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# bcl-2-specific siRNAs restore Gemcitabine sensitivity in human pancreatic cancer cells

**Kinya Okamoto [a, b, #], Matthias Ocker [a, *, #], Daniel Neureiter [c, #], Otto Dietze [c], Steffen Zopf [a], Eckhart G. Hahn [a], Christoph Herold [a]**

[a] *Department of Medicine 1, University Hospital Erlangen, Friedrich-Alexander-University Erlangen-Nuernberg, Erlangen, Germany*
[b] *Second Department of Internal Medicine, Tottori University School of Medicine, Tottori, Japan*
[c] *Insitute of Pathology, Salzburger Landeskliniken, Paracelsus Private Medical University, Salzburg, Austria*

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## Abstract

Gemcitabine has been shown to ameliorate disease related symptoms and to prolong overall survival in pancreatic cancer. Yet, resistance to Gemcitabine is commonly observed in this tumour entity and has been linked to increased expression of anti-apoptotic bcl-2. We therefore investigated if and to what extend silencing of bcl-2 by specific siRNAs (siBCL2) might enhance Gemcitabine effects in human pancreatic carcinoma cells. siBCL2 was transfected into the pancreatic cancer cell line YAP C alone and 72 hrs before co-incubation with different concentrations of Gemcitabine. Total protein and RNA were extracted for Western-blot analysis and quantitative polymerase chain reaction. Pancreatic cancer xenografts in male nude mice were treated intraperitoneally with siBCL2 alone, Gemcitabine and control siRNA or Gemcitabine and siBCL2 for 21 days. Combination of both methods lead to a synergistic induction of apoptosis at otherwise ineffective concentrations of Gemcitabine. Tumour growth suppression was also potentiated by the combined treatment with siBCL2 and Gemcitabine *in vivo* and lead to increased TUNEL positivity. In contrast, non-transformed human foreskin fibroblasts showed only minor responses to this treatment. Our results demonstrate that siRNA-mediated silencing of anti-apoptotic bcl-2 enhances chemotherapy sensitivity in human pancreatic cancer cells *in vitro* and might lead to improved therapy responses in advanced stages of this disease.

**Keywords:** siRNA • RNAi • pancreatic cancer • bcl-2 • gemcitabine

## Introduction

Pancreatic cancer is among the most common causes for cancer related deaths in Western countries and represents about 10% of all gastrointestinal malignancies [1]. Ductal adenocarcinoma represents 90% of all cases and is often diagnosed at an advanced stage with a median survival time of less than 6 months and a 12-month and 5-year survival rate of only 10% and less than 3%, respectively, thus rendering pancreatic cancer one of the neoplasms with the worst prognosis [2, 3]. Only 10–14% of patients are eligible for curative surgery which prolongs median survival to 10–20 months [4, 5]. Various adjuvant, neo-adjuvant, locoregional or

[#] The first three authors contributed equally.

*Correspondence to: Dr Matthias OCKER
Department of Medicine 1, University Erlangen-Nuernberg, Ulmenweg 18, D-91054 Erlangen, Germany.
Tel: +49 9131 8535057
Fax: +49 9131 8535058
E-mail: Matthias.Ocker@med1.imed.uni-erlangen.de

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radio-chemotherapy strategies could not significantly improve overall survival, and still remain palliative [4, 5].

The nucleoside analogue Gemcitabine has been shown to slightly prolong overall survival and to ameliorate disease-related symptoms, especially in combination with other cytotoxic agents, and has become the first-line treatment option for pancreatic cancer [6]. Resistance to Gemcitabine treatment is mainly attributed to an altered apoptotic threshold in pancreatic cancer cells due to increased expression of anti-apoptotic members of the bcl-2 family which stabilize the mitochondrial membrane [7, 8].

Recently, short double-stranded oligoribonoculeotides (siRNAs) have been shown to inhibit the expression of a corresponding target gene in mammals via the biologically conserved mechanism of RNA interference (RNAi) [9,10] where these siRNA (short interfering RNA) molecules are separated into single-strands and incorporated into the RNA induced silencing complex (RISC) which then cleaves the corresponding cellular mRNA [11–13].

Several studies investigated the effect of inhibiting the expression of anti-apoptotic bcl-2 family members (*e.g.* bcl-xL) by using conventional antisense oligonucleotides in different human tumour types (*e.g.* melanoma, gastric cancer etc.) [14–16] and indicate that this treatment strategy might enhance the sensitivity to established chemotherapeutic agents like Gemcitabine in pancreatic cancer, where bcl-2 is over-expressed in approximately 25% of all cases [17, 18].

We have shown previously that transfecting human pancreatic carcinoma cell lines with bcl-2-specific siRNAs specifically inhibits the expression of the cognate target gene, reduces cell proliferation and induces apoptosis *via* the mitochondrial pathway *in vitro* and *in vivo* [19].

In the present study, we investigated if and to what extend bcl-2-specific siRNAs might enhance the anti-proliferative and pro-apoptotic effects of Gemcitabine on pancreatic cancer cells *in vitro* and *in vivo*.

# Material and methods

## Cell culture

YAP C pancreatic adenocarcinoma cells (moderately differentiated, low aggressiveness in xenografts [20]) were cultured on 6-well tissue culture plates (Becton Dickinson, Mannheim, Germany) in RPMI-1640 medium (Biochrom, Berlin, Germany) containing 10% foetal calf serum (FCS, Biochrom), penicillin ($10^7$ U/l) and streptomycin (10 mg/l) at 37°C and 5% $CO_2$. Human foreskin fibroblasts (HF) served as non-malignant controls and were cultured in Dulbecco's modified Eagle's medium (DMEM, Biochrom) with the same supplements. All cell lines were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany).

Gemcitabine was kindly provided by Lilly Research Laboratories (Indianapolis, USA). A stock solution was prepared in dimethyl sulfoxide (DMSO, Sigma, Deisenhofen, Germany) and was further diluted to final working concentrations (0.01–1000 μM) with complete cell culture medium [21]. Cells were assayed as described below after Gemcitabine treatment (0.01–1000 μM) for 24–120 hrs.

## Transfection of siRNA

siRNA was purchased from Qiagen (Hilden, Germany). The sequence of the bcl-2-specific siRNA, named siBCL2, is sense: 5′-UGU GGA UGA CUG AGU ACC UGA-3′ and antisense: 3′-GGA CAC CUA CUG ACU CAU GGA CU-5′. *Silencer*™ Negative Control #1 siRNA (Ambion, Austin, Texas, USA) was used as a non-silencing negative control *in vitro*. For animal experiments, siRNA against the enhanced green fluorescent protein (eGFP) expression plasmid was used as a negative control (sense: 5′-CCA CAU GAA GCA GCA CGA CUU-3′; antisense: 3′-CUG GUG UAC UUC GUC GUG CUG AA-5′) [19].

Transfections were performed at about 70% confluency in six-well plates using oligofectamine (Invitrogen, Carlsbad, California, USA) as described previously [19]. Final concentration of the siRNA was 10 nM. In each experiment, untreated controls and mock-transfected cells, receiving only oligofectamine without siRNA, were included. For combination experiments, cells were transfected with siRNA 72 hrs before Gemcitabine treatment. Cells were assayed 24–144 hrs after transfection.

## Preparation of RNA, cDNA, and quantitative real-time PCR

Total cellular RNA was extracted by use of peqGOLD RNA Pure (Peqlab, Erlangen, Germany) according to the manufacturer's instructions and reverse transcription (RT) was performed as described previously [19].

Relative transcript levels were quantified by real time polymerase chain reaction (PCR) on a LightCycler system (Roche, Mannheim, Germany) as described previously [20]. The sequences of the primers were as follows: bcl-2 forward: 5′-CCT GGT GGA CAA CAT CGC C-3′; reverse: 5′- AAT CAA ACA GAG GCC GCA TGC-3′; GAPDH forward: 5′-GAA GGT GAA GGT CGG AGT C-3′; reverse: 5′-GAA GAT GGT GAT GGG ATT TC-3′. Data were analysed with the LightCycler

software using the proportional second derivative maximum option. To normalise for differences in RNA amounts and variable efficacy of the reverse transcription reactions, results for bcl-2 mRNA were normalised by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels.

## Western-blot anaysis

Trypsinized and washed cells were lysed by adding 100 µl Jie's buffer (20 mM Tris–HCl pH7.4, 10 mM NaCl, 1 mM phenylmethansulfonylfluoride, 5 mM $MgCl_2$, 0.5% NP40, 10 µg/ml protease inhibitor mix [Roche]) and subjected to SDS-PAGE on 14% pre-cast gels (Invitrogen) as described [20]. The separated proteins were transferred to nitrocellulose or polyvinylidene fluoride membranes (GE Healthcare, UK). After blocking overnight at 4°C in a buffer containing PBS, 0.1% Tween 20 and 5% low fat milk powder, membranes were incubated for 90 min with primary antobodies: mouse monoclonal anti-human bcl-2 antibody (1:200, BD Biosciences Pharmingen, San Diego, USA); polyclonal rabbit anti-human bax (1:250, Santa Cruz Biotechnology, Santa Cruz, USA); monoclonal mouse anti-human β-actin (1:5000, Sigma, Taufkirchen, Germany). Membranes were washed three times for 10 min in a buffer containing PBS and 0.1% Tween 20, and then incubated with an appropriate secondary antibody (Sigma) coupled to avidine for 1 hr at room temperature. Reactive bands were detected with the ECL chemiluminescence reagent (Amersham Pharmacia Biotech, Freiburg, Germany).

Obtained images were analyzed using GelScan 5 software (BioSciTec, Frankfurt, Germany).

## Flow cytometric analysis of apoptosis

For quantification of apoptosis, culture supernatants were collected and cells washed two times with PBS, trypsinized and lysed in a hypotonic solution containing 0.1% sodium citrate, 0.1% Triton X-100 and 50 µg/ml propidium iodide (Sigma). Analysis of labelled nuclei was performed on a FACSCalibur fluorescence-activated cell sorter (FACS) using CELLQuest software (both from Becton Dickinson). The percentage of apoptotic cells was determined by measuring the fraction of nuclei with a sub-diploid DNA content.

## Determination of cell viability

Cells were harvested after the incubation period and stained with trypan blue (Biochrom, Germany). The number of unstained (intact) cells was counted in a Neubauer chamber and expressed as relative cell numbers compared to untreated controls (=100%).

## Xenograft model

YAP C pancreatic carcinoma cell lines were harvested and resuspended in sterile physiological NaCl solution. $5 \times 10^6$ YAP C pancreatic carcinoma cells were injected subcutaneously into the flank of 4–6-week-old male NMRI mice (Harlan Winkelmann GmbH, Germany). For each cell line, 5 animals were used. Animals were kept in a light- and temperature-controlled environment and provided with food and water *ad libitum*. Tumour size was determined daily by measurement with a caliper square. Intraperitoneal treatment with siRNA (100 µg/kg/day), Gemcitabine (8 mg/kg/every fourth day) [22] or physiological saline solution (daily) was started when subcutaneous tumours reached a diameter of 7 mm [19, 22]. The Gemcitabine concentraions used represents 1/10 of the standard dose for mice. Animals were sacrificed by cervical dislocation and specimens of tumours were either fixed in 10% phosphate-buffered formalin or snap-frozen in liquid nitrogen. Ethical approval was obtained before the beginning of experiments (621-2531.31-20/01, Government of Lower Franconia, Würzburg, Germany).

## Immunohistochemistry and TUNEL staining

Tumour tissue was fixed with 10% phosphate-buffered formalin and embedded in paraffin. Routine histology (hematoxylin and eosin staining) was performed in order to evaluate basic histomorphological features of the specimens.

Sections were dewaxed, rehydrated and processed by microwave heating in citrate buffer (pH 6.0). Specimens were incubated with primary antibodies (anti-Ki-67, 1.50; anti-bcl-2, 1:100; both from Dako Germany, Hamburg, Germany) overnight at 4°C and visualized using streptavidin–biotin complex (Biogenex, San Ramon, CA, USA) coupled to alkaline phosphatase and developed using 3-hydroxy-2-naphtylacide-2,4-dimethylanilide as substrate. Nuclei were counterstained with hematoxylin. Replacement of primary antibodies by non-immune mouse or rabbit serum (BioGenex, San Ramon, CA, USA) or Tris-buffered saline (pH 7.2) served as negative controls.

TUNEL stainings were performed with the *In situ* Cell Death detection Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. Slides were digitized and analyzed with the Ce2001 Cell Explorer software (BioSciTec, Frankfurt Germany). Quantification (extensity)

and semi-quantification (intensity and distribution) were performed for four independent high power fields in each slide, performed with electronic filtering for respective signals.

### Statistical analysis

Statistical analyses were performed with Microsoft Excel 2003. Significance was calculated using the Student's t-test for paired samples. $P < 0.05$ was regarded as significant.

## Results

### Sensitivity of pancreatic cancer cells to Gemcitabine treatment

Gemcitabine induced apoptosis in YAP C cells at concentrations greater than 0.1 μM. Here, a linear increase in sub-diploid events was observed in FACS analysis, reaching up to 80% after 120 hr for all effective concentrations (0.1–100 μM). Below 0.1 μM, apoptosis levels remained in the range of untreated controls (Fig. 1A). No further increase in sub-G1-events was observed for concentrations higher than 100 μM (not shown).

Non-transformed HF showed a greater resistance to Gemcitabine-induced apoptosis (Fig. 1A). After 120 hr, Gemcitabine induced an apoptosis rate of 65.8%, 52.6%, 24.1% at 100, 10 or 1 μM, respectively, and only 2.4% at 0.1 μM or below.

Incubation with 0.01 μM Gemcitabine did not influence cell number in either YAP C or HF. YAP C showed a moderate response to Gemcitabine at 0.1 μM, reducing the number of viable cells to 50% of untreated controls after 72 hrs and to 30% after 96 and 120 hrs. Higher concentrations (1–100 μM) quickly reduced the cell number to less than 10% of untreated controls (Fig. 1B). In HF, cell number was moderately reduced after incubation with 0.1 μM Gemcitabine until 72 hrs (62.3%) and dropped further until 120 hrs (32.7%). Higher concentrations lead to a rapid reduction in cell number which remained stable until 120 hrs (Fig. 1B).

For further experiments, 0.01 and 1 μM Gemcitabine were used, as these concentrations were either ineffective when applied alone (0.01 μM) or lead to a significant increase in apoptosis and reduction of cell number in tumour cells (1 μM) already after 48 hrs.

### Bcl-2 specific siRNA down-regulates the corresponding mRNA and protein in pancreatic carcinoma cells

Quantitative real time PCR revealed a spontaneous decrease in bcl-2 mRNA levels in mock transfected YAP C cells over the time course of 120 hrs while steady-state levels of the housekeeping gene GAPDH remained relatively unchanged, in accordance with cellular senescence during the time of the experiment [23] (Fig. 2A). bcl-2 specific siRNA (siBCL2) significantly suppressed the level of bcl-2 mRNA (Fig. 2A). The silencing effect increased during the time course of the experiment and reached –142.9 folds of control after 96 hrs. In HF, the maximum silencing effect of siBCL2 reached –3.6 folds after 48 hrs and the silencing effect nearly disappeared after 96 hrs (Fig. 2A).

In YAP C, 1 μM Gemcitabine treatment up-regulated the bcl-2 mRNA level to +8.8 folds of control in maximum (Fig. 2B). In contrast, bcl-2 mRNA levels decreased to –14.5 folds after 120 hrs in HF cells treated with 1 μM Gemcitabine (Fig. 2B). In both of YAP C and HF, the bcl-2 transcriptional levels with 0.01 μM Gemcitabine treatment were within ± 2.0 folds of controls.

Transfection of siBCL2 for YAP C 72 hr before addition of 1 or 0.01 μM Gemcitabine leads to pronounced down regulation of bcl-2 mRNA, reaching –143.0 and – 37.0 folds, respectively (Fig. 2C). In contrast, mock or control siRNA transfections combined with 1 or 0.01 μM Gemcitabine showed only relatively small changes of bcl-2 levels that stayed within the range from –2.9 to +1.7 folds. In HF, the silencing effects of the combination treatment were relatively weaker than in YAP C. siBCL2 with 1 or 0.01 μM Gemcitabine showed a decrease of bcl-2 levels reaching –6.0 and –2.4 folds, respectively (Fig. 2C). The silencing effect was not observed in other treatment groups (+1.1 to +2.0 folds of control).

The results obtained from Western blot also revealed that siBCL2 could inhibit bcl-2 protein expression in YAP C (Fig. 3A). The peak of inhibition was observed after 120 hrs from transfection reaching 37% of control. Pro-apoptotic bax and house keeping β-actin were stably expressed through 120 hrs. In HF there was no distinct difference between controls and treatment groups (Fig. 3A).

In contrast to mRNA levels, 1 μM Gemcitabine treatment tended to down-regulate bcl-2 protein

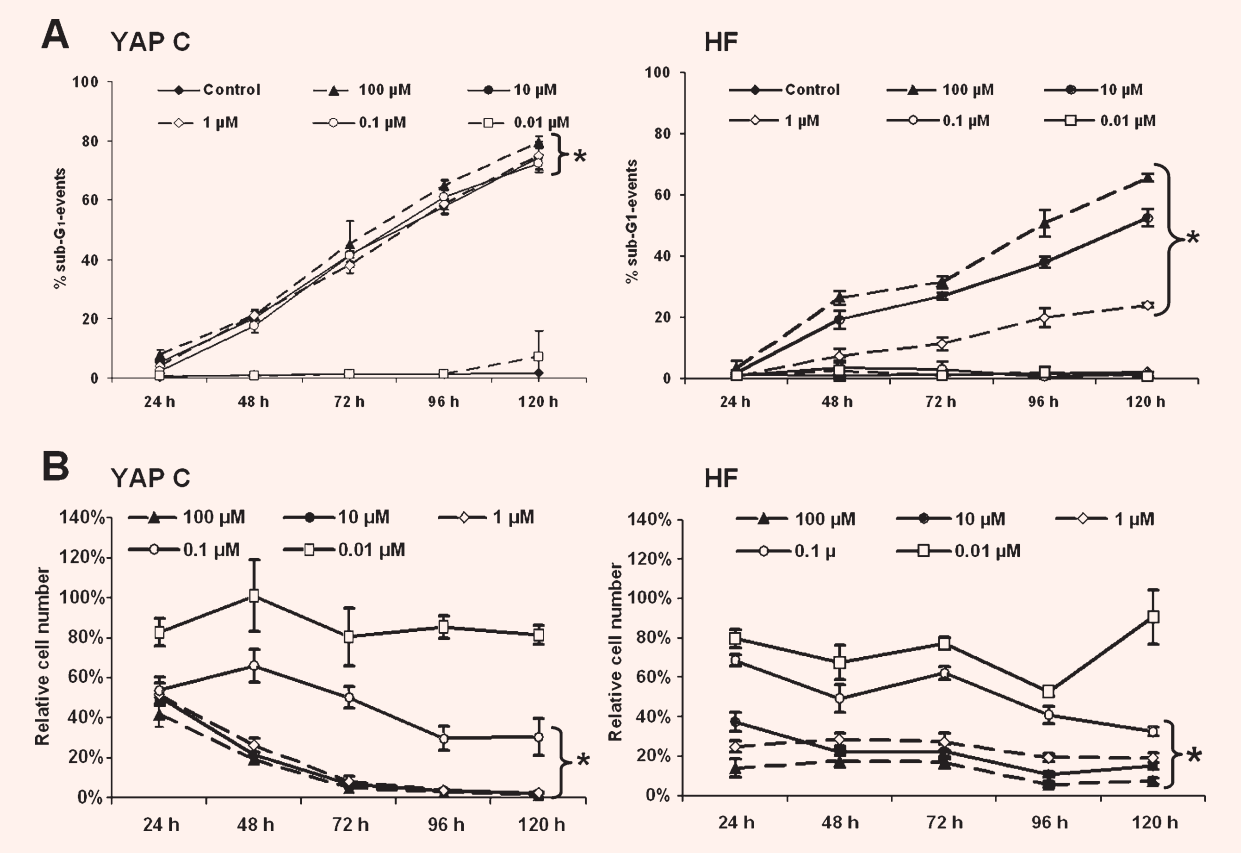


**Fig. 1** Sensitivity of YAP C pancreatic carcinoma cells and non-malignant human foreskin fibroblasts (HF) to Gemcitabine treatment. (**A**) Induction of apoptosis after treatment with different concentrations of Gemcitabine. (**B**) Decrease in cell numbers relative to untreated control (=100%) after incubation with different concentrations of Gemcitabine. Values are mean ± S.E.M. of three independent experiments. *$P < 0.05$ *versus* untreated controls.

expression in YAP C reaching 44% of control at 96 hr (Fig. 3B). 0.01 µM Gemcitabine did not influence the expression of bcl-2 protein. Concerning HF, Gemcitabine treatments did not affect the expression of bcl-2 (Fig. 3B).

The combination treatment of siBCL2 and 1 µM Gemcitabine led to a down regulation of bcl-2 protein expression reaching 47% of control (Fig. 3C). Control siRNA and 1 µM Gemcitabine treatment tended to suppress bcl-2 expression, but the expression of bax in this combinative treatment also decreased and did not lead to change the ratio of bax/bcl-2 towards pro-apoptosis (99% of Control). In HF, the maximal suppression of bcl-2 was 61% of control in siBCL2 and 1 µM Gemcitabine treatment and all of other treatment groups were distributed between 73% and 93% of control (Fig. 3C).

## Transfection of bcl-2-specific siRNA induces apoptosis and reduces pancreatic cancer cell numbers

siBCL2 induced moderate rates of apoptosis in YAP C pancreatic cancer cells at 10 nM, reaching 15.4% after 120 hrs (Fig. 4A). In HF, siBCL2 lead to a transient increase in apoptosis after 72 and 96 hrs (17.1% and 11.6%, respectively) which was not detectable at 120 hrs (Fig. 4A).

YAP C cells showed a constant decrease in cell number beginning 48 hrs after transfection of 10 nM siBCL2. Cell number was reduced to 30.2% of untreated controls after 120 hrs (Fig. 4B). In HF, transfection of 10 nM siBCL2 lead to a minor decrease in cell number, reaching 76% of untreated

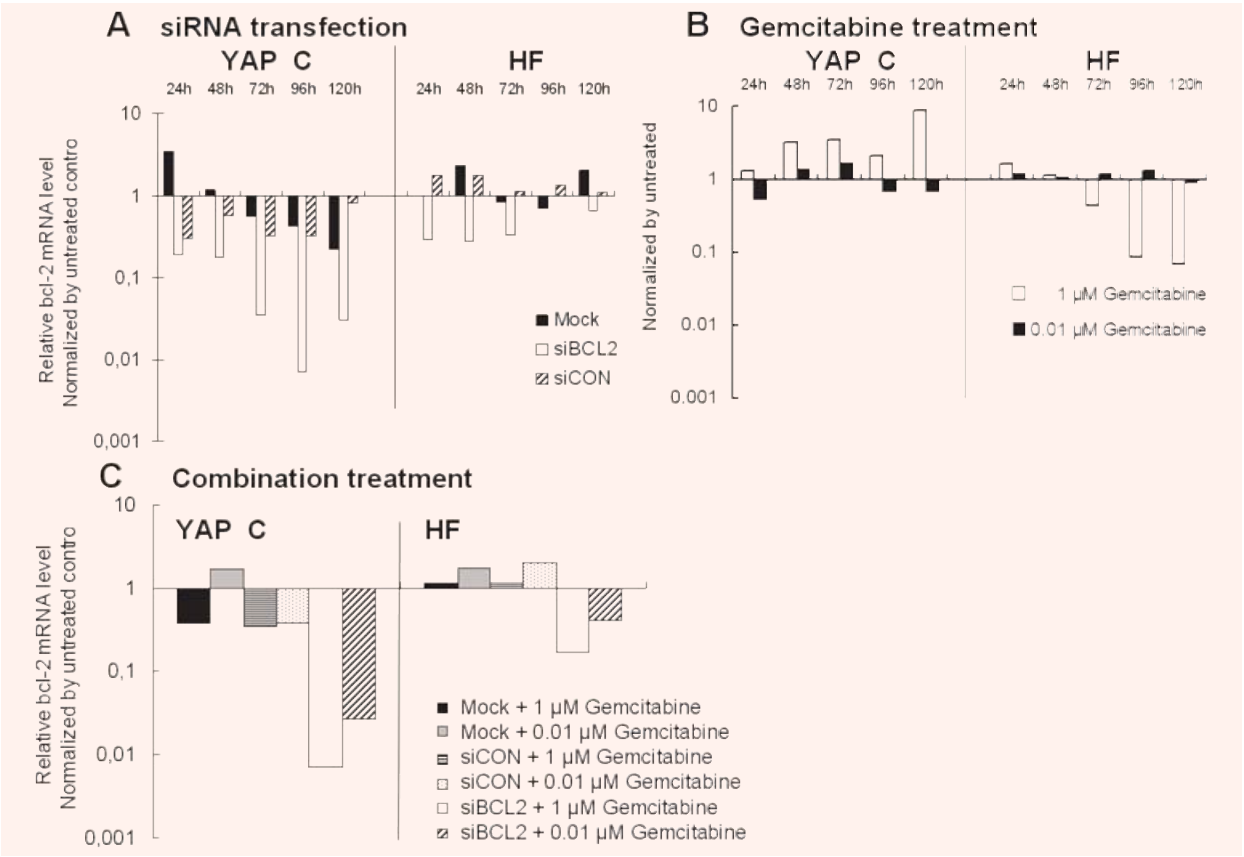


**Fig. 2** bcl-2 mRNA levels (normalized to GAPDH on log tables). (**A**) siRNA transfection: Bcl-2 mRNA level was down-regulated by bcl-2 specific siRNA (siBCL2) in YAP C, but not by control siRNA, with maximum effect 96 hrs after transfection. (**B**) Gemcitabine treatment: 1 µM but not 0.01 µM Gemcitabine treatment up-regulated bcl-2 mRNA levels in YAP C, but down-regulated it in HF. (**C**) Combination treatment: Gemcitabine treatment 72 hrs after siBCL2 transfection leads to pronounced down regulation of bcl-2 mRNA in YAP C compared to control siRNA and Gemcitabine treatment as well as HF controls. Bars represent means of two independent experiments with pooled RNA samples from three independent experimental settings.

controls after 120 hrs. This effect was stable over the time course of the experiment (Fig. 4B). Mock transfections without any siRNA or transfection of the unspecific control siRNA did not significantly alter cell numbers as compared to untreated controls in both cell lines.

## Synergistic effects of bcl-2-specific siRNAs and Gemcitabine in pancreatic cancer cells

Transfection of siRNAs 72 hrs before addition of low concentrations of Gemcitabine leads to a potentiated induction of apoptosis and inhibition of proliferation in YAP C but not HF. While mock transfected YAP C cells did not respond with increased apoptosis when treated with 0.01 µM Gemcitabine, this concentration was sufficient to induce apoptosis levels of nearly 50% in siBCL2 transfected cells. This apoptosis level is significantly higher than in untreated control or mock transfected cells treated with 0.01 µM Gemcitabine ($P < 0.005$, $P < 0.02$, respectively). Compared to Gemcitabine treatment alone, no further increase in apoptosis was detectable at 1 µM (Fig. 5A). Transfection of the control siRNA before adding Gemcitabine lead to increased apoptosis levels at 1 µM, but not at 0.01 µM Gemcitabine, indicating that the apoptosis rates observed here are mere Gemctiabine effects. In contrast, fibroblasts did not

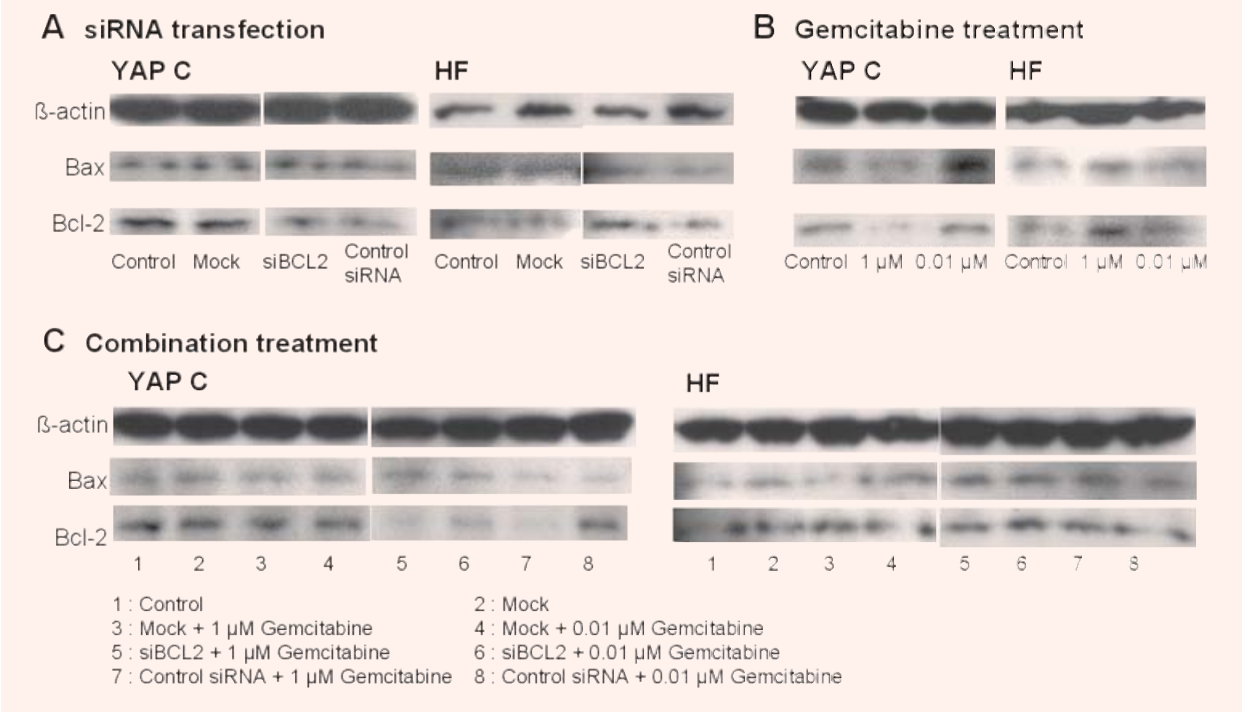


**Fig. 3** Expression of the bcl-2 protein determined by Western blotting. (**A**) siRNA transfection inhibited bcl-2 protein expression of YAP C but not in HF. (**B**) 1 µM Gemcitabine treatment tended to down-regulate bcl-2 protein expression in YAP C in comparison to 0.01 µM Gemcitabine treated YAPC cell lines or controls. (**C**) The combination treatments of siBCL2 and Gemcitabine lead to a pronounced down-regulation of bcl-2 protein in YAP C but not in HF cells.

show elevated levels of apoptosis after treatment with siBCL2 and Gemcitabine alone or in any combination (Fig. 5A).

Similar results were obtained for inhibition of cell proliferation (Fig. 5B). Pre-treatment of mock transfection did not influence Gemcitabine effects in both cells lines. Transfection of 10 nM siBCL2 72 hrs before addition of 0.01 µM Gemcitabine reduced the number of viable YAP C cells by more than 50%. This value was significantly smaller than in untreated controls ($P < 0.002$) and 0.01 µM Gemcitabine treatment and mock transfection ($P < 0.002$), while HF showed only a minor response to this combination therapy with no statistically significant differences (Fig. 5B). 1 µM Gemcitabine was able to reduce the number of viable cells in both cell lines with either siBCL2 or the control siRNA to the levels observed for Gemcitabine monotherapy, indicating that the observed effects here are mainly Gemcitabine effects, too. This view is further corroborated by the finding that control siRNA and 0.01 µM Gemcitabine did not lead to a change in cell number in YAP C and HF.

## siBCL2 and Gemcitabine lead to a synergistic growth inhibition *in vivo*

Previous results showed that the intraperitoneal application of unmodified siRNA suffices to delay growth of pancreatic cancer xenografts in nude mice [19]. Here, mice ($n = 5$/group) were treated with daily intraperitoneal injections of siBCL2 or control siRNA combined with application of Gemcitabine at 1/10 of the standard dose every fourth day. Gemcitabine or control siRNA alone or animals receiving only physiological saline served as controls.

After 21 days, tumours reached a size of 1.8 relative to day 1 in animals receiving saline only. In animals receiving control siRNA, relative tumour size was 1.7, while siBCL2 alone already lead to a reduction in tumour size to 1.6. Gemcitabine alone or combined with control siRNA lead to a significant tumour growth suppression compared with saline control (relative tumour sizes are 1.45 and 1.46, $P < 0.02$ and 0.03, respectively). Tumours of animals receiving

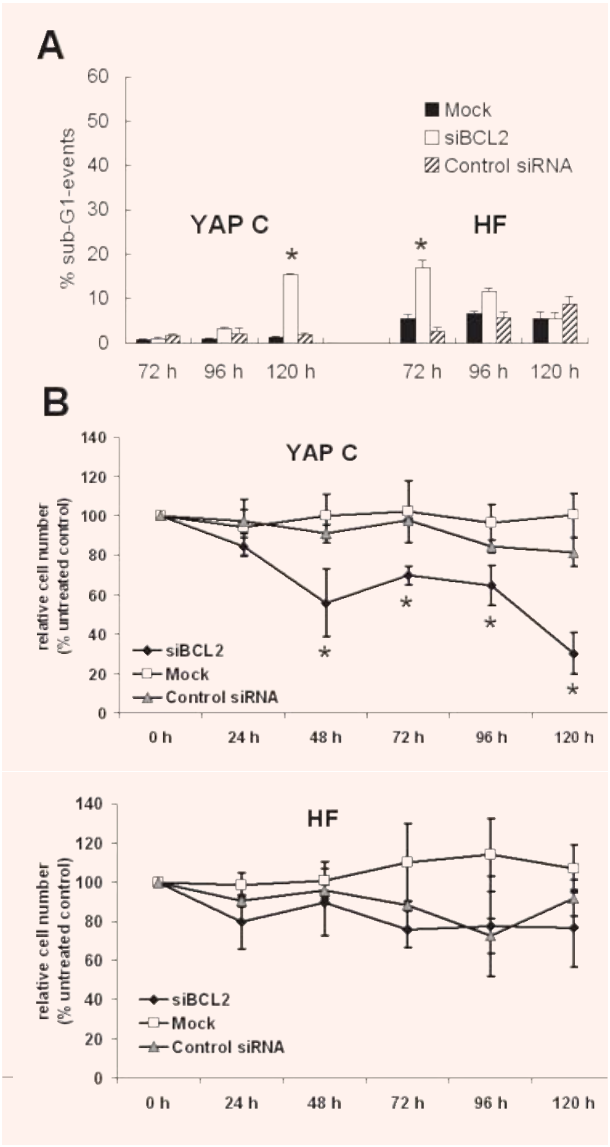


**Fig. 4** Apoptosis rate and numbers of viable cells after siRNA transfection. (**A**) 10 nM siBCL2 lead to a moderate increase in apoptosis in pancreatic cancer cells and in HF while the control siRNA was ineffective in both cell lines. (**B**) siBCL2 lead to a significant reduction in of viable cells in YAP C, while in HF only a moderate but not significant reduction of cell number was observed. Mock or control siRNA transfections did not induce apoptosis or reduce the number of viable cells in both cell lines. Values are mean ± S.E.M. of three independent experiments. *$P < 0.05$ *versus* mock transfected cells and untreated controls.

Gemcitabine and siBCL2 reached a relative tumour size of 1.11 ($P < 0.04$ *versus* Gemcitabine alone and $P < 0.03$ *versus* Gemcitabine and control siRNA; Fig. 6A).

These effects were mediated by a down-regulation of bcl-2 protein in animals receiving siBCL2 and Gemcitabine, while no change of bcl-2 expression was observed in the other groups (Fig. 6B). This combination also lead to increased levels of pro-apoptotic bax, thus shifting the ratio of bax/bcl-2 towards pro-apoptosis, whereas this ratio remained unchanged in the other groups, too.

Immunohistochemistry showed a distinct cytoplasmic expression of bcl-2 in control animals which was almost completely lost in the siBCL2 treated groups and the intensity of bcl-2 expression significantly decreased compared to controls ($P < 0.01$). Gemcitabine alone or in combination with control siRNA only marginally reduced bcl-2 immunohistochemistry with no statistical difference to controls (Fig. 6C upper panels).

The macroscopically observed growth delay was also due to reduced proliferation of tumour cells as demonstrated by PCNA staining (Fig. 6C middle panels). All tumours showed a high proliferation index of 49.2% (saline), 56.9% (Gemcitabine alone) and 52.9% (control siRNA/Gemcitabine), in contrast to 25.6% in the siBCL2/Gemcitabine group. This treatment also lead to increased TUNEL positivity, indicating that the diminished tumour size was also due to induction of cell death (Fig. 6C lower panels). In the siBCL2/Gemcitabine group, TUNEL-positive areas were observed in 3.6% of the examined high power fields compared to 0.8% in saline controls, 1.3% in the Gemcitabine group and 1.5% in the control siRNA/Gemcitabine group, respectively.

## Discussion

Gemcitabine, a deoxycytidine analogue, has been shown to ameliorate disease-related symptoms and to improve survival time [24], especially in combination with other cytotoxic agents (*e.g.* cisplatin [25] or irinotecan [26]). Yet, the development of an effective treatment for pancreatic cancer remains an urgent task. Despite of detailed knowledge of its molecular pathology, little progress has been made regarding overall survival [6]. Previously, we have shown that the pancreatic cancer cell lines YAP C and DAN G respond to siBCL2 with increased apoptosis and diminished proliferation *in vitro* and that pancreatic cancer xenografts are suitable for studying the effect of siBCL2 *in vivo* [19]. Due to the high aggressiveness (early metastases) of DAN G, we now only investigated YAP C with respect of animal protection. Furthermore, YAP C represents a moderately differentiated pancreatic ductal adenocarcinoma with low aggressiveness *in vivo* that is still capable of

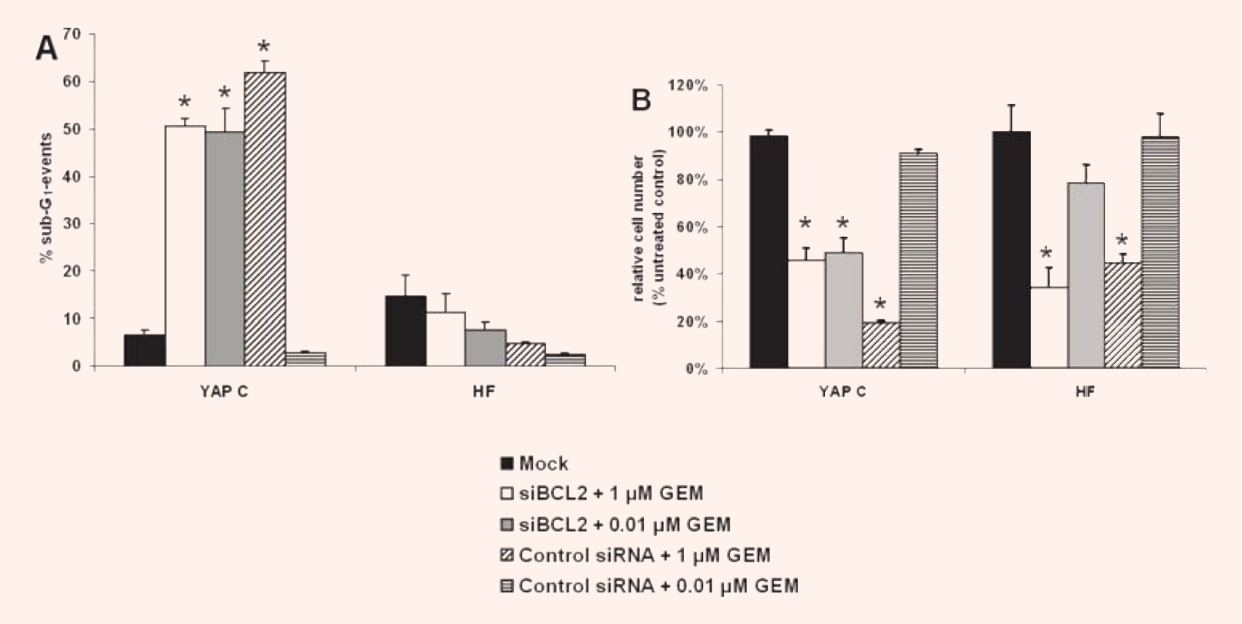


**Fig. 5** Sensitivity of YAP C pancreatic carcinoma cells and HF to combined siRNA and Gemcitabine treatment. (**A**) Apoptosis rate of YAP C, but not of HF, significantly increased after addition of 1 or 0.01 µM Gemcitabine 72 hrs after transfection with 10 nM siBCL2. In combination with control siRNA, only the high concentration of Gemcitabine leads to significant levels of apoptosis, indicating the predominant cytotoxic effect of Gemcitabine at this concentration. (**B**) Addition of 0.01 µM Gemcitabine to siBCL2 reduced the number of viable YAP C cells by more than 50%, while HF showed only a minor response to 0.01 µM Gemcitabine and siBCL2 combination therapy. Combinations with control siRNA and 1 µM Gemcitabine show only the Gemcitabine effect but no further reduction in both cell lines. Results are mean ± S.E.M. of three independent experiments. *P < 0.05 *versus* untreated controls and 0.01 µM Gemcitabine with pre-treatment of mock transfection.

transdifferentiation [20], rendering YAP C representative for early and still treatable stages of pancreatic cancer in human patients.

Known treatment resistance to Gemcitabine has been correlated with the expression of anti-apoptotic members of the bcl-2 protein family in different cancer cell lines [27]. It was thus postulated that the effectiveness of these chemotherapeutic regimens could be enhanced by a targeted down-regulation of bcl-2 or bcl-xL [7, 8, 28]. So far, several studies investigated the effect of conventional DNA antisense oligonucleotides on bcl-2 expression and chemotherapy sensitivity with promising results [7,14–16]. Due to unspecific effects (*e.g.* cytokine release), hepatotoxicity and unsatisfactory pharmacokinetic properties (short half-life *in vivo*, low plasma and tissue concentrations) with the need to introduce chemical modifications (*e.g.* locked nucleic acids) [29, 30], we previously applied the recently discovered RNA interference (RNAi) pathway to silence the expression of bcl-2 in human pancreatic cancer cells: 10 nM of bcl-2-specific siRNAs inhibited growth of pancreatic cancer cell lines *in vitro* and *in vivo* and lead to a specific downregulation of bcl-2 mRNA and protein. This lead to the breakdown of the mitochondrial transmembrane potential $\Delta\Psi_m$ and shifted the bax/bcl-2 ratio towards pro-apoptosis [19].

Resistance to the intrinsic, *i.e.* mitochondrial, pathway of apoptosis induction *via* up-regulation of anti-apoptotic bcl-2 members has been shown to be the main reason for failure of Gemcitabine treatment in pancreatic cancer [7, 8] and other tumours (*e.g.* mesothelioma cells [28]). Accordingly, and in addition to our previous findings [19], we have shown here that transfection of bcl-2-specific siRNAs to pancreatic cancer cells lowers the apoptotic threshold by decreasing levels of the corresponding target protein as demonstrated by FACS analysis and Western blotting, shifting the bax/bcl-2 ratio towards pro-apoptosis. Co-incubation with low micromolar concentrations of Gemcitabine, which were ineffective when applied alone, lead to a synergistic increase in apoptosis induction and a decrease in the number of viable cancer cells, while non-transformed fibroblasts showed only minor responses. At higher concentrations of

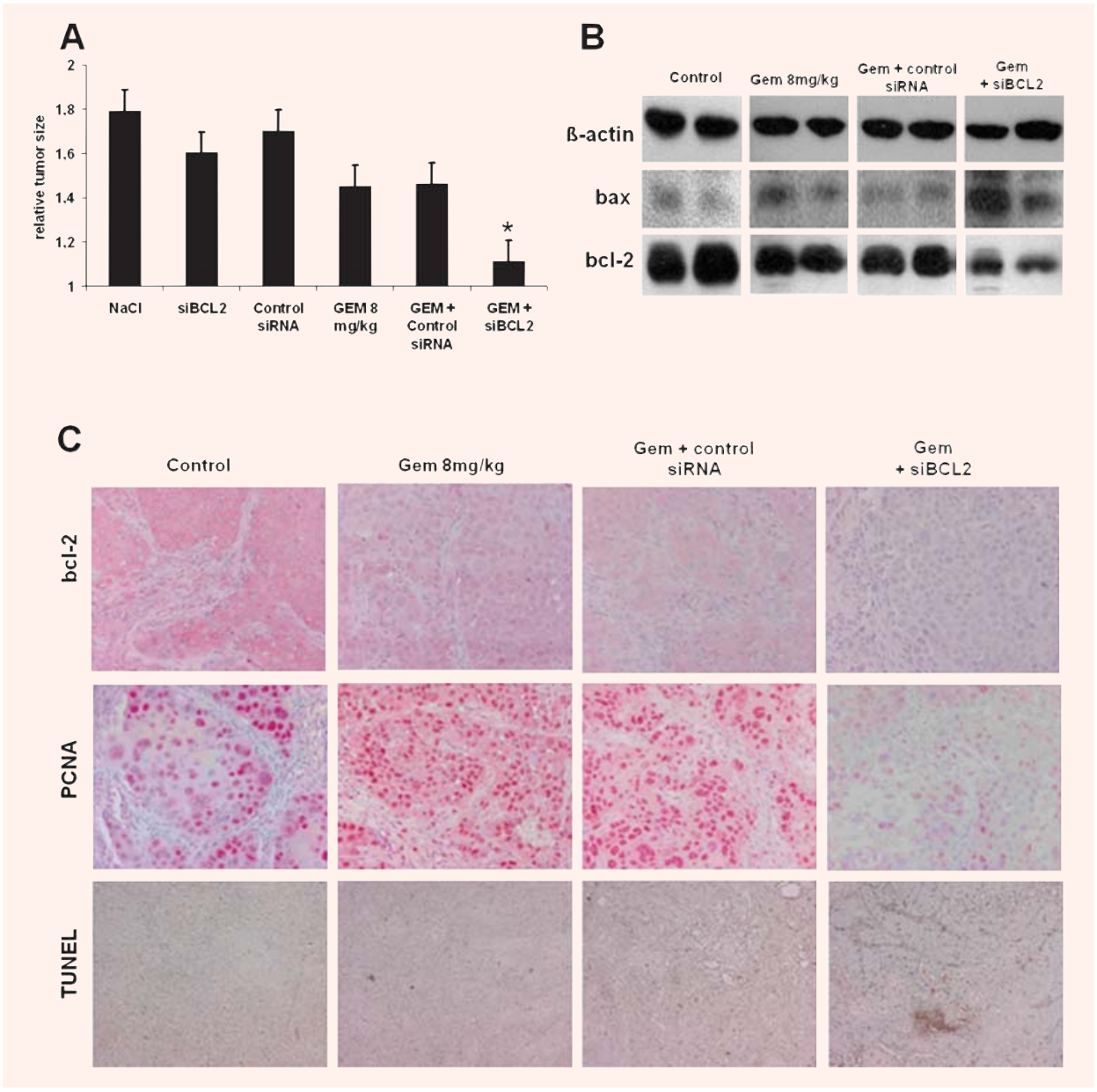


**Fig. 6** Results of pancreatic cancer xenografts in nude mice. (**A**) Growth rate of pancreatic cancer xenografts: After 21 days, single agents (siBCL2, control siRNA or Gemcitabine at 8 mg/kg) as well as the combination of Gemcitabine with control siRNA only lead to a minor decrease in relative tumour size compared to saline controls (1.6, 1.7, 1.45 and 1.46, respectively), while tumour size relative to day 1 reached 1.11 in animals receiving Gemcitabine and siBCL2. $^{*}P < 0.05$ *versus* untreated control or control experiments. (**B**) Western-blot analysis of pancreatic cancer xenografts revealed a down regulation of bcl-2 protein in animals receiving siBCL2 and Gemcitabine, while no change of bcl-2 expression was observed in the other groups. (**C**) Immunohistochemistry staining of pancreatic cancer xenografts. A strong cytoplasmic staining for bcl-2 was detectable in saline controls in contrast to intermediate or little staining in sections from xenografts of Gemcitabine and siBCL2 treated animals. Lowest proliferation index (percentage of PCNA-positive nuclei) was detected in xenografts treated with siBCL2/Gemciatbine (25.6%). The maximum of TUNEL-positive areas were seen in xenografts with siBCL2/Gemcitabine (3.6%). 200 × magnification of bcl-2 and PCNA immunohistochemistry and 100 × for TUNEL staining.

Gemcitabine (1 μM), siBCL2 did not further increase the chemotherapeutic efficacy, indicating that especially the very low concentration of 0.01 μM Gemcitabine should be used for these combination treatments.

1 μM Gemcitabine treatment tended to increase the bcl-2 mRNA level in YAP C whereas it decreased bcl-2 protein levels, while 1 μM Gemcitabine decreased bcl-2 mRNA levels in HF but the change was not reflected to bcl-2 protein level. Several former reports also have shown the discrepancy between mRNA and protein levels of bcl-2 in normal or tumour cells [31]. The mechanism has not been clearly elucidated, Ikeguchi *et al.* reported that cisplatin treatment significantly increased the expression levels of bcl-2 mRNA in PANC-1 cells [32] and Bold *et al.* suggested that the exposure to a cytotoxic agent induces bcl-2 levels in pancreas carcinoma cells [8]. On the other hand, some reports reveal the post-transcriptional downregulation of bcl-2 [33]. For example, 3'-untranslated region of bcl-2 mRNA which contains cis-acting AU-rich elements (ARE) and ARE-binding proteins regulate mRNA stability [34] and Bandyopadhyay *et al.* showed that chemotherapeutic agents reduced bcl-2 steady-state level through inactivation of ARE-binding proteins [35]. Several reports supported the possibility of the poor correlation between protein and RNA in eukaryotic systems, where up to 20-fold changes in protein levels can be seen without corresponding alterations in mRNA abundance, and up to 30-fold changes in mRNA levels without reflection on protein levels [36–38].

Corroborating our *in vitro* results, our *in vivo* results show that the combination treatment of Gemcitabine and bcl-2 specific siRNA gives a clear therapeutic gain over pancreas cancer xenografts. This combination treatment abrogated bcl-2 in pancreas cancer tissues, reduced proliferation of tumour cells and increased the apoptotic index. This result suggests that suppression of bcl-2 by corresponding siRNA is associated with sensitization of pancreatic cancer to apoptotic cell death *in vivo*. It is also worth mentioning that this increase of the anti-tumour effect was not accompanied by an increase of toxicity, since the lack of additive toxicity is an important factor in clinical therapy for advanced cancer patients. The *in vivo* application of unmodified siRNA still represents an urgent problem for clinical application. Several papers use a hydrodynamic tail vein injection in mice, which was not used here due to animal protection laws. We have previously established the method of intraperitoneal application of siRNA dissolved in RNAse free sterile physiological saline solution which seems to provide a suitable vehicle for applying siRNA *in vitro* [19], especially to different tissues of the gastrointestinal tract, while the commonly used phosphate buffered saline seems to offer unfavourable electrochemical conditions for siRNA.

In summary, our results indicate that silencing anti-apoptotic bcl-2 by siRNA specifically sensitizes human pancreatic cancer cells to Gemcitabine treatment at low, otherwise ineffective, concentrations. This treatment strategy might contribute to the therapy of otherwise Gemcitabine-resistant pancreatic cancers *in vivo* and might lower that rate of unwanted chemotherapy effects in patients with advanced diseases.

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