Pancreatic cancer is one of the most common causes of cancer related deaths in Western countries and represents approximately 10% of all gastrointestinal malignancies.1 Ductal adenocarcinoma is responsible for 90% of all cases and is often diagnosed at an advanced stage, with a median survival time of less than six months and five year survival rates of only 10% and less than 3%, respectively. Hence pancreatic cancer has the poorest prognosis of many neoplasms.23 Only 10–14% of patients are eligible for curative surgery which prolongs median survival to 10–20 months.4 Various adjuvant, neoadjuvant, loco-regional, or radiochemotherapy strategies could not significantly improve overall survival, and still remain palliative.1–3 Several genetic risk factors have been identified to date.5 Among these, activating point mutations in codon 12 of the K-ras proto-oncogene can be found in up to 90% of tumour specimens.6 Other factors include mutations of p16INK47, the deleted in colon cancer gene (DCC), p53, and related cell cycle control genes.8–10 Furthermore, the antiapoptotic gene bcl-2 is highly expressed in most pancreatic cancers.11–12 The family of bcl-2 homologues consists of proapoptotic members such as bax, bad, bid, or bim, and anti-apoptotic members such as bcl-2 or bcl-xL which regulate central pathways in cell apoptosis, frequently enhancing the effects of conventional chemotherapy in various malignancies—for example, lymphoma,13 leukemias,14 breast cancer,15 melanoma,16 or gastric cancer.17 Suppression of expression of antiapoptotic bcl-2 has so far been investigated using antisense oligonucleotides.18 However, preclinical and clinical studies did not demonstrate convincing antitumour efficacy due to unspecific effects (for example, complement activation, thrombocytopenia, cytokine release), hepatotoxicity, and the need to introduce chemical modifications (for example, locked nucleic acids) to stabilise these molecules.19–21

We therefore exploited the recently discovered mechanism of specific suppression of a target gene by short double stranded (ds) oligoribonucleotides utilising the RNA interference (RNAi) pathway. RNAi is a highly conserved pathway of cleaving dsRNA (for example, after viral infection) by generating short interfering RNA (siRNA) molecules, duplexes of 21–23 nt in length, which use the RNase III-like enzyme DICER.22–24 Intracellularly, DICER generated or chemically synthesised siRNAs are separated into single stranded (ss) siRNAs, in a process termed endonucleolytic cleavage of dsRNA (for example, after viral infection) by generating short interfering RNA (siRNA) molecules, duplexes of 21–23 nt in length, which use the RNase III-like enzyme DICER.22–24 Intracellularly, DICER generated or chemically synthesised siRNAs are separated into single strands by an ATP dependent helicase and become incorporated into the RNA induced silencing complex (RISC). RISC binds the cognate endogenous mRNA and cleaves the target molecule if the siRNA derived antisense strand is incorporated.25–31 siRNAs and RNAi have been used to silence different target genes in vitro and in model organisms in vivo.12–24 As a therapeutic tool, siRNAs have been used to suppress the replication of several viruses (for example, hepatitis C virus13 or human immunodeficiency virus),32 and expression of inflammatory genes or oncogenes such as c-erbB, for example.33

We therefore studied the pharmacokinetics of parenterally administered siRNA, and determined if, and to what extent, a tumour gene specific siRNA targeted to bcl-2 could contribute to the pharmacology of siRNA to bcl-2. We hypothesised that siRNA may have a clinical benefit to patients with pancreatic cancer.22–23 In addition, we determined if, and to what extent, a tumour gene specific siRNA targeted to bcl-2 could contribute to the pharmacology of siRNA to bcl-2. We hypothesised that siRNA may have a clinical benefit to patients with pancreatic cancer.22–23 In addition, we determined if, and to what extent, a tumour gene specific siRNA targeted to bcl-2 could contribute to the pharmacology of siRNA to bcl-2. We hypothesised that siRNA may have a clinical benefit to patients with pancreatic cancer.22–23 In addition, we determined if, and to what extent, a tumour gene specific siRNA targeted to bcl-2 could contribute to the pharmacology of siRNA to bcl-2. We hypothesised that siRNA may have a clinical benefit to patients with pancreatic cancer.22–23 In addition, we determined if, and to what extent, a tumour gene specific siRNA targeted to bcl-2 could contribute to the pharmacology of siRNA to bcl-2. We hypothesised that siRNA may have a clinical benefit to patients with pancreatic cancer.22–23 In addition, we determined if, and to what extent, a tumour gene specific siRNA targeted to bcl-2 could contribute to the pharmacology of siRNA to bcl-2. We hypothesised that siRNA may have a clinical benefit to patients with pancreatic cancer.22–23 In addition, we determined if, and to what extent, a tumour gene specific siRNA targeted to bcl-2 could contribute to the pharmacology of siRNA to bcl-2. We hypothesised that siRNA may have a clinical benefit to patients with pancreatic cancer.22–23 In addition, we determined if, and to what extent, a tumour gene specific siRNA targeted to bcl-2 could contribute to the pharmacology of siRNA to bcl-2. We hypothesised that siRNA may have a clinical benefit to patients with pancreatic cancer.22–23 In addition, we determined if, and to what extent, a tumour gene specific siRNA targeted to bcl-2 could contribute to the pharmacology of siRNA to bcl-2. We hypothesised that siRNA may have a clinical benefit to patients with pancreatic cancer.22–23 In addition, we determined if, and to what extent, a tumour gene specific siRNA targeted to bcl-2 could contribute to the pharmacology of siRNA to bcl-2. We hypothesised that siRNA may have a clinical benefit to patients with pancreatic cancer.22–23 In addition, we determined if, and to what extent, a tumour gene specific siRNA targeted to bcl-2 could contribute to the pharmacology of siRNA to bcl-2. We hypothesised that siRNA may have a clinical benefit to patients with pancreatic cancer.22–23 In addition, we determined if, and to what extent, a tumour gene specific siRNA targeted to bcl-2 could contribute to the pharmacology of siRNA to bcl-2. We hypothesised that siRNA may have a clinical benefit to patients with pancreatic cancer.22–23 In addition, we determined if, and to what extent, a tumour gene specific siRNA targeted to bcl-2 could contribute to the pharmacology of siRNA to bcl-2. We hypothesised that siRNA may have a clinical benefit to patients with pancreatic cancer.22–23 In addition, we determined if, and to what extent, a tumour gene specific siRNA targeted to bcl-2 could contribute to the pharmacology of siRNA to bcl-2. We hypothesised that siRNA may have a clinical benefit to patients with pancreatic cancer.22–23 In addition, we determined if, and to what extent, a tumour gene specific siRNA targeted to bcl-2 could contribute to the pharmacology of siRNA to bcl-2. We hypothesised that siRNA may have a clinical benefit to patients with pancreatic cancer.22–23 In addition, we determined if, and to what extent, a tumour gene specific siRNA targeted to bcl-2 could...
to the treatment of pancreatic cancer. We also investigated if structural variations in bcl-2-specific siRNA could influence RNAi efficacy in vitro and in vivo.

**MATERIAL AND METHODS**

**Cell culture**

YAP C and DAN G pancreatic adenocarcinoma cells were cultured on six well tissue culture plates (Becton Dickinson, Mannheim, Germany) in RPMI-1640 medium (Biochrom, Berlin, Germany) containing 10% fetal calf serum (FCS, Biochrom), penicillin (107 U/l), and streptomycin (10 mg/l) at 37°C with 5% CO2. Human foreskin fibroblasts served as non-malignant controls and were cultured in Dulbecco’s modified Eagle’s medium (Biochrom) with the same supplements. All cell lines were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany).

**Transfection of siRNA**

siRNA was provided by Ribopharma AG (Kulmbach, Germany) or purchased from Qiagen (Hilden, Germany). Sequences of the five bcl-2 specific and three control siRNAs used are given in Table 1. bcl-2 specific sequences were numbered consecutively.

Transfections were performed at approximately 70% confluence in six well plates using oligofectamine (Invitrogen, Carlsbad, California, USA) according to the manufacturer’s instructions. Briefly, 0.25 x 10^6 cells were seeded in complete growth medium the day before transfection. For each transfection reaction, siRNA-oligofectamine complexes were prepared by mixing oligonucleotides with oligofectamine diluted 1:5 (v/v) with medium directly before addition to the cells. Final concentrations of the siRNA were between 1 and 100 nM. Transfections were performed in 1 ml of serum free medium for four hours. Thereafter, 0.5 ml of medium containing 30% FCS (v/v) was added to achieve 1 ml of serum free medium for four hours. Thereafter, 0.5 ml of medium containing 30% FCS (v/v) was added to achieve complete growth conditions (10% FCS). In each experiment, untreated controls and mock transfected cells, receiving only medium containing 30% FCS (v/v) were cultured in Dulbecco’s modified Eagle’s medium (Biochrom) with the same supplements. All cell lines were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany).

**Determination of cell viability**

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

**Analysis of ΔΨm**

Mitochondrial injury was assessed by JC-1 staining (MoBiTec, Goettingen, Germany). Cells were adjusted to a density of 0.2 x 10^6/ml, trypsinised, washed in PBS, resuspended in 0.5 ml PBS, and kept on ice in the dark until analysis. Analysis was performed by FACS scan, and mitochondrial function was expressed as relative cell numbers compared with untreated controls (= 100%).

**Fluorescence microscopy of FITC labelled siRNA**

siRNA was labelled with fluorescein isothiocyanate (FITC-siRNA, obtained from Ribopharma, Kulmbach, Germany or from the siRNA STARTER KIT, Qiagen, Germany) and transfected to cells seeded on ChamberSlides, as described above. Twenty four hours after transfection, cells were washed in 1 x PBS (Biochrom) and fixed for 30 minutes in ice cold methanol at –20°C. After an additional washing in PBS, nuclei were counterstained with 1 μg/ml propidium iodide (Sigma, Deisenhofen, Germany). 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 subdiploid DNA content.

To assess bcl-2 protein levels by flow cytometry, cells were transfected and harvested as described. Fixation and membrane permeabilisation were performed with Fix and Perm, as recommended by the manufacturer (Caltag Laboratories GmbH, Hamburg, Germany). A FITC conjugated bcl-2 specific antibody (10 μl F7053; Dako, Glostrup, Denmark) was added for 15 minutes at room temperature in the dark. Samples were washed with PBS, centrifuged at 300 g, resuspended in PBS, and kept on ice in the dark until FACS analysis with fluorescence detector FL1-H. Ten thousand events were collected for each sample analysed.

**Table 1** Sequences of short interfering RNAs (siRNAs)

<table>
<thead>
<tr>
<th>siRNA</th>
<th>Sequence</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcl1</td>
<td>5'-CACGGACUCUGCCGCUCGACGGA-3'</td>
<td>Starting position: 1659</td>
</tr>
<tr>
<td>Bcl2</td>
<td>5'-CACGGACUCUGCCGCUCGACGGA-3'</td>
<td>Starting position: 1659</td>
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<tr>
<td>Bcl3</td>
<td>5'-ACGGCUAGCUGUGAAAGGUCC-3'</td>
<td>Starting position: 1982</td>
</tr>
<tr>
<td>Bcl4</td>
<td>5'-UGUGGAACUGAUUGAUGCCG-3'</td>
<td>Starting position: 2047</td>
</tr>
<tr>
<td>Bcl5</td>
<td>5'-UGUGGAACUGAUUGAUGCCG-3'</td>
<td>Starting position: 2047</td>
</tr>
<tr>
<td>Bcl6</td>
<td>5'-UGUGGAACUGAUUGAUGCCG-3'</td>
<td>Starting position: 2047</td>
</tr>
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<td>Bcl7</td>
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<tr>
<td>Bcl8</td>
<td>5'-UGUGGAACUGAUUGAUGCCG-3'</td>
<td>Starting position: 2047</td>
</tr>
<tr>
<td>Control1</td>
<td>5'-GAUGGGAUCCUGCCUCGCGGCAA-3'</td>
<td>Eukaryotic kanamycin resistance protein</td>
</tr>
<tr>
<td>Control2</td>
<td>5'-GCCUUUGUGGAACUGUACGGC-3'</td>
<td>EGFP expression vector</td>
</tr>
<tr>
<td>Control3</td>
<td>5'-GCCUUUGUGGAACUGUACGGC-3'</td>
<td>3' UTR of hepatitis C virus</td>
</tr>
</tbody>
</table>

Starting position indicates position of the first nt of the siRNA sense strand in the bcl-2 mRNA (accession No M13994). Specificity of the sequences was verified by a BLAST search against human and murine databases to minimise unspecific or off target effects.
Promega (Mannheim, Germany), were incubated with 1 mM stopped by 15 minutes heat inactivation at 70 °C and cDNA primers (each at 0.5 μM) polymerase chain reaction (PCR). Sense and antisense primers and probes (table 2) were designed to span exon-intron boundaries, reducing the risk of detecting genomic DNA.

Preparation of RNA, cDNA, and quantitative real time PCR
Total RNA was prepared from 0.25×10⁵ untreated controls or transfected cells, 24–120 hours after transfection using peqGOLD RNA Pure (Peqlab, Erlangen, Germany) according to the manufacturer’s instructions and subsequent phenol-chloroform extraction. RNA integrity was checked electrophoretically and quantified photometrically. The concentration of total RNA was adjusted to 0.1 μg/ml in RNAse free water.

For reverse transcription (RT), 1 μl of a primer mix containing 100 pmol/μl oligo(dT)₁₅ primer and 100 pmol/μl random hexamer primer (5' -NNN NNN -3'), both from Promega (Mannheim, Germany), were incubated with 1 μg RNA and denatured for 10 minutes at 70°C. An RT reaction mix consisting of 5× RT reaction buffer (Invitrogen, Karlsruhe, Germany), 0.1 M DTT (Invitrogen), and a dNTP mix with 10 mM of each dNTP was added together with 40 U RNAsin RNAse inhibitor (Promega) and 100 U Superscript II reverse transcriptase (Invitrogen) and placed on ice for two minutes. Total reaction volume was adjusted to 20 μl with RNAse free water and incubated for 10 minutes at 25°C and cDNA synthesised for one hour at 42°C. The reaction was stopped by 15 minutes heat inactivation at 70°C and cDNA stored at -20°C until use.

Relative transcript levels were quantified by real time RT-polymerase chain reaction (PCR). Sense and antisense primers were designed to span exon-intron boundaries using primer 3'-end and with the quencher molecule TAMRA at the 3'-end. 5'-primer and 3'-primers were designed to span exon-intron boundaries to minimise the amplification of genomic DNA.

Table 2 Primers and probes for quantitative real time polymerase chain reaction

<table>
<thead>
<tr>
<th>Target gene</th>
<th>5'-primer</th>
<th>Probe</th>
<th>3'-primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bax</td>
<td>GTCACACAGAAGGCTGAGCG</td>
<td>GCCAGGGAGGATGATGGGCGCC</td>
<td>TCCGAAAGAAGCTCTCGGG</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>CCGGGATGACGAGTCGAGGC</td>
<td>GCCAGGGAGGATGATGGGCGCC</td>
<td>AATCAAAACAGGGCCGATGC</td>
</tr>
<tr>
<td>β₂-microglobulin</td>
<td>TGACTGCTGTCATGCATGATCCCA</td>
<td>AATCCAAATGGGCGATCTTC</td>
<td></td>
</tr>
</tbody>
</table>

Probes were labelled with the reporter dye FAM at the 5'-end and with the quencher molecule TAMRA at the 3'-end. 5' - and 3'-primers were designed to span exon-intron boundaries to minimise the amplification of genomic DNA.

Xenograft model

Human pancreatic carcinomas established from Panc-1 cells were transplanted subcutaneously to 4–6 week old nude mice (Harlan Winkelmann GmbH, Germany). Animals were housed in a light and temperature controlled environment and provided with food and water ad libitum. Treatment was started when tumours reached 7 mm in diameter. Animals received intraperitoneal injections of 200 μg/kg siRNA dissolved in 120 μl RNAse free physiological NaCl. Control mice were treated with NaCl alone. Tumour size was determined daily by measurement with a calliper square. After 24 days, animals were sacrificed by cervical dislocation and specimens of tumour, liver, lung, kidney, spleen, and bone marrow were either fixed in 10% phosphate buffered formalin or snap frozen in liquid nitrogen for protein extraction, as described below. Ethics approval was obtained for all experiments (621-2531.31-20/01, Government of Lower Franconia, Würzburg, Germany).

Semiquantification of proteins involved in apoptosis

Snap frozen tumour specimens were lysed by adding 2× sample buffer (2 mM N-ethylmaleimide, 2 mM phenyl-methyl-sulfonylfluoride, 4% sodium dodecyl sulfate, 4% dithiothreitol, 20% glycerol, 0.01% bromphenol blue, 2 M urea, 0.01 M sodium ethylenediaminetetra-acid (Na-EDTA), 0.15 M Tris-HCl). DNA was sheared by pipetting up and down for three minutes at room temperature. Samples were boiled at 95°C for 15 minutes centrifuged at 13 000 rpm for 10 minutes, and then subjected to 14% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (Invitrogen). After blocking overnight at room temperature in a buffer containing PBS, 0.1% Tween 20, and 4% low fat milk powder, nitrocellulose membranes were incubated for 90 minutes either with polyclonal rabbit antihuman Bcl-2 (1:400, sc-783) or polyclonal rabbit antihuman Bax (1:400, sc-493; both Santa Cruz Biotechnology, Santa Cruz, California, USA). Membranes were washed three times for 10 minutes in a buffer containing PBS, 0.1% Tween 20, and 4% low fat milk powder, and incubated with an antirabbit IgG coupled to peroxidase (1:1000; Sigma) for one hour at room temperature. Reactive bands were detected with the ECL chemiluminescence reagent (Amersham Pharmacia Biotech, Freiburg, Germany) and analysed using GelScan 5 software (BioSciTec, Frankfurt, Germany). Signals were standardised to β-actin (1:5000; Sigma) content.

Distribution of radioactive labelled siRNA in vivo

siRNA probe bc5 was labelled radioactively with ³²P-UTP and applied as a single bolus in physiological saline by tail vein injection into non-tumour bearing male nude mice at a
concentration of 1 mg/kg, corresponding to 360,000 cpm/μl.

Pure $^{33}$P-UTP (360,000 cpm/μl) dissolved in physiological saline was used as a control. Three animals per group were kept in metabolic cages to collect faeces and urine. Five minutes to 24 hours after injection, animals were sacrificed by heart puncture and total blood samples collected and fixed with RNAlater (Qiagen, Hilden, Germany). Whole bodies were fixed in formalin, as described above, and cut in sagittal and paramedian plains and 5 μm serial sections. Sections were stained with haematoxylin and eosin and analysed for

by heart puncture and total blood samples collected and fixed with RNAlater (Qiagen, Hilden, Germany). Whole bodies were fixed in formalin, as described above, and cut in sagittal and paramedian plains and 5 μm serial sections. Sections were stained with haematoxylin and eosin and analysed for

Figure 1 Short interfering RNA (siRNA) transfection efficacy, as assessed by fluorescence microscopy. Slides were analysed for fluorescein isothiocyanate (FITC) and propidium iodide (PI) fluorescence. Digital images were acquired and merged with OpenLab software for each channel independently. PI stained nuclei were detectable in all analysed slides, while FITC signals were not present in untreated controls (not shown) or mock transfected cells (top row). Cells transfected with 100 nM FITC-siRNA exhibited distinct intracellular and perinuclear staining (both fibroblasts or YAP C cells). No qualitative difference was detectable between siRNA labelled on the sense or antisense strand (not shown).

Figure 2 Induction of apoptosis by bcl-2 specific short interfering RNAs (siRNAs) in pancreatic cancer cells but not in non-transformed foreskin fibroblasts. Shown are apoptosis rates measured as subdiploid events in flow cytometry by propidium iodide staining after transfection with 10 nM bcl-2 specific (A) or non-silencing (C) siRNA in YAP C pancreatic carcinoma cells 72, 96, and 120 hours after transfection. Results for bcl-2 specific siRNAs in foreskin fibroblasts are shown in (B). (D) Results of apoptosis induction by 10 nM bcl-2 specific siRNAs in DAN G pancreatic carcinoma cells 120 hours after transfection. Values are means (SEM) of three independent experiments. *p<0.05.
24 hours in a Fuji FLA-3000 phoshpo and fluorescence imager (Raytest GmbH, Straubenhardt, Germany).

Immunohistochemistry
Tumour tissue was fixed with phosphate buffered 10% formalin and embedded in paraffin. Sections (5 μm) were cut and stored at room temperature until use. Routine histology (haematoxylsin-eosin staining) was performed in order to evaluate basic histomorphological features of the specimens and to determine toxic effects on organs, especially the liver, spleen, kidney, and haematopoietic system. For immunohistochemistry, sections were dewaxed, rehydrated, and processed by microwave heating in citrate buffer (pH 6.0), as described previously. Specimens were incubated with monoclonal primary antibodies (anti-Ki-67, 1:50; anti-bcl-2, 1:100; Dako) overnight at 4°C and visualised using streptavidin-biotin complex (Biogenex, San Ramon, California, USA) coupled to alkaline phosphatase and developed using 3-hydroxy-2-naphthaldehyde-2,4-dimethylandilide as substrate. Nuclei were counterstained with haematoxylin. Replacement of primary antibodies by non-immune mouse or rabbit serum (BioGenex) or Tris buffered saline (pH 7.2) served as negative controls. Slides were digitised and analysed with the Ce2001 Cell Explorer software (BioSciTec, Frankfurt Germany). Semiquantification was performed for four independent high power fields in each slide, using electronic filtering for respective signals.

RESULTS
siRNAs are effectively delivered into pancreatic cancer cells in vitro
To optimise the transfection protocol, siRNA labelled with FITC at either the 5'-end of the sense strand or the 3'-end of the antisense strand was used. Compared with untransfected or mock transfected cells without FITC signals, distinct intracellular fluorescence staining was observed in cells transfected with FITC labelled siRNA. These signals showed intracytoplasmic and perinuclear localisation, in accordance with the current notion of RNAi occurring as a post-transcriptional process. No qualitative difference was observed between sense or antisense labelled siRNA molecules. As this assay was used to confirm cellular uptake of siRNAs, cells were fixed 24 hours after transfection. At this early time point, no morphological signs of cellular apoptosis were found, indicating low toxicity of the transfection reagent used. The transfection protocol used was optimised for transfecting YAP C cells and human foreskin fibroblasts at comparable efficacy (fig 1).

Bcl-2 specific siRNAs selectively induce apoptosis in pancreatic cancer cells
At 10 nM, siRNAs bcl1, bcl3, and bcl11 induced significant levels of apoptosis in YAP C, reaching a maximum of 25.0%, 37.1%, and 15.4% after 120 hours, respectively (fig 2A), which did not increase further using 100 nM siRNA. Apoptosis remained low after transfection of bcl5 and bcl8,
Transfection of bcl-2 specific siRNA reduces pancreatic carcinoma cell numbers

All bcl-2 specific siRNA sequences reduced the relative cell number of YAP C pancreatic carcinoma cells (fig 3A). This effect was most pronounced for bcl3, bcl5, and bcl11 (up to more than 70% after 120 hours), while bcl1 and bcl8 reduced cell proliferation by more than 50%. Reduced cell viability was detectable as early as 24 hours after transfection. In fibroblasts, cell viability was not significantly altered after transfecting either unspecific control siRNAs or bcl-2 specific siRNA sequences (not shown). Cell number remained stable in both cell types over this time course for mock transfected cells and was reduced slightly, but not significantly, after transfection of control siRNAs 1 and 2. The number of viable cells was slightly increased after transfection with control siRNA 3 (fig 3B). In contrast with YAP C, bcl1 did not reduce cell numbers in DAN G at 10 nM after 120 hours (103.2%), while the other bcl-2 specific siRNAs caused a reduction in viable tumour cells, reaching 86.7%, 62.5%, 54.6%, and 47.0%, respectively, after 120 hours (fig 3C).

Bcl-2 specific siRNA leads to rapid downregulation of the corresponding mRNA

Quantitative real time PCR revealed spontaneous decrease in bcl-2 mRNA levels in control or mock transfected cells over the time course of 120 hours while steady state levels of the housekeeping gene β2-microglobulin or the proapoptotic gene bax remained relatively stable (fig 4A, B), in accordance with cellular senescence during the time of the experiment. Transfection of bcl-2 specific siRNA led to a rapid but variable decrease in bcl-2 mRNA levels, with suppression persisting up to 120 hours. Here, bcl8 was most effective, reaching only 8.6% and 4.3%, respectively, after 120 hours. No effect on cell death was observed in cells transfected with 1 nM of any siRNA (data not shown), and even the most effective bcl-2 specific siRNA, bcl3, could not induce apoptosis in non-transformed skin fibroblasts (fig 2B). Furthermore, control siRNAs with no corresponding endogenous target sequences also failed to activate cellular death signalling pathways in pancreatic cancer cells (fig 2C) or fibroblasts (not shown), except for control siRNA 1 which induced some apoptosis in tumour cells. After 120 hours, apoptosis rates of untreated or mock transfected YAP C cells or fibroblasts remained below 2.0%. These results were paralleled in DAN G cells, with untransfected or mock transfected controls reaching only 4.8% and 5.1% apoptosis after 120 hours, respectively, and all bcl-2 specific siRNAs inducing significant levels of cell death even at 10 nM, reaching 18.5%, 12.3%, 11.9%, 27.9%, and 15.8%, respectively (fig 2D).
while for bcl3 and bcl11 time and concentration dependent effects were observed. The proapoptotic counterpart of bcl-2, bax, remained stable in all experiments and showed an increase after transfection of bcl3 and bcl11. Thus, on the mRNA level, the bax/bcl-2 ratio was shifted towards apoptosis induction.

Expression of bcl-2 protein is diminished after transfection of bcl-2 specific siRNA
FACS analysis revealed a significant reduction in bcl-2 protein levels after transfection of bcl5 or bcl11 in a time and dose dependent manner in YAP C cells (fig 5). Untreated controls and mock transfected cells showed high and stable levels of bcl-2 staining, even after 120 hours (>70% of all detected events), while both bcl-2 specific siRNAs at 100 nM reduced bcl-2-FITC signals to 24.3% (bcl5) or 40.9% (bcl11). A minor but significant reduction in bcl-2 protein levels was observed after transfection of 10 nM bcl5 but not of bcl11 (p<0.05).

Downregulation of bcl-2 disturbs mitochondrial membrane potential
Paralleling the reduced expression of bcl-2 protein, the percentage of cells showing red fluorescence (that is, intact mitochondria) decreased compared with untreated or mock transfected cells (fig 6). With 10 nM of bcl5 or bcl11, JC-1 red fluorescence was reduced to 51% and 54.5%, respectively, after 120 hours, and was further reduced after transfection of 100 nM siRNA (15.1% and 32.5%, respectively). Red fluorescence correlated closely with levels of bcl-2 protein, as determined by flow cytometry (r² = 0.9655). These findings indicate that bcl-2 is involved in maintaining mitochondrial membrane integrity and preventing induction of apoptosis.

siRNA rapidly distributes in vivo and is eliminated via the kidney and liver
Pharmacokinetic data using radioactively labelled siRNA were collected in male NMRI-mice. Ten minutes after a single bolus injection into the tail vein, radioactivity was

Figure 7 Distribution of ³²P-labelled short interfering RNA (siRNA) in vivo. Representative phosphoimager analyses of sagittal (left column) and para-sagittal (right column) sections of animals sacrificed 10 minutes, and 2, 8, and 12 hours after intravenous bolus injection of labelled siRNA. Haematoxylin-eosin stainings of whole body sections are shown in the top row. Organs indicated by arrows are brain (B), gut (G), heart/thoracic haematoma (H), kidney (K), liver (L), pancreas (P), spine (S), spleen (SP), and testis (T). Bottom row shows representative sections of animals receiving pure ³²P-UTP without nucleic acid as a control, sacrificed 12 hours after tail vein injection. Here, signals were more prominent in osseous tissues while in the siRNA treated animals, rapid redistribution from blood to kidney to liver and gut was visible.
quickly distributed by blood flow, showing intense signals in the heart and the retrocardial thoracic haematoma resulting from heart puncture (fig 7). Radioactivity was then redistributed to the kidney (two hours) and accumulated finally in the liver and gut wall (eight and 12 hours). In contrast, $^{33}$P-UTP alone showed signals predominantly in osseous tissues such as spine, skull, or the thoracic wall (fig 8, bottom row). These data indicate that a major proportion of siRNA is quickly excreted via the kidneys but is also metabolised and eliminated by the liver. While pancreata were clearly identified in haematoxylin-eosin stainings, unequivocal identification was not possible in phosphoimager analyses due to limitations in optical resolution. Overall, tissue distribution was homogenous, indicating that several target tissues can be reached with nude siRNA.

**Bcl-2 specific siRNA delays growth of pancreatic cancer xenografts in vivo**

To assess the efficacy of siRNA mediated gene silencing in vivo, mice ($n = 11$ for bcl-2, $n = 8$ for controls) bearing human pancreatic carcinoma xenografts in NMR mice was determined by daily measurement with a standardised calliper square. Data represent mean (SD) tumour volume from animals treated with daily intraperitoneal injections of 200 μg/kg bcl-2 specific siRNA bcl5, control siRNA 1, or vehicle (physiological saline, NaCl), beginning at day 1. (B) Immunohistochemical analysis of formalin fixed paraffin embedded tumour specimens revealed decreased expression of Ki-67 in animals treated with siRNA against bcl-2 but not in control mice. Expression of bcl-2 was decreased by approximately 56% in animals treated with siRNA against the target gene versus physiological saline controls (NaCl). (C) Western blot analysis of xenograft samples revealed specific downregulation of bcl-2 protein in all animals treated with bcl5, in two of four animals treated with control siRNA 1, and in one of three mice from the NaCl group. Proapoptotic bax was detected in all samples; β-actin served as a loading control. (D) Semiquantification of western blot results. Mean (SEM) densitometry values per group relative to NaCl treated animals (broken line). *p < 0.05 versus NaCl treated animals.
xenograft specimens revealed specific downregulation of bcl-2 in three of four of the bcl5 treated animals while proapoptotic bax and the housekeeping protein β-actin remained unchanged (fig 8C). However, as yet unidentified factors may contribute to decreased tumour cell proliferation also. Thus in animals treated with control siRNA or with physiological saline, two of four also showed loss of bcl-2 whereas in others expression of the target gene remained elevated, indicating different endogenous expression of bcl-2 due to clonal expansion of low or high expressing tumour cells. Overall densitometric analysis showed a reduction in bcl-2 expression in the bcl5 treated group to a mean of 6.0% of expression in the NaCl group (p<0.05, fig 8D). In the control group, bcl-2 expression reached 70.0% of the saline control. Proapoptotic bax remained stable at a mean of 124% (bcl5) and 170% (control siRNA), shifting the bax/bcl-2 ratio to 20.7 in animals treated with bcl-2 specific siRNA compared with 2.5 in NaCl controls.

**DISCUSSION**

Efficient and well tolerated therapy for pancreatic cancer is urgently needed. Specific downregulation of genes that prevent apoptosis or chemotherapeutic sensitivity by interfering with the expression of target genes was therefore pursued. In this study we report that chemically modified siRNAs can enhance their silencing activities. 58–60 Thus separation of the siRNA duplex into sense and antisense strands is mediated by an ATP dependent helicase. Dependent on which end of the duplex is more accessible for the enzyme, either the desired antisense strand (unwinding from the 3'-end of the sense strand) or the unwanted sense strand (unwinding from the 5'-end of the sense strand) is predominantly incorporated into RISC. When the sense strand is incorporated, no cleavage of the targeted mRNA is observed, which explains the ineffectiveness of some siRNAs. Strand separation from the 3'-end can be facilitated by selecting for A/U base pairs in positions 15–19, as has been done for all of our bcl-2 specific siRNAs (table 1). Further improvement in siRNA design (for example, by inserting mismatches in the nt 15–19 region of the duplex or chemical variations to the phosphate backbone) might further enhance strand separation and lead to increased silencing activity. 56-58

siRNAs have been used to suppress expression of reporter genes under experimental conditions in mice. 59–61 Ours is the first report on silencing of an endogenous target gene in a solid cancer after systemic administration of unmodified siRNAs in vivo, investigating the therapeutic utility of siRNA in the treatment of these disease entities. Furthermore, we showed that siRNAs are homogenously distributed in all organs of adult mice after intraperitoneal administration followed by renal clearance and a second hepatic elimination phase. Chemical modification of siRNA duplexes can further improve tissue targeting (for example, by tagging to RGD peptides for increased cellular uptake) and in vivo stability. 56-61
more studies are needed to assess the pharmacokinetics and improve tissue specific targeting of certain genes with siRNA. Enhancing cleavage of bcl-2 mRNA by siRNA may contribute to the treatment of pancreatic cancer by improving chemotherapy sensitivity of this otherwise resistant carcinoma.

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EDITOR’S QUIZ: GI SNAPSHOT

Answer
From question on page 1262
Gastroscopy showed a bulge at the posterior wall of the fundus. The computed tomography scan identified a homogeneous cystic lesion of the spleen, 6×8 cm in diameter, of liquid density. Endoscopic ultrasound showed an anechoic cyst with a thin wall and septas. She underwent splenectomy. At histology (fig 4), the cystic wall consisted of fibrous tissue covered with a non-keratinised squamous lining. The final diagnosis was epidermoid cyst.

Non-parasitic splenic cysts (NPSC) are uncommon. The first classifications were performed by Fowler and Martin. Morgenstern proposed a new classification based on the gross appearance and type of cellular lining. Seventy five per cent of NPSC are pseudocysts with only fibrous tissue lining, with a history of trauma in most people. The remainder are true cysts with cellular lining, including epidermoid cysts, and are thought to be congenital. Non-operative treatment is recommended for small asymptomatic cysts.

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Variants of bcl-2 specific siRNA for silencing antiapoptotic bcl-2 in pancreatic cancer

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