A novel antisense oligonucleotide inhibiting several antiapoptotic Bcl-2 family members induces apoptosis and enhances chemosensitivity in androgen-independent human prostate cancer PC3 cells

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Abstract

Bcl-2 and Bcl-xL are associated with treatment resistance and progression in many cancers, including prostate cancer. The objective of this study was to determine whether a novel bispecific antisense oligonucleotide targeting both Bcl-2 and Bcl-xL induces apoptosis and enhances chemosensitivity in androgen-independent PC3 prostate cancer cells. An antisense oligonucleotide with complete sequence identity to Bcl-2 and three-base mismatches to Bcl-xL selected from five antisense oligonucleotides targeting various regions with high homology between Bcl-2 and Bcl-xL was found to be the most potent inhibitor of both Bcl-2 and Bcl-xL expression in PC3 cells. This selected Bcl-2/Bcl-xL bispecific antisense oligonucleotide reduced mRNA and protein levels in a dose-dependent manner, reducing Bcl-2 and Bcl-xL protein levels to 12% and 19%, respectively. Interestingly, Mcl-1 was down-regulated as well, although levels of Bax, Bad, or Bak were not altered after treatment with this bispecific antisense oligonucleotide. Indirect down-regulation of inhibitor of apoptosis (IAP) family, including XIAP, cIAP-1 and cIAP-2, via second mitochondria-derived activator of caspases was also observed after bispecific antisense oligonucleotide treatment. Executioner caspase-3, caspase-6, and caspase-7 were shown to be involved in apoptosis induced by bispecific antisense oligonucleotide. This Bcl-2/Bcl-xL bispecific antisense oligonucleotide also enhanced paclitaxel chemosensitivity in PC3 cells, reducing the IC50 of paclitaxel by >90%. These findings illustrate that combined suppression of antiapoptotic Bcl-2 family members using this antisense oligonucleotide could be an attractive strategy for inhibiting cancer progression through alteration of the apoptotic rheostat in androgen-independent prostate cancer.

Introduction

Prostate cancer is the most commonly diagnosed malignancy and second leading cause of cancer-related death in men in North America. Although androgen withdrawal is the only effective therapy for men with advanced disease, progression to a lethal and untreatable stage of androgen independence unfortunately occurs within a few years in almost all cases (1). Two phase III trials recently reported survival benefit in advanced hormone-refractory prostate cancer using docetaxel-based regimens (2, 3). Despite these recent promising data, androgen-independent disease remains the main obstacle to improving the survival and quality of life in patients with advanced prostate cancer, and novel therapeutic strategies targeting the molecular basis of progression to androgen independence of prostate cancer are required.

Results of numerous scientific and clinical studies link altered expression of apoptosis-regulatory proteins with the development of hormone resistance of prostate cancer (4). Among them, the Bcl-2 family of genes, which share sequence homology domains, plays a key role in the regulation of apoptotic cell death induced by a wide variety of therapeutic stimuli (5). These genes can form homodimers and/or heterodimers that modulate one another’s function, whereby their relative concentrations function as a rheostat for the apoptotic program (6). The Bcl-2 family is characterized by the presence of Bcl-2 homology domains and fall into two main groups: antiapoptotic proteins, such as Bcl-2, Bcl-xL, Bcl-w, Mcl-1, and A1, and proapoptotic proteins, such as Bax, Bak, Bad, Bid, and Bcl-xS (5). Of them, Bcl-2 and Bcl-xL have been well characterized as potential genes involved in the apoptotic pathway. The Bcl-2 gene, initially recognized
as the proto-oncogene translocated to the immunoglobulin heavy chain locus in human follicular B-cell lymphoma cells, is the prototype of a novel class of oncogenes that contributes to neoplastic progression by enhancing tumor cell survival through inhibition of apoptotic cell death (7). The Bcl-x gene encodes two proteins, a long form (Bcl-xL) and a short form (Bcl-xS), through an alternative splicing mechanism. Bcl-xL, which displays remarkable amino acid and overall structural homology to Bcl-2, effectively blocks apoptosis, whereas Bcl-xS, lacking 63 amino acids contained within Bcl-xL, is a dominant inhibitor of Bcl-2 activity and thereby acts as a proapoptotic factor (8). In prostate cancer, experimental and clinical studies link Bcl-2 and Bcl-xL to androgen independence through inhibition of apoptotic cell death. Moreover, overexpression of these genes is associated with resistance to several therapeutic stimuli, including androgen ablation, cytotoxic chemotherapies, and radiotherapy (9–13). In tumors where Bcl-2 and Bcl-xL are co-expressed, it is difficult to predict which is more biologically important for cell survival and a more relevant target for gene therapy. Furthermore, tumor cells can switch expression from Bcl-2 to Bcl-xL (14). Collectively, these findings suggest that simultaneous down-regulation of Bcl-2 and Bcl-xL genes could be an attractive approach for inhibiting progression of prostate cancer.

Antisense oligodeoxynucleotides are chemically modified stretches of ssDNA that are complementary to mRNA regions of a target gene and effectively inhibit gene expression by forming RNA/DNA duplexes (15). Off-target or nonspecific effects include immune stimulation by CpG motifs or G quartets or antisense oligonucleotide-protein interactions. Second-generation antisense oligonucleotides with a phosphorothioate backbone modified at the 2’-position of the deoxyribose show enhanced RNA-binding affinity compared with their first-generation 2’-deoxy counterparts. Moreover, owing to the central 2’-deoxy gap, these antisense compounds are still capable of activating RNase H. Recently, several antisense oligonucleotides specifically targeted against genes involved in neoplastic progression have been evaluated both in vitro and in vivo as potential therapeutic agents (4, 16–18). However, because various genes are involved in tumor progression, it is reasonable to use multitarget or combination strategies when developing anticancer regimens. In fact, combined use of antisense oligonucleotides with other compounds, such as chemotherapeutic agents, produce more potent antitumor effects in some tumor model systems compared with antisense oligonucleotide alone (19–22).

Recently, we reported the efficacy of monospecific antisense oligonucleotides targeting Bcl-2 or Bcl-xL as a potent inducer of apoptosis using several prostate cancer models (12, 21). Moreover, the 2’-methoxyethoxy gapmer antisense oligonucleotide 4625 targeting a region of high homology shared by Bcl-2 and Bcl-xL can induce apoptotic cell death in various kinds of tumor cells (22–27). In the present study, we investigated the ability of a 2’-methoxyethoxy gapmer of antisense oligonucleotide 4625 (ISIS 279228) to suppress the expression of Bcl-2 and Bcl-xL, induce apoptotic cell death, and enhance paclitaxel chemosensitivity in androgen-independent human prostate cancer PC3 cells.

### Materials and Methods

#### Tumor Cell Lines

PC3 and DU145 cells, purchased from the American Type Culture Collection (Rockville, MD), were maintained in DMEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 5% fetal bovine serum. LNCaP and LAPC-4 cells, kindly provided by Dr. Leland W.K. Cheng (Emory University, Atlanta, GA) and Dr. Rob Reuter (University of California at Los Angeles, Los Angeles, CA), respectively, were maintained in RPMI 1640 with 5% fetal bovine serum.

#### Human Prostate Tissue Microarray Preparation

Archival formalin-fixed, paraffin-embedded human prostate tumor specimens were used to construct a human prostate cancer tissue array. A total of 125 specimens was obtained from benign prostate (n = 23), hormone prostate cancer (n = 85), and bone metastasis (n = 17) tissues. The benign samples were obtained from transition zone biopsies of radical prostatectomy specimens. Two samples from each tumor specimens (diameter, 0.6 mm) were taken from preselected regions of individual paraffin-embedded donor blocks and precisely arrayed into a new recipient paraffin block with a tissue arrayer (Beecher Instrument, Silver Spring, MD). After the block construction was completed, 5-μm sections were cut with a microtome by use of an adhesive-coated tape sectioning system (Intrumedics, Hackensack, NJ) to support the adhesion of the array elements.

Mounted tissues on the slides were rehydrated, and endogenous peroxidase activity was blocked with methanol containing H2O2. Bovine serum albumin (Promega, Madison, WI) was applied for 1 hour at room temperature to block the nonspecific binding sites on the slides, which were then incubated in a humidified chamber overnight at 4°C with a 1:50 dilution (200 μg/mL) of a mouse anti-human monoclonal Bcl-2 antibody (DAKO Corp., Carpinteria, CA) and 1:200 dilution (50 μg/mL) of a mouse anti-human monoclonal Bcl-xL antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). After primary incubation, tissue was washed thrice with PBS and incubated with a horseradish peroxidase–conjugated goat anti-mouse IgG secondary antibody (DAKO) used at a dilution for 30 minutes at 25°C. The antigen was visualized by subsequent 5-minute incubation with diaminobenzidine tetrahydrochloride before counterstaining with hematoxylin. Negative control slide was processed in an identical fashion to that above, with the substitution of normal goat nonimmune serum for the primary antiserum. The staining intensity of cytoplasmic Bcl-2 and Bcl-xL was evaluated and scored by a pathologist (L.F.) and the Image Plus software (Media Cybernetics, Silver Spring, MD). Specimens were graded from 0 to +4 intensity, representing the range from no staining to heavy staining. All comparisons of staining intensity were made at ×400 magnification.
**Chemotherapeutic Agents**

Paclitaxel was purchased from Sigma Chemical Co. (St. Louis, MO). Stock solutions of paclitaxel (1 mg/mL) were diluted with PBS to the required concentrations before each in vitro experiment.

**Antisense Oligonucleotides**

The 2′-methoxyethoxy-modified gapmer antisense oligonucleotides used in this study were generously supplied by Dr. Brett P. Monia (ISIS Pharmaceuticals, Carlsbad, CA). All oligonucleotides displayed a length purity higher than 95% with a phosphodiester content lower than 0.3%. The sequences of five Bcl-2/Bcl-xL bispicific antisense oligonucleotides were as follows (italicized letters represent nucleotides with 2′-methoxyethoxy modification): ISIS 16012 5′-CCAGCGCGTTCCTCCTGGA-3′, ISIS 16013 5′-TAGAGTTCCACAAAGATATC-3′, ISIS 22782 5′-CAAAAGTATCCCCAGCGGCCG-3′, ISIS 279227 5′-AAAGTATCCCCAGCGGCCGTT-3′, and ISIS 279228 5′-AAGGATCCCGGCCTCGGTGTTTGTGGAGTTCTT-3′. ISIS 16012 is complementary to nucleotides 679 to 698 of Bcl-xL and has two mismatches to nucleotides 592 to 616 of Bcl-2. ISIS 16013 is complementary to nucleotides 699 to 718 of Bcl-xL and has three mismatches to nucleotides 617 to 636 of Bcl-2. ISIS 22782 is complementary to nucleotides 689 to 708 of Bcl-xL and has three mismatches to nucleotides 607 to 626 of Bcl-2. ISIS 279227 is complementary to nucleotides 687 to 706 of Bcl-xL and has three mismatches to nucleotides 605 to 624 of Bcl-2. ISIS 279228 is complementary to nucleotides 650 to 624 of Bcl-2 and has three mismatches to nucleotides 687 to 706 of Bcl-xL mRNA. ISIS 124565 with the sequence 5′-CAGCGCTGACAACAGTTTCAT-3′ was used as a scrambled control oligonucleotide. A BLAST search of the National Center for Biotechnology Information database revealed no homology of these sequences to other known human genes.

**Antisense Oligonucleotide Treatment of Cells**

Lipofectin, a cationic lipid (Life Technologies), was used to increase antisense oligonucleotide uptake into cells. PC3 cells were treated with various concentrations of antisense oligonucleotide after a preincubation for 30 minutes with 3 μg/mL Lipofectin in serum-free Opti-MEM (Life Technologies). Four hours after the beginning of incubation, the medium was replaced with the standard culture medium described above.

**Real-time PCR**

Total RNA was isolated from cultured cells using the acid-guanidinium thiocyanate-phenol-chloroform method. For cDNA synthesis, total RNA (2 μg) was reversetranscribed for 1 hour at 37 °C in 20-μL reactions containing 50 mmol/L Tris (pH 8.3), 75 mmol/L KCl, 3 mmol/L MgCl₂, 500 mmol/L each dATP, dGTP, dCTP, and dTTP, 7.5 mg/mL random hexamers (Promega), 10 mmol/L DTT, and 200 Molony murine leukemia virus reverse transcriptase (Life Technologies). The Applied Biosystems 7000 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA) was used for real-time PCR amplification according to the Taqman Universal PCR Master Mix protocol (28). Relative quantification of gene expression was done as described in the manual using rRNA as an internal standard and the comparative threshold cycle method. For Bcl-2 amplification, primers with the sequences 5′-CATGTGTGAGAGCCCTCCTCA-3′ and 5′-GCTGTCAAGTTACATCA-3′ were used together with a 5′-CCTGGTGACAAATCCGGCCCTGT-3′ Taqman probe. For Bcl-xL amplification, primers with the sequences 5′-TCTTGTGCTCTTACGAGG-3′ and 5′-GGTGCCATGTGGCCCTT-3′ were used together with a 5′-ACAGTGCAGCCGGAGAGAAGA-3′ Taqman probe. For Mcl-1 amplification, primers with the sequences 5′-AAGGATGGTGGAGGGTCC-3′ and 5′-GCAAAAACCCCGACGACCAT-3′ were used together with a 5′-CATGTAGAGACCTAGAAGGTGCATCAGG-3′ Taqman probe. The Bcl-2, Bcl-xL, and Mcl-1 probes were labeled at the 5′ end with the reporter dye molecule 6-carboxyfluorescein and at the 3′ end with the quencher dye molecule 6-carboxytetramethylrhodamine. A BLAST search of the National Center for Biotechnology Information database revealed no homology of the primer and probe sequences to other known human genes.

**Western Blot Analysis**

Samples containing equal amounts of protein (40 μg) from lysates of the cultured PC3 cells were electrophoresed on a SDS-polyacrylamide gel and transferred to nitrocellulose filter. The filters were blocked in PBS containing 5% nonfat milk powder for 1 hour and incubated overnight at 4°C with a 1:200 diluted anti-human Bcl-2 mouse monoclonal antibody, 1:200 diluted anti-human Bcl-xL mouse monoclonal antibody, 1:500 diluted anti-human Mcl-1 rabbit monoclonal antibody (Santa Cruz Biotechnology), 1:1,000 diluted anti-human Bax rabbit monoclonal antibody (Cell Signaling Technology, Inc., Beverly, MA), 1:100 diluted anti-human Bad mouse monoclonal antibody (Santa Cruz Biotechnology), 1:100 diluted anti-human Bak mouse monoclonal antibody (Calbiochem, San Diego, CA), 1:1,000 diluted anti-human survivin mouse monoclonal antibody, 1:1,000 diluted anti-human X-linked inhibitor of apoptosis (IAP) rabbit polyclonal antibody (Cell Signaling Technology), 1:1,000 diluted anti-human cIAP-1 rabbit polyclonal antibody, 1:500 diluted anti-human cIAP-2 rabbit polyclonal antibody (R&D Systems, Inc., Minneapolis, MN), 1:1,000 diluted anti-human second mitochondria-derived activator of caspases (Smac) rabbit polyclonal antibody (Oncogene Research Products, San Diego, CA), anti-human caspase-3 rabbit monoclonal antibody (Oncogene Research Products, San Diego, CA), anti-human caspase-7 rabbit monoclonal antibody (Oncogene Research Products, San Diego, CA), anti-human cleaved lamin A (Asp²⁵⁰) rabbit polyclonal antibody, anti-human caspase-7 rabbit monoclonal antibody (Cell Signaling Technology), and 1:2,000 diluted anti-human vinculin mouse monoclonal antibody (Sigma). The filters were then incubated for 45 minutes with horseradish peroxidase–conjugated antirabbit IgG antibody (Promega) or anti-rabbit IgG antibody (DAKO). Visualization of the immunocomplexes was done by enhanced chemiluminescence with the use of the enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Piscataway, NJ) followed by exposure to X-ray films.
Measurement of Cell Growth

Cell growth was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described previously (29). Cells were plated in 96-well plates at 4,000 per well, allowed to attach 24 hours, and treated once daily with various concentrations of antisense oligonucleotide for 2 days. After 72 hours of incubation, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent was added (40 μL/well of a 10 mg/mL solution in PBS) to each well followed by incubation for 4 hours at 37°C. The formazan crystals were then dissolved in DMSO. The absorbance was determined with a microculture plate reader (Becton Dickinson Labware, Franklin Lakes, NJ) at 570 nm. Absorbance values were normalized to the values obtained from the vehicle-treated cells to determine the percentage of survival. Each assay was done in triplicate.

Determination of Cell Viability

Cell viability was determined based on trypan blue exclusion analysis. Cells were plated in six-well plates at 100,000 per well, allowed to attach 24 hours, and treated once daily with concentration of 200 nmol/L antisense oligonucleotide for 2 days. At different time points after treatment, cells were incubated with 0.4% trypan blue in PBS for 5 minutes at room temperature, and the percentage of dead cells was calculated by counting the numbers of dead and viable cells using phase-contrast microscopy.

Measurement of Apoptosis

Apoptotic cells were identified by their subdiploid DNA content using flow cytometry analysis. Forty-eight hours after antisense oligonucleotide treatment with or without paclitaxel, PC3 cells were analyzed for DNA content as described previously (30). Briefly, cells were washed with PBS and resuspended in 250 μL of 70% ethanol. After overnight incubation at 4°C, cells were washed in PBS and incubated with 1 μg/mL RNase A (Sigma) for 30 minutes at room temperature. Cells were then resuspended in 1 mL propidium iodide staining solution (50 μg/mL propidium iodide). After incubation for 30 minutes at room temperature, cell cycle analysis was done using a dual laser flow cytometer (Beckman Coulter EPICS Elite, Beckman, Inc., Miami, FL).

Statistical Analysis

The χ² test was used to analyze differences between proportions. Other data were analyzed by Student’s t-test. The level of statistical significance was set at P < 0.05, and all statistical calculations were done using Statview 5.0 software (Abacus Concepts, Inc., Berkeley, CA).

Results

Expression of Bcl-2 and Bcl-xL in Prostate Cancer

To quantify Bcl-2 and Bcl-xL mRNA and protein levels, real-time PCR and Western blotting analyses, respectively, were done in PC3, DU145, LNCaP, and LAPC-4. Although all prostate cancer cell lines expressed Bcl-2 and Bcl-xL, their basal expression levels varied (data not shown). In PC3 cells, abundant expression of Bcl-xL mRNA was detected, whereas Bcl-2 mRNA expression is comparatively low.

Human prostate specimens from benign, primary prostate cancer with or without pretreatment with neoadjuvant hormone therapy and from hormone-refractory lesions were used to construct a tissue microarray and stained with Bcl-2 and Bcl-xL antibody. Expression of Bcl-2 was found in 7% of hormone-naive primary prostate cancer specimens and 35% of hormone-refractory lesions. Differences in the incidence of Bcl-2 expression between primary prostate cancer and

Figure 1. Expression of Bcl-2 and Bcl-xL in prostate cancer. Human prostate specimens from benign and cancer tissues before or after androgen ablation were used to construct a tissue microarray and stained with Bcl-2 and Bcl-xL antibody. Representative results from benign prostate, prostate cancer, and bone metastatic lesion are presented.
hormone-refractory lesions were significant \((P < 0.05)\). On the other hand, Bcl-xL was expressed in all prostate cancer specimens. A high Bcl-xL expression was found in 35\% of prostate cancer, and differences in Bcl-xL staining between benign prostate and prostate cancer were significant \((P < 0.05)\). Representative examples of immunohistochemical staining of benign prostatic hyperplasia, prostate cancer, and bone metastatic lesion specimens are shown in Fig. 1.

**Screening for Active Antisense Oligonucleotide Sequences Targeting the Bcl-2 and Bcl-xL Genes**

To identify potent antisense oligonucleotides capable of simultaneously inhibiting Bcl-2 and Bcl-xL gene expression in PC3 cells, five antisense oligonucleotides designed to hybridize with various regions of high homology comprising nucleotides 605 to 624 and 687 to 706 of the Bcl-2 and Bcl-xL genes, respectively, were screened. Real-time PCR and Western blot analyses were done to evaluate the effects of treatment with these antisense oligonucleotides on Bcl-2 and Bcl-xL expression in PC3 cells. The different antisense oligonucleotides exhibited varied degrees of activity (data not shown). The most potent antisense oligonucleotide identified in this series was ISIS 279228, with sequence corresponding to antisense oligonucleotide 4625, reducing Bcl-2 and Bcl-xL mRNA levels to 10\% and 30\% of baseline levels, respectively. At the protein level, ISIS 279228 also reduced Bcl-2 and Bcl-xL expression to 10\% and 20\% of baseline levels, respectively (data not shown). Based on these data, ISIS 279228 (antisense oligonucleotide 4625) was identified as the most potent Bcl-2/Bcl-xL bispecific antisense oligonucleotide and therefore used in subsequent experiments.

To further investigate whether the effects of this Bcl-2/Bcl-xL bispecific antisense oligonucleotide were dose dependent, Bcl-2 and Bcl-xL mRNA and protein levels were quantified after treatment with various doses of Bcl-2/Bcl-xL bispecific antisense oligonucleotide by real-time PCR and Western blot analyses, respectively. Dose-dependent suppression of both Bcl-2 and Bcl-xL by ISIS 279228 is illustrated in Fig. 2A and B. As shown in Fig. 2A, daily treatment of PC3 cells with ISIS 279228 (100, 500, or 1,000 nmol/L) for 2 days reduced Bcl-2 and Bcl-xL mRNA levels by 40\%, 11\%, or 10\% and 75\%, 35\%, or 32\%, respectively. Figure 2B illustrates similar dose-dependent suppression of Bcl-2 and Bcl-xL protein levels after bispecific antisense oligonucleotide treatment.

**Down-Regulation of Mcl-1 in PC3 Cells by ISIS 279228**

To investigate whether treatment of cells with Bcl-2/Bcl-xL bispecific antisense oligonucleotide alters expression levels of other Bcl-2-related proteins, Western blot analyses were done. Whereas the expression of proapoptotic genes...
Bax, Bad, and Bak was not significantly altered, bispecific antisense oligonucleotide treatment markedly decreased expression of the antiapoptotic gene Mcl-1 (Fig. 3A). These changes in Mcl-1 expression were confirmed to be dose dependent at both mRNA and protein levels (Fig. 3B and C).

**Indirect Down-Regulation of IAP Family and Activation of Caspases by ISIS 279228**

Because Bcl-2 and Bcl-xL can affect expression of IAP family members, including survivin, XIAP, cIAP-1, and cIAP-2, by preventing release of Smac from mitochondria (31), we assessed the effects of ISIS 279228 on various IAP family members. The levels of IAP family were significantly reduced (Fig. 4A), especially XIAP, cIAP-1, and cIAP-2, to <10% of controls. We next examined whether Smac is released from mitochondria into the cytoplasm after ISIS 279228 treatment. As shown in Fig. 4B, Smac was detected in the cytosolic fraction in cells treated with Bcl-2/Bcl-xL bispecific antisense oligonucleotide but not in cells treated with control oligonucleotide.

To further examine the mechanism of cell death in PC3 cells after treatment with Bcl-2/Bcl-xL bispecific antisense oligonucleotide, activation of executioner caspases (caspase-3, caspase-6, and caspase-7) was assessed by Western blot analyses. The full-length caspase-3 (35 kDa) was observed in all samples examined; however, cleaved large fragments of caspase-3 (17 and 19 kDa) were detected only after antisense oligonucleotide treatment (Fig. 4C). Lamin A is cleaved by caspase-6 and thus served as a marker for caspase-6 activation (32). After ISIS 279228 treatment, cleaved lamin A was evidently observed. Activation of caspase-7 was further confirmed by induction of cleaved caspase-7 in PC3 cells after ISIS 279228 treatment. These findings suggest the involvement of caspase-3, caspase-6, and caspase-7 in cell death induced by ISIS 279228. We further evaluated the effects of this antisense oligonucleotide on cleavage of poly(ADP-ribose) polymerase protein, a substrate of caspase activated during the process of apoptotic execution. The intact poly(ADP-ribose) polymerase (116 kDa) was observed in all samples examined; however, the 85-kDa poly(ADP-ribose) polymerase cleavage fragment was detected only after treatment with ISIS 279228 (Fig. 4C).

**Effect of ISIS 279228 on PC3 Cell Growth and Viability**

Figure 5A shows that treatment of PC3 cells with ISIS 279228 results in a dose-dependent inhibition of cell growth. The control oligonucleotide also revealed a certain degree of cytotoxicity; however, it was considerably less than that of ISIS 279228. To determine whether inhibition of cell growth by ISIS 279228 was due to increased cell death,
a trypan blue exclusion assay was done. As shown in Fig. 5B, ISIS 279228 produced a time-dependent increase in cell death. In contrast, little cell death was observed using control oligonucleotides at this dose. ISIS 279228 also enhanced paclitaxel chemosensitivity, reducing the IC50 of paclitaxel by >90% from 100 to <10 nmol/L (Fig. 5C). Flow cytometric analyses indicated increased fraction of cells (sub-G0-G1) undergoing apoptosis after ISIS 279228 compared with controls with or without paclitaxel (P < 0.01). ISIS 279228 increased the apoptotic fraction from 14.9% to 39.1% without paclitaxel and from 32.6% to 59.3% with paclitaxel (data not shown).

**Discussion**

Close association between increased expression of the antiapoptotic genes Bcl-2 and Bcl-xL and progression of many malignant tumors is frequently observed. Although the relative expression levels of Bcl-2 and Bcl-xL vary depending on the type and stage of the tumor, accumulating evidence shows that Bcl-2 and Bcl-xL overexpression protects cancer cells from apoptosis induced by several types of therapies, thereby accelerating disease progression. Although Bcl-2 and Bcl-xL are considered to be functionally indistinguishable, evidence supports distinct biological roles of these proteins for protection from apoptosis induced by different cytotoxic stimuli (33, 34). In the present study, the human prostate cancer cell lines PC3, DU145, LNCaP, and LAPC-4 express different amounts of these two antiapoptotic proteins. In addition, we showed that Bcl-2 levels were significantly higher in hormone-refractory prostate cancer compared with hormone-naive primary prostate cancers, and the incidence of high staining intensity of Bcl-xL in prostate cancer was significantly higher than that in benign prostate. In tumors where Bcl-2 and Bcl-xL are coexpressed, it is difficult to predict which of the two proteins is more critical for survival; moreover, some types of tumor cells are able to switch expression from Bcl-2 to Bcl-xL (14, 35).

The limited efficacy of cytotoxic chemotherapy remains a major obstacle for the treatment of patients with advanced hormone-refractory prostate cancer. Various reasons, including inherent chemoresistance, pharmaceutical mechanism of chemotherapeutic action, and inability of elderly patients to tolerate its toxicity, contribute to modest activity of conventional cytotoxic chemotherapy in these patients. Several previous studies suggest that the chemoresistant phenotype in hormone-refractory prostate cancer is attributable, in part, to high levels of antiapoptotic genes, including Bcl-2, Bcl-xL, and clusterin, all of which are increased after androgen ablation and remain constitutively overexpressed in androgen-independent tumors (36–38). Therapeutic strategies that target the molecular basis of chemoresistance of prostate cancer may improve survival. Recent preclinical studies provided proof-of-principle evidence that targeting antiapoptotic genes using antisense
oligonucleotide can enhance apoptosis induced by conventional cytotoxic chemotherapy (4, 20, 21), whereas a phase I study reported that combining an antisense Bcl-2 oligonucleotide (Genasense) plus mitoxantrone in patients with metastatic hormone-refractory prostate cancer was well tolerated (39). A recently reported phase II trial in men with metastatic hormone-refractory prostate cancer combined G3139 with docetaxel, and responses were noted in 4 of 12 (33%) patients with measurable disease and a >50% reduction in prostate-specific antigen was measured in 15 of 27 (56%) patients (40). The current status of Genasense in prostate and other cancers was recently reviewed (41).

In this study, we set out to screen for potent and sequence-specific antisense oligonucleotides targeting both human Bcl-2 and Bcl-xL genes. The ability to simultaneously down-regulate Bcl-2 and Bcl-xL expression may have a broader applicability in cancer therapy than do Bcl-2 and Bcl-xL monospecific oligonucleotides. An antisense oligonucleotide with complete sequence identity to Bcl-2 and three-base mismatches to Bcl-xL was found to be the most potent inhibitor of the five antisense oligonucleotides targeting various regions of both Bcl-2 and Bcl-xL genes in PC3 cells. This sequence corresponds to Bcl-2/Bcl-xL bispecific antisense oligonucleotide 4625 designed by Zangemeister-Wittke et al. (22). A first-generation phosphorothioate antisense oligonucleotide 4625 is a potent inducer of apoptosis in several neoplastic cell types, including human lung cancer (22), breast carcinoma (23, 24), colorectal carcinoma (23), melanoma (25), and glioblastoma (26). To further evaluate the functional role of Bcl-2 and Bcl-xL in hormone-refractory prostate cancer, we assessed the apoptotic effects of this Bcl-2/Bcl-xL bispecific antisense oligonucleotide in PC3 cells.

ISIS 279228 is the identical sequence to antisense oligonucleotide 4625 but incorporates a second-generation antisense oligonucleotide backbone, modified at its 3' and 5' ends by a 2'-methoxyethoxy. In this study, the ISIS 279228 inhibited Bcl-2 and Bcl-xL expression in PC3 cells in a dose-dependent manner with significant reduction in cell growth. Flow cytometry analysis indicated that reduced cell numbers after Bcl-2/Bcl-xL bispecific antisense oligonucleotide treatment resulted mainly from increased apoptotic cell death.

Simultaneous down-regulation of both Bcl-2 and Bcl-xL protein expression by a single antisense oligonucleotide has been accomplished by taking advantage of the low stringency requirements of RNase H. Interestingly, Mcl-1 was also down-regulated in a dose-dependent manner, whereas levels of Bax, Bad, and Bak were not altered significantly. Mcl-1 is a potent antiapoptotic gene and
possesses Bcl-2 homology domains (BH1–BH3) like other Bcl-2 family members (42, 43). Furthermore, the intracellular distribution of Mcl-1 seems to be more widespread than Bcl-2, as Mcl-1 is also distributed over a variety of nonmitochondrial compartments in addition to its predominant localization on mitochondrial membranes (44). Immunocytochemical studies by Krajewski et al. (45) showed that Mcl-1 expression was highly expressed and correlated with Gleason score and the presence of metastases in prostate cancer. These findings suggest that Mcl-1 may be important in preventing cell death in prostate cancer as well. Accordingly, an antisense oligonucleotide that inhibits not only Bcl-2 and Bcl-xL but also Mcl-1 expression may be more preferable as treatment for prostate cancer.

Recent studies have reported that Bcl-2 and Bcl-xL inhibit the release of both cytochrome c and Smac, both of which are closely associated with induction of apoptosis (31). Smac is normally localized to mitochondria but, like cytochrome c, is released into the cytosol during the early stages of apoptosis, where it seems to promote caspase activity by inhibiting IAP family genes. Therefore, by inhibiting the release of Smac, Bcl-2 and Bcl-xL can prevent the inactivation of IAP family genes and inhibit cell death. IAP family genes inhibit the activation of caspase-3, caspase-7, and caspase-9 and thereby contribute to apoptotic resistance in prostate cancer (46). In the present study, we showed that IAP family genes were indirectly down-regulated by Bcl-2/Bcl-xL bispecific antisense oligonucleotide treatment via Smac, leading to activation of caspases and subsequent apoptosis.

With the recent survival advantage of docetaxel-based regimens in hormone-refractory prostate cancer (2, 3), future strategies to improve survival would aim to enhance sensitivity to conventional chemotherapy by reduction of anti-apoptotic gene-mediated chemoresistance. In the present study, we showed that Bcl-2/Bcl-xL bispecific antisense oligonucleotide enhanced paclitaxel chemosensitivity in PC3 cells, reducing the IC50 of paclitaxel by >90% through enhanced induction of apoptotic cell death. Collectively, these findings support the evaluation of combined treatment regimens using Bcl-2/Bcl-xL bispecific antisense oligonucleotide and with taxane chemotherapy for prostate cancer.

In conclusion, Bcl-2/Bcl-xL bispecific antisense oligonucleotide treatment results in effective induction of apoptotic cell death in androgen-independent human prostate cancer PC3 cells. This results, at least in part, by direct suppression of three potential antiapoptotic genes, Bcl-2, Bcl-xL, and Mcl-1, as well as indirect inactivation of IAP family genes, survivin, XIAP, cIAP-1, and cIAP-2, via Smac. Moreover, sensitivity of PC3 cells to paclitaxel is significantly enhanced by Bcl-2/Bcl-xL bispecific antisense oligonucleotide treatment, providing additional preclinical support for this combined regimen for patients with hormone-refractory prostate cancer.

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A novel antisense oligonucleotide inhibiting several antiapoptotic Bcl-2 family members induces apoptosis and enhances chemosensitivity in androgen-independent human prostate cancer PC3 cells

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