Bcl-xL in Prostate Cancer Cells: Effects of Overexpression and Down-Regulation on Chemosensitivity

Irina Lebedeva, Robert Rando, Joshua Ojwang, Paul Cossum, and C. A. Stein

Abstract

Both Bcl-xL and Bcl-2, antiapoptotic members of the Bcl family, are found in prostate cancer cell lines. Although these proteins may have similar antiapoptotic functions, it is not clear to what extent each serves as an antiapoptotic effector in prostate cancer cells. We engineered LNCaP and PC-3 cells to overexpress Bcl-xL protein and demonstrated that this desensitized them to the effects of cytotoxic chemotherapy. We then used two “antisense” strategies to down-regulate Bcl-xL protein expression in the parental lines. The first strategy used C5-propynylated phosphorothioate-phosphodiester oligonucleotides and co-down-regulated both Bcl-xL and Bcl-2; the second strategy used isosequential “gap-mer” phosphorothioate-oligonucleotides containing 2′-O-methyl oligoribonucleotides at the 3′ and 5′ termini. In this case, only Bcl-xL protein expression was affected. The most active oligonucleotides of both types decreased the level of Bcl-xL protein expression to 5–30% of the control level. Multiple controls were inactive. Experiments combining oligonucleotide treatment with cytotoxic chemotherapeutic agents (paclitaxel, docetaxel, etoposide, vinblastine, carboplatin, and mitoxantrone) demonstrated a marked increase in the sensitivity of these prostate cancer cells. However, the increase in chemosensitivity in PC-3 cells was statistically identical (except for mitoxantrone) for both “antisense” strategies, indicating that basal expression of Bcl-2, in contrast to that of Bcl-xL, may play little cytoprotective role in these cells.

Introduction

Several pathways have been described that regulate programmed cell death (apoptosis). One of these implicates Bcl-2 family proteins as critical in apoptosis commitment. The Bcl-2 family includes several homologous proteins that may be either pro- or antiapoptotic. Bcl-2 and Bcl-xL are antiapoptotic, whereas Bax promotes apoptosis. Together, these proteins form a complex network of heterodimers and homodimers that govern the relative sensitivity of a cell to potentially apoptotic stimuli, such as cytotoxic chemotherapy.

Bcl-x is a relatively new member of the Bcl-2 family of apoptosis-related proteins, and its expression has been detected in a range of normal tissues, particularly in the central nervous system and thymus. Immunocytochemical studies have demonstrated that the Bcl-x protein can be detected in numerous tumor cell lines as well. Expression has been found in the epithelial cells of the normal prostate (1), where it is speculated that it contributes to the hormone-dependent control of programmed cell death. Subsequently, Krajewski et al. (2) found that 64 of 64 (100%) cases of adenocarcinoma of the prostate stained positively for Bcl-x protein. Staining intensity seemed to be correlated with increasing Gleason score and the presence of metastases. Because Bcl-xS, a proapoptotic protein, appeared not to be expressed either in prostatic tumors or in prostate cell lines in tissue culture, this staining most likely originated solely from the Bcl-xL protein. Similarly, Bcl-2 expression is also found quite extensively in primary prostate cancer specimens (3), although it has been observed in only 33% of samples of prostate tumors obtained from the bone marrow of patients with hormone-refractory disease (4). At the present time, it appears that although Bcl-xL and Bcl-2 may have similar antiapoptotic functions, it is not clear why both are co-expressed in at least some prostate cancer cells, although it is possible that overexpression of Bcl-xL and Bcl-2 protein might be factors enabling prostate cancer cells to survive in an androgen-deprived environment (5). Forced overexpression of Bcl-2 has been used in many experiments to demonstrate chemodensitization of prostate cancer cells (5–7). However, it is unclear whether the function of Bcl-2 at forcibly overexpressed intracellular levels bears a resemblance to its basal function.

Both Bcl-2 and Bcl-xL may prevent apoptosis by similar mechanisms. Bcl-xL (like Bcl-2) clearly acts to regulate mitochondrial membrane potential and volume (8) and can block the apoptosis-inducing release of cytochrome c and apoptosis inhibitory factor into the cytoplasm (8–13). In addition, Bcl-2 and Bcl-xL also may suppress apoptosis in a cytochrome c-independent manner (14), perhaps because of their ability to inhibit cytoxin-induced caspase-3 activity and subsequent poly(ADP-ribose) polymerase cleavage and lamin B1 degradation (15, 16).

The $M_r$ 25,600 Bcl-xL protein has been shown in a number of cell lines to be a potent protector of cellular apoptosis induced by aneuploid agents (9). When transferred into the murine interleukin 3-dependent prolymphocytic line FL5.12, Bcl-xL greatly reduced the proapoptotic effects of bleomycin, cisplatin, etoposide, vincristine, and doxorubicin (17). In U937 cells, inhibitory effects on camptothecin-induced apoptosis were shown to be dependent on the intracellular concentration of Bcl-xL protein. Diminution of cell death in response to etoposide, vinblastine, paclitaxel, and cisplatin were also observed (18, 19). Bcl-xL can also block cellular apoptosis in settings in which Bcl-2 is ineffectual (20). Furthermore, several agents, such as butyrate in human fibroblasts (21) and paclitaxel in LNCaP and PC-3 human prostate cancer cells (22), induce apoptosis that can be correlated with substantial decreases in the expression of Bcl-xL protein, and Bcl-xL protein expression, but not Bcl-2 or Bax expression, has been shown to decrease prior to the onset of apoptosis in CTLL-2 cells (23, 24). Finally, intracellular cleavage of Bcl-xL by caspase-3-like proteases has been associated with increased rates of cell death (23).

In this work, we demonstrate that an increase in Bcl-xL expression in LNCaP and PC-3 prostate cancer cells desensitizes them to the effects of cytotoxic chemotherapy. We used the directed oligonucleotide (“antisense”) biotechnology to down-regulate the expression of Bcl-xL in these cells. Our results demonstrate that the sensitivity of prostate cancer cells to a wide variety of cytotoxic agents can be markedly increased by this strategy. On the basis of the use of the two antisense strategies, this report suggests that basal Bcl-2 protein expression may not play a major role in the antiapoptotic phenotype in prostate cancer cell lines when treated with cytotoxic agents.

Materials and Methods

Reagents. Paclitaxel, etoposide, and carboplatin are products of Bristol-Myers Squibb (Princeton, NJ). Docetaxel is a product of Rhone-Poulenc Rorer (Collegeville, PA). Mitoxantrone was from Immunex (Seattle, WA), and

Received 1/21/00; accepted 9/1/00.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom requests for reprints should be addressed, at Columbia University, 630 West 168 Street, New York, NY 10032. Phone: (212) 305-3606; Fax: (212) 305-7348; E-mail: stein@cuccfa.ccc.columbia.edu.
vinblastine was from Eli Lilly (Indianapolis, IN). Bacterial lipopolysaccharide was from Sigma Chemical Co. (St. Louis, MO).

Cell Culture and Transformation of Cell Lines with Bcl-xL Plasmid. Human PC-3 and LNCaP prostate cancer cell lines were obtained from American Type Culture Collection (Rockville, MD). They were grown in RPMI 1640 (Life Technologies, Inc., Grand Island, NY), containing 10% (v/v) heat inactivated (56°C) fetal bovine serum FBS2 (Life Technologies, Inc., Grand Island, NY), supplemented by 1% nonessential amino acids, 1% pyruvate, 100 units/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate. All cell lines were cultured in 5% CO₂ atmosphere.

LNCaP cells were transfected with the neomycin-selectable pSFFV/Bcl-xL plasmid (obtained from Dr. Stanley Korsmeyer, Washington University School of Medicine, St. Louis, MO) or with a control, neomycin-resistant expression vector pSFFV. Aliquots containing 10 μg of plasmid and 5 μg of Lipofectamine (Life Technologies, Inc., Gaithersburg, MD) in serum-free OPTI-MEM (Life Technologies, Inc.) were added to cultured cells. The transfection medium was replaced 4 h later with RPMI-10% FBS medium. Individual colonies were selected from these plates after approximately 3–4 weeks of routine maintenance in RPMI-10% FBS containing 0.6 mg/ml G418 sulfate (Geneticin; Life Technologies, Inc.). The Bcl-xL-transformed LNCaP cells were cultured in RPMI-10% FBS, supplemented with 0.3 mg/ml G418 sulfate in 5% CO₂ atmosphere.

Oligonucleotides. In the present study, we have used a directed oligonucleotide (“antisense”) strategy to down-regulate Bcl-xL protein expression in prostate cancer cell lines. To maximize efficacy, we initially screened 20 different oligonucleotide sequences (10 20-mers and 10 18-mers) complementary to the Bcl-xL mRNA. Because at the present time no computer algorithm has sufficient predictive power of activity, sequences were randomly chosen and are shown in Fig. 1. To reduce nonsequence specificity, phosphorothioate linkages were used. Phosphodiester linkages were retained 5’ to each pyrimidine. Phosphodiester linkages were retained 5’ to each pyrimidine, because in this setting they are almost as nuclease resistant as a phosphorothioate linkage (25, 26). Isosequential oligonucleotides were also synthesized with the C5-propyne modification. Oligonucleotides in Fig. 1 with even numbers have unmodified heterocyclic bases, whereas those with odd numbers contained a C-propynyl modification at each pyrimidine.

Oligonucleotides were screened for their antisense activity to down-regulate Bcl-xL protein expression using Western blot analysis. All oligonucleotides were prepared and purified as described previously (27) and were at least 95% full-length when analyzed by reverse-phase high-pressure liquid chromatography.

Treatment of Cells with Oligonucleotide-Porphyrin Complexes. LNCaP and PC-3 prostate cells were seeded the day before the experiment in six-well plates at a density of 3 x 10⁴ cells/well to be 80–90% confluent on the day of the experiment.

Western Blot Analysis. Cells treated with oligonucleotide-porphyrin complex were washed twice with cold PBS and lysed on ice for 30 min in 100 μl of cold RIPA buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% SDS, 1% NP40, and 0.5% sodium deoxycholate] with freshly added 0.1 mg/ml phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 1 mg/ml aprotinin. Cell debris was removed by centrifugation at 14,000 x g for 10 min at 4°C. Protein concentrations were determined using the Bio-Rad protein assay system (Bio-Rad Laboratories, Richmond, CA).

Aliquots of cell extracts containing 20–50 mg of total protein were resolved in 12% SDS-PAGE and transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA). Filters were blocked for 1 h at room temperature in Blotto A [5% nonfat milk powder in TBS-T: 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% Tween 20] and then incubated for 1 h at room temperature in Blotto B containing a 1:200 dilution of either rabbit anti-Bcl-xL, anti-Bax, or anti-Bak polyclonal antibodies (all from Santa Cruz Biotechnology Inc., Santa Cruz, CA). After washing in TBS-T buffer (three times for 5 min each time, at room temperature), filters were incubated for 45 min at room temperature in Blotto B containing a 1:10,000 dilution of corresponding peroxidase-conjugated, antirabbit secondary antibody (Amer- sham, Arlington Heights, IL). To detect Bcl-2, a 1:500 dilution of an anti-Bcl-2 mouse mAb (Dako, Carpinteria, CA) was added in 1% BSA/TBS. After washing in TBS-T buffer (three times for 5 min each time, at room tempera-
tute), filters were incubated for 45 min at room temperature in 1% BSA/TBS buffer containing a 1:3,000 dilution of peroxidase-conjugated, antinouse secondary antibody (Amersham). After washing in TBS-T, enhanced chemiluminescence was performed according the recommendation of the manufacturer. Bcl-xL protein expression, as determined by Western blot analysis, was quantitatively analyzed via laser-scanning densitometry using NIH Image Version 1.61 software. All results were calculated as a percentage of Bcl-xL protein expression in treated versus untreated cells.

RNA Isolation and Northern Blot Analysis. Total RNA was isolated from 5 x 10^6 cells using an RNaseq Mini kit from Qiagen (Santa Clarita, CA). Forty μg of total RNA were electrophoresed on a 1.2% denaturing agarose gel containing formaldehyde, transferred to Nytran-Plus nylon membranes (Schleicher & Schuell, Keene, NH), and covalently bound to a membrane by UV irradiation. The 0.5-kbp GAPDH probe was PCR generated. The Bcl-xL (Schleicher & Schuell, Keene, NH), and covalently bound to a membrane by containing formaldehyde, transferred to Nytran-Plus nylon membranes

The cytotoxicity of the combinations of drugs with oligonucleotides was determined by measurement of cell viability by use of the MTT assay. LNCaP or PC-3 cells were seeded in 96-well tissue culture plates and treated the next day with oligonucleotides and drugs at the indicated concentrations as described in “Results.” Drug-containing medium was then removed, and the cells were washed once with PBS. RPMI/10% FCS containing 0.5 mg/ml MTT (Sigma) was added to each well. The cells were incubated at 37°C for 4 h and then an equal amount of solubilization solution (0.04N HCl in isopropyl alcohol) was added to each well and mixed thoroughly to dissolve the crystals of MTT formazan. After all of the crystals were dissolved, the plates were read on a Dynatech MR600 Microplate Reader at 540 nm. Statistical analysis of the results of the MTT experiments was performed using the Analysis ToolPack provided by Microsoft Excel. A Student two-sample t test, assuming unequal variances, was used to determine the equality of the means of two samples. The confidence level (α) was 0.05.

RESULTS

Increased Expression of Bcl-xL in LNCaP and PC-3 Cells Desensitizes Them to Cytotoxic Chemotherapy. Stable, Bcl-xL-overexpressing LNCaP and PC-3 cells were constructed as described above. As shown in Fig. 2A, the LNCaP-Bcl-xL line expresses ~15-fold more Bcl-xL protein than does the empty-vector LNCaP/neo. Similarly, PC-3-Bcl-xL cells express 4–5-fold more Bcl-xL protein than PC-3/neo cells. In neither cell line were levels of Bcl-2 or Bax proteins altered (data not shown). The effects of Bcl-xL overexpression on the sensitivity of these cell lines to cytotoxic chemotherapeutic agents is shown in Fig. 2B. MTT determinations were performed after 48 h of continuous treatment with the cytotoxic agents. Under these conditions, cellular viability after treatment with either paclitaxel or mitoxantrone (chosen because both are clinically active) is decreased by approximately 40–50% in the overexpressing cell line versus either the wild-type cells or the LNCaP/neo cells. This decrease is statistically significant (P < 10^-2). It should be noted that at concentrations of paclitaxel >100 nM, Bcl-xL-dependent chemosensitization was no longer observed because the drug was equally toxic to the overexpressing, neo-transfected, and wild-type cells.

Statistically significant (P < 10^-2) Bcl-xL-dependent chemosensitization was also observed in the PC-3/Bcl-xL cells versus the PC-3 wild-type and PC-3-neo cells (Fig. 2C; MTT assays were performed after 72 h of continuous drug treatment). Taken together, these data strongly suggest that Bcl-xL impacts greatly on the chemosensitivity of prostate cancer cells in tissue culture.

Down-Regulation of Bcl-xL Expression by Chimeric, C5-Pro- pynlylated Oligonucleotides. Because of unacceptable cytotoxicity, we could not use cationic lipids, such as Lipofectin, as delivery agents...
in prostate cancer cells. Instead, we used two cationic porphyrins, TMP and TAP, to deliver the oligonucleotides (27). For unclear reasons, however, the porphyrin carrier required for maximal antisense activity was prostate cancer cell-line dependent. For LNCaP cells, only TAP but not TMP was effective, whereas the converse was true for PC-3 cells, where only TMP was active. For LNCaP cells, optimally active complexes were formed at concentrations of 3 μM TAP/1 μM oligonucleotide; for PC-3 cells, 7 μM TMP/2 μM oligonucleotide was required.

Down-regulation of the expression of Bcl-xL protein by C5-propyne-modified chimeric oligonucleotides was demonstrated by Western blotting, as shown in Fig. 3A. A $M_r$ 19,000 band that would correspond to the Bcl-xS protein could not be detected in either of these cell lines. Equal loading of protein samples was achieved by measurement of protein concentrations by the Bradford assay (Bio-Rad Laboratories). In some cases, equal protein loading was verified by examination of actin expression. These results correlated exactly with protein concentration determination. In LNCaP cells, the most active sequences were 29, 41, 57, and 61. Other oligomers presented in Fig. 3A, such as 33, 45, 38, and 53 presented here, showed little or no activity. The full spectrum of oligonucleotide activity (or lack thereof) is shown in Fig. 3B (LNCaP cells) and Fig. 3C (PC-3 cells). In PC-3 cells, optimal results were obtained with 41, and 29 and 61 were also active. In general, oligomers not containing C5-propynylated pyrimidine residues were not as active as the substituted molecules. Compared with the untreated cells or cells treated with inactive oligonucleotides, the most active oligonucleotides (Figs. 4 and 5) consistently decreased the level of Bcl-xL expression to 5–30% of the control level. Oligonucleotide treatment also did not cause any reduction in the total amount of cellular protein, nor, as demonstrated by MTT assay, did it inhibit cellular growth.

We then evaluated Bcl-xL mRNA expression in treated and untreated cells by Northern analysis, using a full-length Bcl-xL cDNA fragment as a probe. Additional Western blots for the most active oligomers are presented in Fig. 4, along with the corresponding Northern blots. Down-regulation of bcl-x mRNA levels occurs congruently with down-regulation of Bcl-xL protein expression, thus supporting an RNase H-based mechanism of action. In LNCaP, 41 is perhaps the most active oligomer, with both 29 and 41 being highly active in PC-3 cells.

**Antisense Bcl-xL Oligonucleotides Also Down-Regulate Bcl-2 but not Bax or Bak.** The ability of cell to undergo apoptosis is determined by the ratio between anti- and proapoptotic members of the Bcl-2 family (28). We therefore investigated whether treatment of cells with antisense Bcl-xL oligonucleotides affects these Bcl-xL-related proteins (Fig. 6). Oligonucleotides 29, 41, and 43, when delivered with TMP to PC-3 cells, caused down-regulation of both antiapoptotic proteins Bcl-xL and Bcl-2 but appeared to up-regulate Bax protein expression. Bak protein expression, however, was not changed after treatment. In LNCaP cells, however, the diminution in Bcl-2 expression after treatment with 41 was not as great as in PC-3 cells, but neither Bax nor Bak protein expression was affected. Table 1 presents the results on Bcl-xL, Bcl-2, and Bax protein expression after treatment of LNCaP and PC-3 cells with the most active C5-propynylated oligonucleotides. It should be noted that in several instances (e.g., with oligomers 57 and 61), although Bcl-xL and Bcl-2 expression decreased, Bax expression also decreased. Additional evidence for the co-down-regulation of Bcl-2 and Bcl-xL in LNCaP and PC-3 cells by 41 is provided in Fig. 6. Western blots for Bcl-2 and Bcl-xL demonstrate the previously demonstrated down-regulation by 41; a Northern blot demonstrates that Bcl-2 mRNA expression is down-regulated as well, whereas the expression of GAPDH mRNA is unchanged.

**Down-Regulation of Bcl-xL (and Bcl-2) Increases Sensitivity of PC-3 Prostate Cancer Cells to Multiple Chemotherapeutic Agents.** As shown in Fig. 7, after 3 days of drug treatment which followed 5 h of antisense oligonucleotide treatment, cell viability was significantly decreased, as determined by MTT assay. (It should be kept in mind that the active oligonucleotides also down-regulate Bcl-2 expression.) For the most active oligomer, 41, MTT expression (compared with drug-only treated cells; for all experiments, $n = 5$) in the presence of 0.1 μM mitoxantrone was decreased 39.8 ± 4.3% ($P < 10^{-10}$); in the presence of 25 nm paclitaxel, 34 ± 6.2% ($P < 10^{-8}$); in the presence of 50 nm Taxotere, by 33.1 ± 4.8% ($P < 10^{-2}$); in the presence of 10 μM carbolipin, by 47 ± 7.2% ($P < 10^{-7}$); and by 49.9 ± 2.0% in the presence of etoposide ($P < 10^{-7}$). Oligomers 57 and 61, also highly statistically significant ($P < 10^{-7}$), increased sensitivity to these cytotoxic agents but did not
demonstrate equal loading of RNA.

active oligonucleotides are shown. Northern blots of GAPDH mRNA are shown to tend to be as active as antibodies and with anti-Bcl-2 mAb. performed as described in the text, using polyclonal anti-Bcl-x, anti-Bax, and anti-Bak total protein was harvested. Western blots of 20 g of total protein and mRNA were harvested 20 h later. Bcl-xL and Bcl-2 Western and Northern blots are prepared as described in the text. Total protein and mRNA were harvested 20 h later. Western blotting was performed with a polyclonal anti-Bcl-x antibody and with an anti-Bcl-2 mAb and with anti-Bak antibodies and with anti-Bcl-2 mAb.

northern analysis did not demonstrate any decrease in the expression of Bcl-xL mRNA, even by the most active oligomers (Fig. 9). It is thus very unlikely that the down-regulation of Bcl-xL protein expression by 41m is Rnase H dependent. Nevertheless, treatment of both LNCaP and PC-3 cells with the most active oligomers (41m for LNCaP and 41m or 57m for PC-3 cells) led to significant chemo-sensitization (P < 10^{-4}) to a large number of cytotoxic agents when compared with cells treated only with drugs (Fig. 10). These agents included docetaxel (25 nm; 25.4 ± 2.9%, P < 10^{-5}), paclitaxel (25 nm; 46.9 ± 4.3%, P < 10^{-4}), vinblastine (1 μM; 70.6 ± 4.6%, P < 10^{-5}), etoposide (10 μM; 47.2 ± 1.9%, P < 10^{-3}), and carboplatin (10 μM; 47.9 ± 11.3%, P = 10^{-3}) in PC-3 cells. 41mut and 57mut, which contain four mutated bases, were ineffective (i.e., identical to control). Similar, statistically significant (P < 10^{-4}) sensitization to taxanexes, velban and mitoxantrone, were also observed in LNCaP cells with 41m (10 nm paclitaxel, 33.5 ± 4.4%; 20 nm docetaxel, 43.7 ± 5.7%; 1 μM mitoxantrone, 49.7 ± 4.2%; 1 μM carboplatin, 47.9 ± 11.3%).

Fig. 6. Possible irrelevant cleavage of the Bcl-2 mRNA by active C5-propyne-modified oligonucleotides in prostate cancer cell lines. LNCaP and PC-3 cells were treated and prepared as described in the text. Total protein and mRNA were harvested 20 h later. Western blotting was performed with a polyclonal anti-Bcl-x antibody and with an anti-Bcl-2 mAb as described in "Materials and Methods." Bcl-xL, Bcl-2, and GAPDH mRNA levels were determined by Northern blotting with Bcl-xL and Bcl-2 cDNA and GAPDH PCR 32P-labeled probes. Bcl-xL and Bcl-2 Western and Northern blots are shown for 41, the most active oligonucleotide. GAPDH mRNA Northern blot demonstrates equal loading of RNA.

Table 1 Regulation of Bcl family protein expression in prostate cancer cell lines by the most active C5-propyne chimeric phosphodiester-phosphorothioate oligodeoxynucleotides

<table>
<thead>
<tr>
<th>PC-3 cell line</th>
<th>2 μm C5-propyne oligo, 7 μm TAP</th>
<th>LNCaP Cell Line</th>
<th>1 μm C5-propyne oligo, 3 μm TAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcl-xL</td>
<td>Bax</td>
<td>Bcl-2</td>
<td>Bcl-xL</td>
</tr>
<tr>
<td>29</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>41</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>43</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>57</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>61</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
vinblastine, 46.5 ± 5.4%), whereas 41mut was ineffective. In contrast, PC-3 cells were not sensitized to the cytotoxic effects of all-trans-retinoic acid after treatment with 41m.

**DISCUSSION**

A major function of Bcl-xL and Bcl-2 is to protect mitochondrial function and to prevent release from the mitochondrion of the proapoptotic factors, apoptosis inhibitory factor and cytochrome c (28). Overexpression of Bcl-xL or Bcl-2 in diverse cell lines reduces the release of these factors (8, 11, 12) in response to various apoptosis effector molecules and desensitizes them to the effects of cytotoxic chemotherapy. Similarly, as demonstrated here, LNCaP and PC-3 prostate cancer cells engineered to overexpress Bcl-xL protein are desensitized to the growth-suppressive effects of cytotoxic agents.

Both LNCaP and PC-3 cells endogenously express Bcl-2 protein (29, 30). Although Bcl-2 and Bcl-xL appear to “repress a common pathway of cell death” (31) and in certain circumstances appear to be functionally equivalent (32, 33), some evidence indicates that under other circumstances, their sites of action may be distinct. For example, in clones of FL5.12 lymphoid cells containing approximately equal amounts of both Bcl-2 and Bcl-xL protein, the latter provided significantly greater protection against etoposide and teniposide, and against three S-phase agents including methotrexate, 5-fluorouracil, and hydroxyurea (34). In WEHI-231.7 JM lymphoid cells overexpressing Bcl-xL, the apoptotic response to cyclosporin A, FK-506, and rapamycin was blocked (20). In contrast, apoptosis was not blocked when this line was engineered to overexpress Bcl-2. In addition, Bcl-xL, but not Bcl-2, could protect MCF-7 cells by preventing ceramide accumulation induced either by tumor necrosis factor-α or camptothecin (35).

Previous experiments have also demonstrated that down-regulation of Bcl-xL expression can result in chemosensitization in at least some cell types. 20-mer antisense phosphorothioate oligonucleotides (0.45 μM) delivered by the lipid N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammoniummethyl sulfate to eosinophils blocked granulocyte/macrophage-colony stimulating factor or interleukin 5-induced Bcl-xL up-regulation and inhibited the
interaction (maximize the yield of active oligonucleotides, we improved the and target each of these fragments individually. Subsequently, to divide the targeted mRNA sequence into a series of 18–20-mers to find active antisense oligonucleotide sequences is to manually these are not critical proteins for basal viability of these cells.

Bcl-2) expression has been dramatically reduced indicates that significant apoptosis in prostate cancer cells in which Bcl-xL (and 524–530, respectively. Of course, for any oligonucleotide, the sensitivity of the cells to staurosporine and ceramide were also increased, which is an interesting observation in light of the data of El-Assaad et al. (35) that Bcl-xL does not appear to protect MCF-7 breast cancer cells from ceramide-induced apoptosis. The lack of significant apoptosis in prostate cancer cells in which Bcl-xL (and Bcl-2) expression has been dramatically reduced indicates that these are not critical proteins for basal viability of these cells.

It has been demonstrated (37–39) that the best way at this time to find active antisense oligonucleotide sequences is to manually divide the targeted mRNA sequence into a series of 18–20-mers and target each of these fragments individually. Subsequently, to maximize the yield of active oligonucleotides, we improved the interaction (i.e., raised the $T_m$) between the oligomer and the target by using a C5-propyne substituent at each pyrimidine moiety (40–42). This modification promotes oligonucleotide base stacking (42), which appears to favor duplex formation. As expected, in the absence of this modification, the extent of antisense down-regulation decreased even for the most active species. Furthermore, to decrease the nonsequence specificity, which is such a prominent feature of the behavior of phosphorothioate linkages (43, 44), we retained phosphodiester linkages 5’ to each purine residue, at which location they are resistant to nuclease (25, 26), as opposed to those 5’ to pyrimidine, which are highly sensitive. However, the C5-propyne-modified all-phosphorothioate 41 down-regulated Bcl-xL protein expression with identical efficacy as the chimeric 41. Because the effects on Bcl-xL protein expression (not shown) of the more nonspecific all-phosphorothioate C5-propynylated oligomers were identical to those of the chimerics, which have reduced phosphorothioate content, it seems logical to assume that the effects of the chimerics were not solely attributable to non-specificity.

Treatment of either LNCaP or PC-3 cells with porphyrin (TAP for LNCaP and TMP for PC-3 cells) complexes of the active C5-propyne chimeric oligonucleotides led to co-down-regulation of Bcl-xL and Bcl-2 protein expression but did not lead to significant (>10%) spontaneous cellular apoptosis. This contrasts with the apoptosis observed in diverse cell types (including prostate cancer cell lines) with G3139 (45), an all-phosphorothioate 18-mer targeted to the initiation codon region of Bcl-2. In light of our observations, the strongly proapoptotic effects of G3139 indicate that down-regulation of Bcl-2 protein expression by this molecule may be necessary, but is not sufficient, for apoptosis initiation; an additional, perhaps necessary toxic insult may be provided by the lipid carrier (Lipofectin). An additional possibility is that G3139 can down-regulate the expression of perhaps many additional genes via the process of “irrelevant cleavage” (46). Because of the “low stringency,” nonselective mechanism of action of RNase H, sequence homology of the antisense oligonucleotide with nontargeted mRNAs may lead to their cleavage. Indeed, this process is probably responsible for the co-down-regulation of Bcl-xL and Bcl-2 by the C-5-propynylated chimeric oligomers presented in this work. In the study presented here, the GAGTC five-base motif found in the antisense Bcl-xL oligomer 41 is also found at both 3290–3294 and 4554–4558 nucleotides in the Bcl-2 mRNA. Similarly, oligomers 29 and 57, which down-regulate both Bcl-xL and Bcl-2 in PC-3 cells, have a region of seven-base homology (TG-GTTGA for 29 and GGAGATG for 57) at nucleotides 2532–2538 and 524–530, respectively. Of course, for any oligonucleotide, partially or fully complementary, to elicit RNase H activity it must hybridize to its complement; and it is also true that many if not
most complementary sequences are either not accessible to sites on the target because of mRNA secondary structure and folding, or even if accessible, may not form sufficiently stable complexes to be recognized and cleaved by RNase H (47). Interestingly, the antisense Bcl-2 oligomer G3139 has a single seven-base (and several five-base) regions of contiguous homology with the Bcl-x mRNA, sufficient overlap for RNase H competency (48). Furthermore, preliminary results obtained from the treatment of RCC-E kidney cancer cells by G3139 demonstrate co-down-regulation of Bcl-2 and Bcl-xL protein expression.3

Down-regulation of Bcl-xL and Bcl-2 protein expression by a single oligonucleotide, achieved by taking advantage of the “low stringency” requirements of RNase H, has been accomplished recently (49). Such a bispecific oligonucleotide induced apoptosis in the SW2 small cell lung cancer cell line, where Bcl-2 has been shown to be a significant antiapoptotic effector. However, in other cell lines, where Bcl-xL was found to be the important antiapoptotic effector, the bispecific antisense oligonucleotide was not a more efficient inducer of apoptosis than was an antisense oligonucleotide that down-regulates Bcl-xL expression only. These results correlate exactly with our own. However, the use of RNase H-competent oligonucleotides to co-down-regulate both Bcl-2 and Bcl-xL may affect the expression of other, as yet unknown, genes because of irrelevant cleavage. It is entirely possible that these unintended effects may also contribute to the observed chemosensitization. A reduction in RNase H activity, and thus in irrelevant cleavage, can be obtained by eliminating RNase H-competent deoxyribose linkages at the molecular termini and substituting them with non-RNase H-competent 2′-O-methyl “gap-mers.” In this work, as in others (50, 51), in such “gap-mers” a central region of phosphorothioate oligonucleotide was included to retain some, albeit reduced, RNase H activity. As predicted, in part because of reduced irrelevant cleavage, neither 41m nor 57m down-regulated the expression of Bcl-2 protein. However, despite the ability of these oligonucleotides to down-regulate Bcl-xL protein expression, Northern analysis (Fig. 9) did not demonstrate any decline in Bcl-xL mRNA expression, demonstrating that this decrease in protein expression was non-RNase H dependent. Nevertheless, the fact that this down-regulation occurred in the absence of a diminution in mRNA expression is surprising in light of the activity of the 80S ribosome (52, 53), which in theory should have unwound the mRNA-“gap-mer” oligonucleotide duplex. In addition, the eight-base core phosphorothioate region should certainly have been sufficient for RNase H competency. Therefore, in one scenario, it is possible that the duplex does not come into contact with RNase H, which implies (among other possibilities) that this oligonucleotide may alter intracellular mRNA transport. Alternatively, the duplex, perhaps because of steric considerations, may not be recognized by RNase H.

Using a two-tailed, two-sample t test with unequal variances, no statistically significant difference was observed in MTT activity in PC-3 cells after treatments with 41 (co-down-regulation of Bcl-xL and Bcl-2) or 41m (down-regulation of Bcl-xL, no effect on Bcl-2) and either etoposide or carboplatin (P > 0.8). In addition, 41m in combination with paclitaxel was actually more inhibitory than 41 with paclitaxel (P = 10−8). In sharp contrast, however, 41m, in contrast to 41, did not sensitize PC-3 cells to mitoxantrone at all. These data imply that basal Bcl-2 expression, which in any case is much lower (at least 5–10-fold) in PC-3 cells than is Bcl-xL basal expression, is in general not highly protective to cytotoxic agents (mitoxantrone being an exception), and of the two antiapoptotic proteins, Bcl-xL is far more protective to a range of cytotoxics. This may not be the case, however, in cell lines engineered to overexpress Bcl-2 (5) or in other cell lines. In summary, our data suggest that Bcl-xL is an extremely important protein in the set of pro- and antiapoptotic effector molecules that exist in prostate cancer cells, and the relative sensitivity of these cells to cytotoxic chemotherapy seems to be a direct function of the level of its expression. Our results also suggest that Bcl-2 may not be an optimum target in prostate cancer cells. In addition, experiments presented here further highlight the value of the antisense biotechnology, when used in what is hopefully an appropriate and rigorous manner, in the validation of gene function.

REFERENCES


3 M. Wilenshchik, N. Bander and C. A. Stein, unpublished data.

32. Huang, D. C., Cory, S., and Strasser, A. Bcl-2, Bcl-XL and adenovirus protein E1B19kD are functionally equivalent in their ability to inhibit cell death. Oncogene, 12: 21718–21721, 1995.


Bcl-xL in Prostate Cancer Cells: Effects of Overexpression and Down-Regulation on Chemosensitivity

Irina Lebedeva, Robert Rando, Joshua Ojwang, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/60/21/6052

Cited articles
This article cites 50 articles, 31 of which you can access for free at:
http://cancerres.aacrjournals.org/content/60/21/6052.full.html#ref-list-1

Citing articles
This article has been cited by 33 HighWire-hosted articles. Access the articles at:
/content/60/21/6052.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.