezomib) were used alone. Potentiation of gemcitabine-induced apoptosis by bortezomib in pancreatic cells was validated by procaspase-3 activity and PARP cleavage. Moreover, in our results, we first found that bortezomib, but not gemcitabine, was able to induce DRAK1 in both cell lines, providing a molecular link between proteasome inhibition and activation of DAP kinase-related apoptosis-inducing kinase 1.

Sanjo *et al.*¹⁴ identified two additional members of the DAP kinase family (designated as DRAK1 and DRAK2) whose catalytic domains are related to that of DAP kinase, a serine/threonine kinase involved in apoptosis. Both DRAKs are composed of an N-terminal catalytic domain and a C-terminal domain responsible for the regulation of kinase activity. The kinase activity of DRAK1 is significantly stronger than that of DRAK2. Overexpression of DRAK1 and DRAK2 induced the morphological changes of apoptosis, as did DAP kinase and ZIP kinase¹⁴. It was previously shown that the cell apoptosis gene DRAK1 has an interesting expression pattern after exposure to etoposide in MeWo cells, and down-regulation of DRAK1 was observed in L-OHP-resistant cells by microarray experiments. A positive relationship between DRAK1 and L-OHP or etoposide resistance in human cancer cell lines was suggested, implying that the lower expression of DRAK1 might be associated with drug resistance^{24, 25}.

In our studies, PCR and western blot analysis showed that DRAK1 was rarely expressed in gemcitabine-sensi-

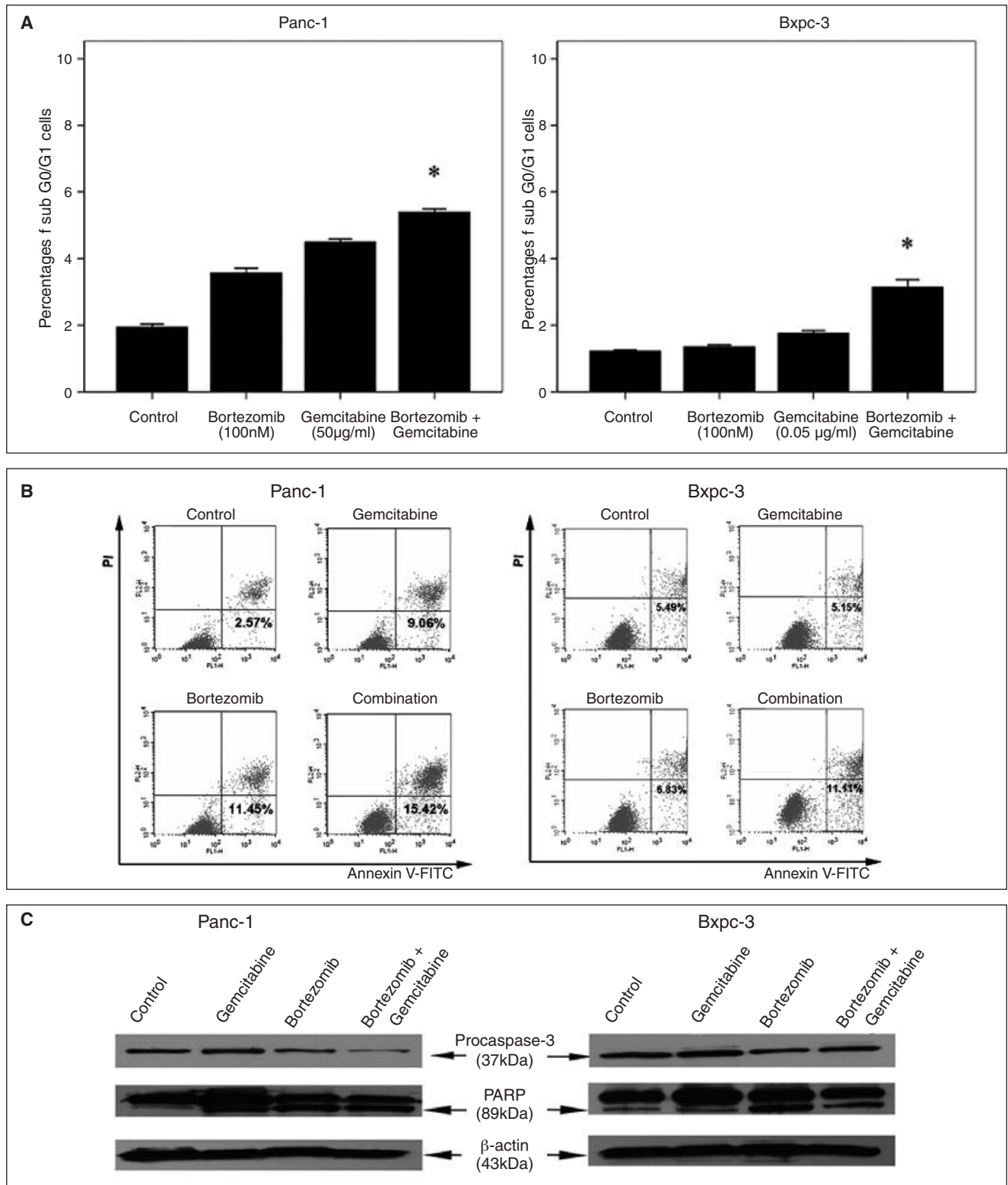


Figure 2 - Effects of bortezomib and gemcitabine on apoptosis of pancreatic cancer cells. Cell lines (panc-1 and bxp-3) were treated with 100 nM of bortezomib, 50 µg/ml or 0.05 µg/ml of gemcitabine or the indicated combinations of drugs for 24 h. A) Cell cycle analysis was performed by PI staining and FACS analysis as described in *Materials and methods*. Sub-G₀/G₁ population of cells was calculated. Columns, mean of three experiments; bars, SE. **P* <0.05, by comparison with respective group. B) Apoptosis rate analysis was performed by Annexin V/propidium iodide flow cytometry as described in *Materials and methods*. Number in each panel is percentage of apoptotic cells. Data are shown from one of three experiments with similar results. C) Cell lysates were subjected to immunoblotting with an antibody that recognizes procaspase-3 (37 kDa) and the cleaved form of PARP (89 kDa). β-actin immunoblotting confirmed equal loading of lanes.

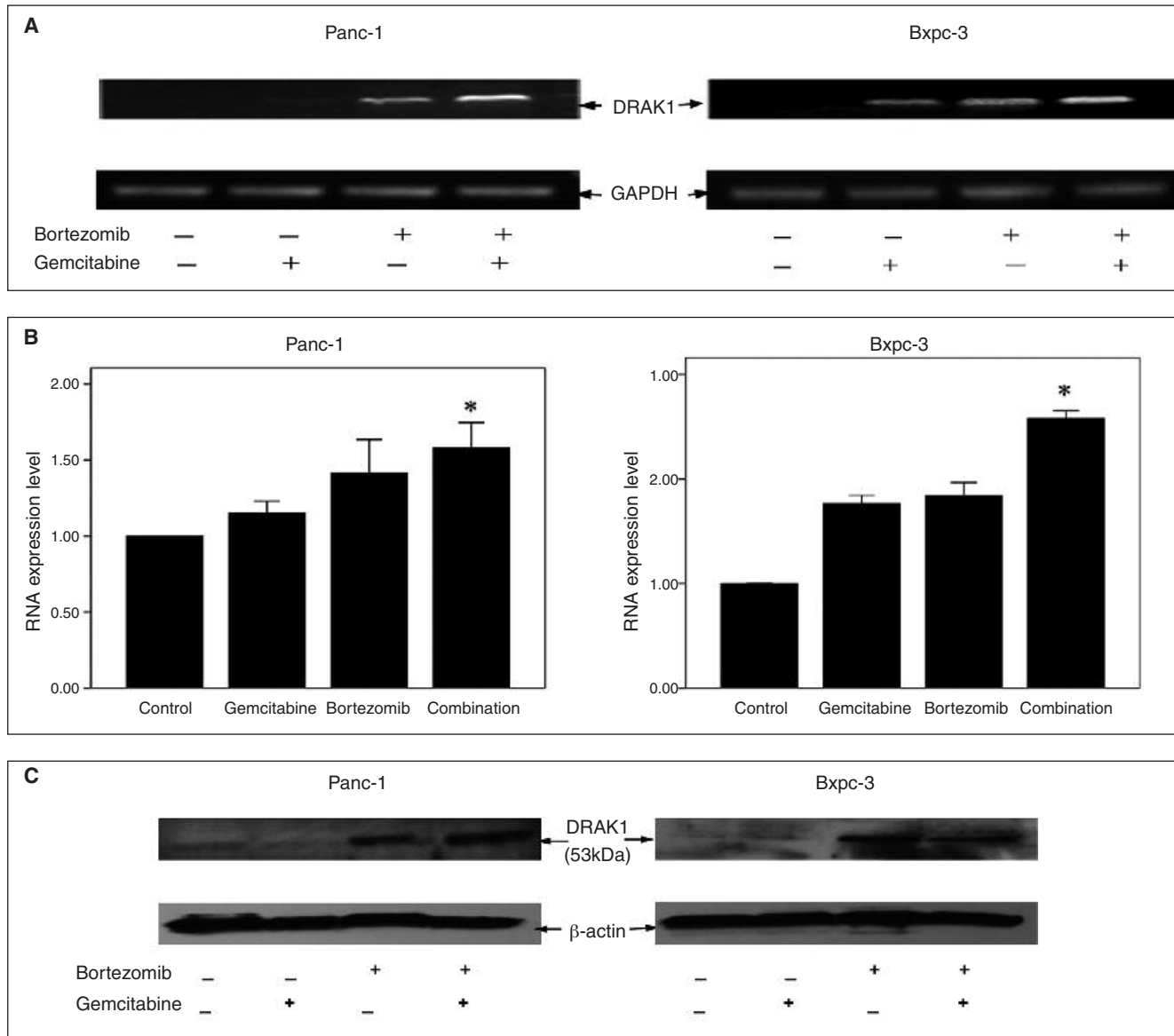


Figure 3 - Effects of bortezomib and gemcitabine on the expression of DRAK1. panc-1 and bxp-3 cells were treated with gemcitabine (50 $\mu\text{g}/\text{ml}$, 0.05 $\mu\text{g}/\text{ml}$, respectively), 100 nM bortezomib, and the two-drug combination for 24 h. mRNA was investigated by using A) RT-PCR and B) qRT-PCR. GAPDH was used as a control for amplification. Data are presented as mean \pm SE of three independent experiments after standardization to GAPDH. * $P < 0.05$, by comparison with respective group. C) Total protein levels of DRAK1 were investigated after treatment with gemcitabine (50 $\mu\text{g}/\text{ml}$, 0.05 $\mu\text{g}/\text{ml}$), 100 nM bortezomib, and the two-drug combination for 24 h. Western immunoblotting for β -actin protein was performed as loading control representative.

tive cell lines (bxpc-3) and gemcitabine-resistant (panc-1) cell lines. The mRNA levels of DRAK1 could be significantly upregulated by treatment with bortezomib in both cell lines. In contrast to bortezomib, mRNA levels of DRAK1 were only induced by gemcitabine in bxp-3 cell lines, suggesting that DRAK1 may be related to with gemcitabine resistance. Compared with either treatment, co-treatment with bortezomib and gemcitabine markedly enhanced the mRNA levels of DRAK1 in both cell lines. On the one hand, no significant change in DRAK1 was observed by western blot analysis between

treatment with combination therapy and treatment with bortezomib alone in bxp-3 cell lines. On the other hand, we found that the combination of bortezomib with gemcitabine strongly upregulated DRAK1 in gemcitabine-resistant (panc-1) cell lines, indicating that the ability of bortezomib to upregulate DRAK1 might be an important mechanism by which it was able to enhance gemcitabine-inducing apoptosis and overcome gemcitabine resistance in pancreatic cancer cells.

In conclusion, results from our study first indicate that bortezomib can potentiate the apoptotic effect of gemc-

itabine and overcome gemcitabine resistance in pancreatic cancer cells by inducing DRAK1. Up-regulation of DRAK1 could provide new strategies to treat pancreatic cancer, especially gemcitabine-resistant pancreatic cancer. Although prospective trials are needed to determine the actual benefits, DRAK1 might be a new target gene in the therapeutic course of pancreatic cancer.

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