Cellular Response to an Antisense-mediated Shift of Bcl-x Pre-mRNA Splicing and Antineoplastic Agents*

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Overexpression of Bcl-xL, an anti-apoptotic member of the Bcl-2 family, negatively correlates with the sensitivity of various cancers to chemotherapeutic agents. We show here that high levels of expression of Bcl-xL promoted apoptosis of cells treated with an antisense oligonucleotide (5′Bcl-x AS) that shifts the splicing pattern of Bcl-x pre-mRNA from the anti-apoptotic variant, Bcl-xL, to the pro-apoptotic variant, Bcl-xS. This surprising finding illustrates the advantage of antisense-induced modulation of alternative splicing versus down-regulation of targeted genes. It also suggests a specificity of the oligonucleotide effects since non-cancerous cells with low levels of Bcl-xL should resist the treatment. 5′Bcl-x AS sensitized cells to several antineoplastic agents and radiation and was effective in promoting apoptosis of MCF-7/ADR cells, a breast cancer cell line resistant to doxorubicin via overexpression of the mdr1 gene. Efficacy of 5′Bcl-x AS combined with chemotherapeutic agents in the PC3 prostate cancer cell line may be translated to clinical prostate cancer since recurrent prostate cancer tissue samples expressed higher levels of Bcl-xL than benign prostate tissue. Treatment with 5′Bcl-x AS may enhance the efficacy of standard anti-cancer regimens and should be explored, especially in recurrent prostate cancer.

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Cancers not completely eradicated by surgery or radiation (localized therapy) may escape control by chemotherapy (systemic therapy) because some cancer cells, especially those resistant to apoptosis, survive treatment (1, 2). For example, prostate cancer that recurs after potentially curative therapy, or that presents in an advanced stage, is palliated with androgen-deprivation therapy. Within several years most recur as androgen-independent, metastatic disease that leads to death. Recently, chemotherapeutic regimens have been developed that allow palliation in most patients. While such treatments may lead to re-remissions of 1 year or more, they have not proven to increase survival (3–5).

Chemotherapeutic resistance usually arises due to overexpression of anti-apoptotic proteins such as Bcl-2 and Bcl-xL (2, 6–8). Bcl-2 is regarded as one of the most important proteins protecting cancer cells from apoptosis and, to date, may be the most highly studied member of the Bcl-2 family. However, in an examination of 60 different cell lines from the National Cancer Institute, Bcl-xL was shown to provide equivalent or greater protection against cytotoxic agents than Bcl-2. Higher levels of Bcl-xL correlated with decreased cellular sensitivity toward a variety of chemotherapeutic reagents; there was no such correlation for Bcl-2 (6). Other studies have shown that high levels of Bcl-xL contributed to increased risk of metastasis in breast cancer (9) and protected cancer cells from chemotherapeutic agents (10, 11). In addition, cancer cells were sensitized to various apoptosis-inducing agents if Bcl-xL levels were decreased (12, 13).

Bcl-xL and Bcl-xS are splice variants produced by alternative splicing of Bcl-x pre-mRNA (14). While Bcl-xL is anti-apoptotic, Bcl-xS has been shown to induce cell death (15, 16) and sensitize cancer cells to chemotherapeutic agents (17–20). Bcl-xL inhibits the anti-apoptotic effects of Bcl-xL and Bcl-2, possibly by forming heteroduplexes with these proteins (21) and/or by acting as a dominant negative gene product (22). Decreasing Bcl-xL and increasing Bcl-xS levels may initiate pro-apoptotic events through various cellular mechanisms that, alone or in synergy with the action of antineoplastic agents, lead to cell death.

We have shown previously that a 2′-O-methyl-oligoribonucleoside phosphorothioate (5′Bcl-x AS)1 targeted to the downstream alternative 5′-splice site in exon 2 of Bcl-x pre-mRNA shifted splicing from the Bcl-xL to Bcl-xS splice variants; this treatment decreased the levels of Bcl-xL and increased the levels of Bcl-xS proteins (23). The shift in splicing induced cell death in oligonucleotide-treated PC3 prostate cancer cells and to a lesser extent in MCF-7 breast cancer cells. In A549 lung epithelial cells, a similar treatment alone was ineffective; cell death resulted only from co-administration of radiation or cisplatin (24). These findings prompted us to investigate the differences in cellular responses as a result of oligonucleotide-induced modification of Bcl-x pre-mRNA splicing. We found that the endogenous level of Bcl-x is the main factor that determines the extent of cell death induced by 5′Bcl-x AS. Treatment of PC3 and MCF-7 cells (two cell lines that express different levels of Bcl-xL) with 5′Bcl-x AS sensitized both cell lines to various chemotherapeutic agents and radiation and increased cell death at lower doses of these agents. Finally, prostate cancer expressed higher levels of Bcl-xL protein than benign prostate. These results suggest that

1 The abbreviations used are: 5′Bcl-x AS, antisense oligonucleotide targeted to the downstream alternative 5′-splice site of Bcl-x pre-mRNA; 5′-FdU, 5-fluorodeoxyuridine; 5-FU, 5-fluorouracil; ASO, antisense oligonucleotide; FCS, fetal calf serum; ER, estrogen receptor; RPA, RNase protection assay; RT, reverse transcription; Gy, Gray; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MEM, modified essential medium.
Cells Lines and Prostate Tissue Samples—The treated human cancer cell lines were from prostate (PC3, DU145), breast (MCF-7, MDA-MB-231, BT-549, Hs578T) and cervical (HeLa) cancers. They included four p53 mutant cells (PC3, DU145, MDA-MB-231, Hs578T) (25–28) and three p53-positive cells (MCF-7, BT549, HeLa) (25, 28–30). Among the breast cancer cell lines, two were ER-negative (MDA-MB-231, Hs578T) (28) and two were ER-positive (MCF-7, BT549) (28, 29). PC3 and DU145 were Androgen-insensitive prostate cancer cell lines. All cell lines were originally from the ATCC and grown in a humidified incubator with 5% CO2 at 37 °C. All cells were cultured in the presence of penicillin/streptomycin or, for HeLa cells gentamycin/kanamycin, in the following media. PC3: DMEM/F12 (Dulbecco’s Modified Eagle’s Medium), 10% fetal calf serum (FCS); MCF-7, MDA-MB-231, Hs578T: MEM (modified essential medium), 10% FCS, 1× sodium pyruvate (Invitrogen), 1× non-essential amino acids (Sigma), 10 µg/ml insulin (Invitrogen); MDA-MB-231 and Hs578T: Dulbecco’s Modified Eagle’s medium, 10% FCS, insulin (10 µg/ml); BT-549: RPMI 1640 (Invitrogen), 10% FCS, insulin (1 µg/ml); HeLa: MEM, 5% FCS, 5% horse serum, l-glutamine (2 mM); Invitrogen); and DU145: MEM, 10% FCS, 1× sodium pyruvate, 1× non-essential amino acids. Twenty-four hours prior to oligonucleotide treatment, all cells were plated in 1 ml of media in 24-well plates at a density of 7 × 104 per well.

Research specimens were recovered from prostate tissue stored in liquid nitrogen. Androgen-independent prostate cancer had been obtained by transurethral resection from 10 men who presented with urinary retention from recurrent prostate cancer 7–92 months after androgen deprivation therapy. Histologic examination revealed poorly differentiated prostate cancer (Gleason scores 8–10) that represented an average of 92% (ranged from 72–99%) of the cross-sectional area of the tissue sections. Ten specimens of benign prostate tissue had been obtained from portions of adenoma removed at prostatectomy; absence of cancer was confirmed by frozen section.

Bcl-x AS-transfected PC3 cells: 1000 at 0 Gy, 2000 at 2 Gy, 4000 at 4 Gy. After irradiation, cells were cultured, and colonies were stained with 5% methylene blue (Sigma) in 50% ethanol for 10 min. Colonies larger than 50 cells were counted. For chemotherapeutic dose-response experiments, re-plated, oligonucleotide-treated cells were treated for 24 h with the chemotherapeutic agents. After treatment with chemotherapeutic agents, the cells were washed with HBSS ( Hank’s Buffered Saline Solution, Invitrogen), and fresh medium was added. The remainder of the procedure was the same.

Bcl-xL Western Blot—Total protein was prepared by lysing cells (one well of a 24-well plate) or lysing prostate tumor tissues (200 mg tissue sections finely ground to a powder) in radioimmune precipitation assay (RIPA) buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate), and a mixture of protease inhibitors (15 µl for every 1 ml of RIPA buffer, Sigma). 20 µg of total protein were electrophoresed on a 15% SDS-polyacrylamide gel and electrotransferred to polyvinylidene difluoride membranes. Blots were probed with Bcl-xL (1:1000 dilution; Transduction Laboratories, Lexington, KY) followed by a horseradish peroxidase (HRP)-conjugated secondary antibody (1:3000 dilution; Bio-Rad). Bcl-xL migrated at ~30 kDa. Equal loading and transfer were confirmed by staining the membranes with Ponceau S (Sigma) and blotting with β-tubulin antibody (1:4000 dilution; Sigma) followed by an HRP-conjugated secondary antibody (1:5000 dilution; Bio-Rad); β-tubulin protein migrated at ~55 kDa. Protein was visualized with ECL Plus (Amersham Biosciences) treatment.

RNA Protection Assay (RPA)—Untreated cells were analyzed for levels of Bcl-xL, Bcl-xS, Bax, Bak, Bcl-2, Mcl-1, and GAPDH genes with a multiprobe template set (hAPO-2; BD Pharmingen, San Diego, CA) and RPA II RNA protection assay kit (Ambion, Inc., Austin, TX). Reactions were carried out according to the manufacturers’ protocols.

Statistical Analysis—Prism (Graph Pad) software was used to generate dose response curves, calculate LC50 values, and for other statistical analyses indicated in the figure and table legends.

RESULTS

Cell Death Affected by a Shift in Splicing from Bcl-xL to Bcl-xS—To shift the alternative splicing pathway of Bcl-x pre-mRNA from Bcl-xL to Bcl-xS, seven different cell lines were treated with 5′Bcl-x AS antisense oligonucleotide targeted to the downstream alternative 5′-splice site of exon 2 (Fig. 1) and delivered to the cells with the aid of DMRIE-C cationic lipid reagent. The treated cell lines originated from prostate (PC3, DU145), breast (MCF-7, MDA-MB-231, BT-549, Hs578T), and cervical (HeLa) cancers and represented distinct genetic backgrounds (see “Experimental Procedures”).

RT-PCR analysis of total RNA from untreated cells showed that in all cell lines Bcl-xL mRNA was essentially the only expressed splice variant; Bcl-xS was barely or not at all detectable (Fig. 2A, lane 1). Since the uptake of the lipid-oligonucleotide complex or the oligonucleotide antisense activity may vary in different cells, for each cell line, the oligonucleotide concentration was adjusted such that the splicing was shifted
randomized oligonucleotide. In this and subsequent figures, error bars represent the S.D. from at least three independent experiments. Mutant (M) and wild type (W) p53 and ER status are indicated below the graph.

**Endogenous Levels of Bcl-xL Determine the Cellular Response to 5′Bcl-x AS**—Since the extent of Bcl-xL/xS splicing modification was normalized to approximately the same 50–60% level (Fig. 2A) it appeared that other factors must have contributed to the variability of the cellular response to 5′Bcl-x AS treatment. No clear correlation was found between susceptibility to 5′Bcl-x AS treatment and the level of expression of functional p53 or ER genes; this indicated that Bcl-xL/Bcl-xS effects are p53 (32, 33) and ER-independent (Fig. 2B). Furthermore, there was no correlation between 5′Bcl-x AS susceptibility, and the levels of expression of several Bcl-2 family members (Bak, Mcl-1, Bcl-2, and Bax) determined by RPA of total RNA from the seven cell lines (Fig. 3).

To further address this issue, an examination of the levels of Bcl-xL mRNA was carried out by RPA. The results showed that the levels of Bcl-xL were highest in PC3 cells, followed by MDA 231, DU145, Hs578T, MCF-7, BT549 and lowest in HeLa cells (Fig. 4, A and B). Analysis of Bcl-xL protein by immunoblotting with anti-Bcl-xL antibody established the same rank order of Bcl-xL expression levels (Fig. 4, C and D). There was a high degree of correlation (p value of < 0.0001 and \( r^2 = 0.9601 \), by Pearson correlation) between the levels of Bcl-xL protein in untreated cell lines and death of 5′Bcl-x AS-treated cells, indicating that cells containing higher levels of Bcl-xL were more susceptible to 5′Bcl-x AS oligonucleotide treatment.

This counterintuitive result, that increased expression of anti-apoptotic Bcl-xL at the same time facilitates cell death of 5′Bcl-x AS-treated cells, is best explained by the data illustrated in Fig. 5, A and B. The seven different cell lines were treated with 5′Bcl-x AS at concentrations indicated in Fig. 2A that resulted in 50–60% shift in Bcl-x pre-mRNA splicing. Despite the fact that the relative amounts of Bcl-xL/xS mRNAs were the same in all cell lines (i.e. the ratio of Bcl-xL to -xS was ~50–60%), RPA of total RNA with a Bcl-xL-specific probe showed that the absolute levels of Bcl-xS mRNA varied substantially (Fig. 5). PC3 cells had the highest and HeLa cells the lowest content of this RNA, consistent with the expression levels of Bcl-xL and not the extent of the shift in splicing. These data suggest that highly expressing cells such as PC3 cells have high levels of Bcl-x pre-mRNA, which when spliced produced large amounts of Bcl-xL mRNA. When targeted with 5′Bcl-x AS oligonucleotide splicing of Bcl-x pre-mRNA resulted in large amounts of Bcl-xS mRNA (Fig. 5) and presumably Bcl-xS protein. Previously observed differences in the level of Bcl-xS protein in oligonucleotide-treated PC3 and MCF-7 cells support this conclusion (23).

**5′Bcl-x AS Sensitizes MCF-7 and PC3 Cells to Antineoplastic Treatments**—The 5′Bcl-x AS-induced shift in splicing may be less effective against cancers with low Bcl-x expression levels.
Thus, we sought to determine if the applicability of this approach could be extended to more resistant cells if the 5'H11032Bcl-x AS treatment is combined with conventional antineoplastic agents. The experiments were carried out on the MCF-7 breast cancer cell line, a cell line relatively resistant to oligonucleotide treatment, and the oligonucleotide-susceptible PC3 prostate cancer cell line. Five apoptosis-inducing agents, cisplatin, doxorubicin, 5-FU, 5-FdU, and etoposide, which exert their cytotoxic effects through different mechanisms, were selected for these experiments. All of these chemotherapeutic agents are a part of the standard set of anticancer agents included in the National Cancer Institute’s drug screen (6).

Dose response curves were generated for MCF-7 cells treated with 0.1 and 0.4 μM 5’Bcl-x AS followed by chemotherapeutic agents. 0.4 μM random oligonucleotide-transfected or mock-transfected cells served as negative controls. 0.1 and 0.4 μM 5’Bcl-x AS alone resulted in ~35 and 50% shift in splicing and 59 and 38% viability, respectively (data not shown). The latter values were normalized to 100% in order to determine the LC50 of the different drugs (see “Experimental Methods”). Examples of the experimental data for cisplatin and doxorubicin are illustrated in Fig. 6, A and B. The summary of the data for all the drugs and MCF-7 and PC3 cells is in Tables I and II.

For MCF-7 cells, the 0.4 μM concentration of 5’Bcl-x AS markedly decreased the LC50 values for cisplatin (>5-fold) and doxorubicin (>6-fold) (Table I). Although the oligonucleotide also sensitized the cells to a statistically significant degree to 5-FdU, the effect was not dose-dependent (see “Discussion”); the effect was even lower for etoposide. The shift in Bcl-x pre-mRNA splicing did not alter the sensitivity of MCF-7 cells to 5-FU. Treatment of PC3 cells with 5’Bcl-x AS at concentra-
tions of 0.01 and 0.08 μM led to a 35 and 55% shift in Bcl-x pre-mRNA splicing and, respectively, to 58 and 25% viability (data not shown). Addition of cisplatin and 5-FdU to oligonucleotide-treated (0.08 μM 5′Bcl-x AS) PC3 cells led to a 10-fold decrease in LC50 of these drugs. The LC50 values of etoposide, 5-FU, and doxorubicin were 2-3-fold lower in 5′Bcl-x AS (0.08 μM)-treated PC3 cells than in that control cells. For the latter three drugs, oligonucleotide dose dependence was not found.

The effects of the oligonucleotide and antineoplastic treatments on cell viability in all the experiments were assayed in long term colony formation assays in tissue culture plates. Thus, it could be argued that there is no evidence that these treatments led to cell death by increasing apoptosis. We have shown previously that the shift in Bcl-xL/xS splicing induced apoptosis in PC3 and MCF-7 cells (23). We confirmed that the combination of 5′Bcl-x AS with cisplatin or 5-FdU for PC3 cells and with doxorubicin for MCF-7 cells induced PARP cleavage (poly(ADP)-ribose polymerase, an indicator of apoptosis) to a greater extent than each agent alone, as expected (data not shown).

Soft agar colony formation tests were carried out to confirm that the oligonucleotide/drug treatments caused cell death and not merely reduced the ability of the treated cells to attach to the culture plate. The 5′Bcl-x AS-transfected PC3 and MCF-7 cells were treated with cisplatin, doxorubicin, 5-FU, and 5-FdU, and etoposide at the LC50 concentrations of these drugs shown in Tables I and II. Colony formation in soft agar and the calculated effects of the treatments on cell viability closely mirrored those obtained in the plate-based clonogenic assay (data not shown). Thus, the combined results of the PARP and soft agar assays indicate that the above treatments increased apoptotic cell death.

5′Bcl-x AS Sensitizes MCF-7 and PC3 Cells to Radiation—Overexpression of Bcl-xL is an important factor in mediating radioresistance (34) whereas cells with lower levels of Bcl-xL are more sensitive to radiation-induced apoptosis (35). Furthermore, it was found that radiation down-regulates Bcl-xL in MCF-7 cells (36). Thus, it seemed likely that the oligonucleotide-induced shift in Bcl-xL/Bcl-xS splicing would sensitize cancer cells to radiation-induced apoptosis. Transfection of MCF-7 and PC3 cells with 5′Bcl-x AS (0.1 and 0.4 μM) for 5′Bcl-x AS and 0.01 and 0.08 μM for PC3 cells, followed by exposure to 1–4 Gy doses of radiation, resulted in a statistically significant reduction of cell viability (Fig. 7, A and B). At 2 Gy and 0.4 μM 5′Bcl-x AS oligonucleotide, MCF-7 cell viability was reduced to 24%, compared with 40% for control oligonucleotide-transfected cells. At the highest dose (4 Gy) the viability was further reduced in a dose-dependent fashion to 5.8 and 3.4% for 0.1 and 0.4 μM Bcl-x AS, respectively, compared with 14.5% for control oligonucleotide-transfected cells.

PC3 cells were found to be more sensitive to the combined oligonucleotide-radiation treatment. Cell viability was reduced close to 2-fold even at low doses (0.01 μM oligonucleotide and 1 Gy radiation). Under these conditions viability of the cells was lower than that of control cells irradiated at 2-Gy dose (Fig. 7B). As the radiation dose increased, the effects of the shift in Bcl-xL/Bcl-xS splicing became less pronounced; at 4 Gy there was no further sensitization, presumably because the radiation alone induced massive cell death.

5′Bcl-x AS Induces Cell Death in the Multidrug-resistant Cell Line, MCF-7/ADR—Since treatment of cancer cells with chemotherapeutic agents may select resistant cells, we sought to...
determine if the oligonucleotide-induced shift in Bcl-xL/xS splicing caused apoptosis in chemotherapy-resistant cells. MCF-7/ADR cells, a p53 mutant (25) breast cancer cell line, are highly resistant to apoptosis induced by chemotherapeutic agents such as doxorubicin (37). Overexpression of the \textit{mdr1} gene, which codes for P-glycoprotein, is the principal mechanism of the chemoresistance for these cells (38–41). Treatment of MCF-7/ADR cells with 5\textsuperscript{B}cl-x AS oligonucleotides resulted in a dose-dependent shift in splicing from Bcl-xL to Bcl-xS (Fig. 8A). The EC\textsubscript{50} of 5\textsuperscript{B}cl-x AS (0.08 \mu M) was comparable to its \textit{EC}\textsubscript{50} in PC3 cells (0.08 \mu M) and 5-fold lower than in parent MCF-7 cells (0.4 \mu M). This effect appears to be due to increased uptake of the oligonucleotide-DMRIE-C complex into the nuclei (data not shown, see “Discussion”). The shift in splicing led to a dose-dependent decrease in the viability of the MCF-7/ADR cells (Fig. 8B). Although the 50\% shift in Bcl-xL/xS splicing was achieved at a 5\textsuperscript{B}cl-x AS concentration lower than that in MCF-7 cells, decreases in cell viability were comparable in the two cell lines (compare Figs. 2B and 8B).

In order to examine this observation in more detail, the level of Bcl-xL protein in MCF-7/ADR cells was determined and plotted versus cell viability and compared with the other cell lines studied. The level of Bcl-xL was similar to that of the parent MCF-7 cells (Fig. 8C). The decrease in cell viability was similar and agreed with the results obtained for other cell lines (Fig. 8C, \( p < 0.0001 \) and \( r^2 = 0.9480 \) by Pearson correlation). Thus, despite apparent changes in the oligonucleotide uptake resulting in increased sensitivity of Bcl-xL/xS splicing to oligonucleotide treatment, the decrease in cell viability remained unchanged suggesting that it depended only on the endogenous level of Bcl-x pre-mRNA as reflected in the levels of Bcl-xL protein.

High Expression of Bcl-xL in Prostate Cancer—Since the androgen-insensitive prostate cancer cell lines, PC3 and DU145, had among the highest levels of Bcl-xL, we tested if clinical specimens of prostate cancer recurrent after androgen deprivation therapy exhibited increased expression of this gene. Immunoblot analysis of prostate cancer and benign prostate samples showed significant differences in the levels of Bcl-xL between the two groups (\( p = 0.0012 \), 2-tailed Student’s \( t \) test; Fig. 9). This suggests that Bcl-xL may play a role in the progression of prostate cancer and that modulation of its expression may be a means of controlling that progression.

\textbf{Fig. 7.} Treatment with 5\textsuperscript{B}cl-x AS sensitizes MCF-7 and PC3 cells to radiation. \textbf{A,} MCF-7 cells; \textbf{B,} PC3 cells. Clonogenic assay of cells transfected with the oligonucleotides at concentrations indicated in the figure followed by radiation at 1–4 Gy. Cell viability of irradiated cells is expressed as percent colonies formed after treatment and normalized versus control cells treated with the oligonucleotide only. Asterisk indicates statistically significant difference versus control cells (\( p < 0.05 \); one-way analysis of variance with Tukey post-hoc test).

\section*{Cellular Response to Bcl-x AS}

\begin{table}[h]
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\begin{tabular}{|l|l|l|l|l|l|}
\hline
Cell line & Treatment & Etoposide & 5-FU & Cisplatin & 5-FdU & Doxorubicin \\
\hline
\hline
MCF-7 & Mock & 0.94 & 1.4 & 0.063 & 5.5 & 0.26 \\
& 0.4 \mu M Control & 0.84 & 1.1 & 0.061 & 5.4 & 0.29 \\
& 0.1 \mu M 5Bcl-x AS & 0.48 & 1.0 & 0.056 \textsuperscript{a} & 1.8 \textsuperscript{a} & 0.19 \textsuperscript{a} \\
& 0.4 \mu M 5Bcl-x AS & 0.49 \textsuperscript{a} & 0.96 & 0.012 \textsuperscript{a} & 1.4 \textsuperscript{a} & 0.041 \textsuperscript{a} \\
\hline
LC\textsubscript{50} decrease (0.4 \mu M) & 1.9 & 1.1 (NS) & 5.3 & 3.9 & 6.3 \\
5\textsuperscript{B}cl-x AS vs. Mock: \\
\hline
\end{tabular}
\caption{LC\textsubscript{50} results for MCF7 cells transfected with 5\textsuperscript{B}cl-x AS}
\end{table}

\begin{table}[h]
\centering
\begin{tabular}{|l|l|l|l|l|l|}
\hline
Cell line & Treatment & Etoposide & 5-FU & Cisplatin & 5-FdU & Doxorubicin \\
\hline
\hline
PC3 & Mock & 2.2 & 12.2 & 0.19 & 5.8 & 0.26 \\
& 0.08 \mu M Control & 2.1 & 13.0 & 0.15 & 4.9 & 0.029 \\
& 0.01 \mu M 5Bcl-x & 0.67 \textsuperscript{a} & 5.0 \textsuperscript{a} & 0.13 \textsuperscript{a} & 1.5 \textsuperscript{a} & 0.017 \textsuperscript{a} \\
& 0.08 \mu M 5Bcl-x & 0.62 \textsuperscript{a} & 3.7 \textsuperscript{a} & 0.02 \textsuperscript{a} & 0.56 \textsuperscript{a} & 0.016 \textsuperscript{a} \\
\hline
LC\textsubscript{50} decrease (0.08 \mu M) & 3.5 & 3.3 & 9.5 & 10.4 & 1.9 \\
5\textsuperscript{B}cl-x AS vs. Mock: \\
\hline
\end{tabular}
\caption{LC\textsubscript{50} results for PC3 cells transfected with 5\textsuperscript{B}cl-x AS}
\end{table}
DISCUSSION

Several recent studies showed that antisense oligonucleotide-mediated down-regulation of expression of Bcl-xL and other anti-apoptotic genes enhanced apoptosis with and without additional treatment with chemotherapeutic drugs (13, 18, 42–47). In these approaches, the higher the expression of the target mRNA, the less effective were the oligonucleotides. In the work reported here, the opposite was true; the higher the expression of Bcl-xL, the more pronounced the effects of the 5’Bcl-x AS oligonucleotide. These results show the power of oligonucleotide modification of splicing and bode well for the specificity of this approach.

The main advantage of splicing modification, especially in the context of opposing Bcl-xL and -xS splice variants, is that for every pre-mRNA molecule targeted with the antisense oligonucleotide one molecule of anti-apoptotic Bcl-xL is replaced with one molecule of pro-apoptotic Bcl-xS. The observations that antisense down-regulation of Bcl-xL was not very effective (23), or even promoted chemoresistance in some cases (48), suggest that the key contributor to 5’Bcl-x AS oligonucleotide-induced apoptosis was newly generated Bcl-xS. Importantly, as shown here, this splice variant was effective regardless of the expression profile of the targeted cells. This notion is well illustrated by the lack of correlation of 5’Bcl-x AS-induced cell death with the levels of Bcl-2, Bak, Bax, Mcl-1 apoptosis genes, p53 status, estrogen receptor status (for breast cancer cells), and mdr1 gene expression (MCF-7/ADR cells). Apparent lack of impact of estrogen receptor status is particularly interesting since estradiol, acting via estrogen receptors, has been shown to activate anti-apoptotic pathways (49). Here, treatment of MCF-7 ER-positive breast cancer cells and Hs578T ER-negative breast cancer cells with equivalent doses of 5’Bcl-x AS resulted in similar levels of cell death. Furthermore, previous results showed that culturing MCF-7 cells in estradiol-free media did not enhance the apoptotic effects of 5’Bcl-x AS treatment (23). Thus, it appears that high expression of Bcl-xS is able to override several different anti-apoptotic pathways. These findings may be exploited as a prognostic tool to identify tumors that are most likely to benefit from 5’Bcl-x AS treatment. It is therefore encouraging that prostate cancer has higher levels of Bcl-xL compared with benign prostate (Fig. 9) or lower grade tumors (50). Furthermore, 5’Bcl-x AS should be quite specific as a drug since non-cancerous cells, that typically express low levels of Bcl-xL, should be relatively resistant to the treatment. While data presented in this paper suggest that the endogenous level of Bcl-xL is a major factor in several cell lines, the role of other factors in different cell lines cannot be ruled out. For example, cells may degrade the oligonucleotide faster, have different rates of mRNA turnover, varying expression levels of other apoptotic genes (such as caspases), or varying levels of proteins in pathways that interact with Bcl-xL and/or Bcl-xS function (e.g. PKC- and MEK-dependent pathways that regulate Bcl-xL expression, Refs. 51 and 52, and JNK, which phosphorylates Bcl-xL, Ref. 49).

The oligonucleotide-induced shift in splicing alone was able to cause significant cell death in PC3 cells and was even more effective in combination with chemotherapeutic agents, partic-
ularly with cisplatin and 5-FdU. Similarly, in MCF-7 cells the combination of cisplatin, 5-FdU, or doxorubicin with 5′-Bcl-x AS oligonucleotide was more effective than each agent alone. This sensitization of cells indicates that in clinical treatments the concentration of the toxic antineoplastic agents can be lowered up to 10-fold if, for example, the results with PC3 cells and cisplatin and 5-FdU could be recapitulated in prostate cancer patients. Since in clinical trials, similarly modified oligonucleotides were found to be relatively non-toxic (53, 54), overall toxicity of the treatment would be reduced.

The specific mechanisms responsible for frequently observed variations in the degree of sensitization to the different chemotherapeutic agents (55, 56) are not entirely clear. The five tested chemotherapeutic drugs, as well as radiation, damage DNA and induce apoptosis (57, 58). Yet, they varied in the ways they acted in combination with 5′-Bcl-x AS treatment. For example 5′-Bcl-x AS treatment effectively sensitized the cells to 5-FdU but not to 5-FU. The obvious difference between these two drugs is that although both compounds incorporate into DNA and affect its function, 5-FU is also incorporated into RNA where it interferes with several processes including splicing (59). To follow this lead, we have tested the effects of all the drugs on the shift in splicing of Bcl-xL to Bcl-xS. Neither 5-FU, nor for that matter the remaining drugs, had any clear effect on splicing of Bcl-x pre-mRNA, as determined by RT-PCR of total RNA of treated MCF-7 and PC3 cells (data not shown). On the other hand, higher sensitivity of MCF-7 (wild type p53) versus PC3 (mutant p53) cells to doxorubicin is consistent with the observation that doxorubicin is most effective in cells with wild type p53 (60). Even technical details such as order of addition of drugs may affect their interactions (61).

Kano et al. (62) found that in various cancer cell lines paclitaxel and cisplatin and 5-FdU could be recapitulated in prostate cancer lines, and Drs. Barry Goz and John Cidlowski (NIEHS) for reading this article. We thank Elizabeth Smith for technical assistance.

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