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-propylated 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 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-xL protein. Staining intensity seemed to be correlated with increasing Gleason score and the presence of metastases. Because Bcl-xL, 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 chemodesensitization 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\textsubscript{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 antineoplastic 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 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 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

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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@ccfca.cc.columbia.edu.

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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 FBS (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 and were routinely passaged when 90–95% confluent.

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 plasmid (obtained from Dr. Stanley Korsmeyer, Washington University School of Medicine, St. Louis, MO). Transfections were performed with Lipofectin (Life Technologies, Inc., Gaithersburg, MD) in serum-free OPTI-MEM (Life Technologies, Inc.) or with a control, neomycin-resistant expression plasmid (obtained from Dr. Stanley Korsmeyer, Washington University School of Medicine, St. Louis, MO) or with a control, neomycin-resistant expression plasmid (obtained from Dr. Stanley Korsmeyer, Washington University School of Medicine, St. Louis, MO).

Fig. 1. Antisense oligonucleotides used in this study. Shown are the targeted Bcl-xL mRNA sites (top) and the antisense sequences (bottom). C5-propyne-modified bases are given in capital letters.*, phosphorothioate internucleotide bonds.

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 × 10⁵ cells/well to be 80–90% confluent on the day of the experiment.

Oligonucleotides were delivered to the cells complexed with catonic porphyrins TMP and TAP (PC-3 and LNCaP cells, respectively). Oligonucleotide was premixed with TAP or TMP in OPTI-MEM medium to the final concentrations described in “Results” for each cell line. A complex was formed for 15 min at room temperature; cells were washed once with OPTI-MEM and treated with prepared complex for 5 h at 37°C. Then cells were washed once with RPMI-10% FBS and were allowed to recover in RPMI-10% FBS for another 20 h. At this time, cells were washed twice with PBS, and then protein and/or mRNA were extracted as described and analyzed.

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% NP-40, 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 × 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 by 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 A containing a 1:200 dilution of goat anti-Orials or anti-Rabbit polyclonal antibodies (both 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 A containing a 1:10,000 dilution of corresponding peroxidase-conjugated, anti-rabbit secondary antibody (Amer sham, Arlington Heights, IL). To detect Bcl-2, a 1:500 dilution of anti-Bcl-2 mouse mAb (Dako, Carpinteria, CA) was used in 1% BSA/TBS. After washing in TBS-T buffer (three times for 5 min each time), at room tempera-
Bcl-xL IN PROSTATE CANCER CELLS

Increased Expression of Bcl-xL in LNCaP and PC-3 Cells

RNA Isolation and Northern Blot Analysis. Total RNA was isolated from 5 × 10⁶ cells using an RNeasy 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 containing formaldehyde, transferred to Nytran-Plus nylon membranes by using the Analysis ToolPack provided by Microsoft Excel. A Student two-sample test, assuming unequal variances, was performed using the Analysis ToolPack provided by Microsoft Excel. A Student two-sample test, assuming unequal variances, was performed using the Analysis ToolPack provided by Microsoft Excel.

**RESULTS**

Increased Expression of Bcl-xL in LNCaP and PC-3 Cells

**Down-Regulation of Bcl-xL Expression by Chimeric, C5-Prodynylated Oligonucleotides.** Because of unacceptable cytotoxicity, we could not use cationic lipids, such as Lipofectin, as delivery agents.
Down-regulation of the expression of Bcl-xL protein by C5-propynylated chimeric oligonucleotides was demonstrated by Western blotting, as shown in Fig. 3A. (A $M_s$ 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 affected 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 absence of 0.1 $\mu$M mitoxantrone was decreased 39.8 ± 4.3% ($P < 10^{-10}$); in the presence of 25 nm paclitaxel, 34 ± 6.2% ($P < 10^{-5}$); in the presence of 50 nm Taxotere, by 33.1 ± 4.8% ($P < 10^{-7}$); in the presence of 10 $\mu$M carboplatin, by 47 ± 7.2% ($P < 10^{-7}$); and by 49.9 ± 2.0% in the presence of etoposide ($P < 10^{-7}$). Oligonucleotides 57 and 61, also highly statistically significant ($P < 10^{-7}$), increased sensitivity to these cytotoxic agents but did not
tend to be as active as 41, perhaps because these two also inhibit expression of Bax protein (Table 1). However, neither the C5-propynylated controls 45 and 47 nor the non-C5-propynylated species 34, 38, 46, or 62 produced any significant change in cellular viability.

2'-O-Methyl “Gap-Mers” Down-Regulate Bcl-xL Protein but not mRNA Expression in Prostate Cancer Cell Lines. To reduce presumed RNase H-dependent irrelevant cleavage, we decreased the number of nucleotides that could elicit RNase H activity. 2'-O-Methyloligoribonucleotides are not substrates for RNase H; a seven-base contiguous region of oligodeoxyribonucleotide phosphorothioates was retained in the center of the molecule, in theory to ensure appropriate RNase H cleavage of the targeted mRNA. These oligonucleotides, as well as their mismatched control sequences, are given in Table 2. As shown in Fig. 8, these gap-mers down-regulated the expression of Bcl-xL protein (41m and 57m are the most active). Bcl-2 expression, as expected, was not dramatically decreased, and Bax expression was unchanged after treatment. The four-base mutant oligomers did not down-regulate either Bcl-xL or Bcl-2 expression.

However, 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 chemosensitization ($P < 10^{-4}$) 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 taxanes, 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, 4.3%)

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

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<tr>
<th>PC-3 cell line</th>
<th>LNCaP Cell Line</th>
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<tr>
<td>2 μm C5-propyne oligo, 7 μm TMP</td>
<td>1 μm C5-propyne oligo, 3 μm TAP</td>
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<td>Bcl-xL</td>
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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, anti-Bax, and anti-Bak antibodies and with anti-Bcl-2 mAb. Northern blots of GAPDH mRNA are shown for 25 kDa. Northern blots of GAPDH mRNA demonstrate equal loading of RNA.
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-dioleoyloxy)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 significantly, oligomers 29 and 57, which down-regulate both 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 nucleases (25, 26), as opposed to those 5’ to pyrimidine, which are highly sensitive. However, the C5-propyne-modified all-phosphorothioate 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 partially or fully complementary, to elicit RNase H activity it must hybridize to its complement; and it is also true that many if not 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, perhaps 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 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 biotechnol-

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Bcl-xL IN PROSTATE CANCER CELLS


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