Abstract. Antisense oligonucleotides (oligos) have been employed against prostate cancer in both in vivo and in vitro models. While most oligos contain a single mRNA binding site, our laboratory has developed bispecifics directed towards two. Previous work has determined that when oligos are used to suppress the expression of individual proteins in highly regulated physiologic processes, additional proteins can be affected. These non-targeted (non-specific effects) regulators can compensate for proteins specifically targeted, reverse the intended effect and promote tumor growth. To evaluate specific and compensatory non-specific effects on growth inhibition of LNCaP cells employing mono- and bispecific oligos directed against BCL-2, LNCaP cells were incubated in the presence of oligos specifically directed against BCL-2 [the second binding site was directed against epidermal growth factor receptor (EGFR)] and compared to lipofectin containing controls. Significant, but comparable, growth inhibition was produced by mono- and bispecific forms. Employing RT-PCR to determine BCL-2 expression, mRNA suppression approached 100% for each oligo type: monospecific MR4 (directed only against BCL-2), 100%; and bispecifics MR24 and MR42, 86% and 100% respectively. Based upon inhibition of in vivo growth and BCL-2 expression, bispecific antisense oligos directed against EGFR and BCL-2 mRNAs are at least as effective as a monospecific directed towards BCL-2. To identify a compensatory response to evade apoptosis in the presence of BCL-2 suppression, levels of mRNA encoding non-targeted BAX, caspase-3 and clusterin were evaluated. We initially found that specific suppression of the apoptosis inhibitor BCL-2 in LNCaP cells does not affect (non-targeted) BAX expression and (non-targeted) caspase-3 expression was suppressed. This suggested that tumor cell variants develop which resist apoptosis through diminished expression of this promoter. This study suggests that compensatory changes in the regulation of apoptosis may not be widespread or be limited to apoptosis promoters (caspase-3), since the expression of the non-targeted apoptosis inhibitor clusterin is not affected. Should BCL-2 suppression be clinically employed with antisense oligos, it may only require maintenance (or replacement) of caspase-3 activity.

Gene therapy for cancer is a complex process because for it to work multiple physiologic pathways (and interacting proteins) must be simultaneously regulated (or replaced). For gene products which are overexpressed, methods to suppress activity have been developed, including the use of antisense oligonucleotides (oligos). While most oligos contain a single mRNA binding site, our laboratory has also developed bispecifics directed towards two. For the treatment of prostate cancer, some oligos (developed by Oncogenex Pharmaceuticals) have entered clinical trials (OGX-011); others follow in preclinical development (OGX-225). An oligo (Trabedersen) which targets transforming growth factor beta-2 has recently entered trials for treating patients with malignant melanoma, colorectal cancer, pancreatic cancer and highgrade glioblastoma (2).

Additional antisense oligos have been employed in both in vivo and in vivo prostate cancer models (3-10) targeting protein growth factors, androgens (oligos directed against 5-alpha reductase), receptors which bind stimulating factors and various...
oncogenes (11). Previous work in our laboratory, employing prostatic LNCaP and PC-3, as well as breast-derived MCF-7 and T98G glioblastoma cell lines (12-15) show statistically significant effectiveness of mono- and bispecific oligos directed against the autocrine loop consisting of transforming growth factor-alpha (TGF-α) and its binding site the epidermal growth factor receptor (EGFR), as well as apoptosis inhibitors BCL-2 and clusterin. Bispecifics directed against the combination of EGFR and BCL-2 as well as BCL-2 and clusterin were also effective alone and significantly enhanced when administered with therapeutics Rapamycin or Taxol (15).

Oligos provide a specific, relatively non-toxic method for translational arrest, and almost all are directed against single gene products (monospecific). While most assessments of activity quantitate the inhibition of in vivo growth, more specific methods use the polymerase chain reaction (PCR) to measure protein encoding mRNA.

Since most tumors are characterized by the enhanced expression of many proteins, growth is unlikely to be inhibited by knockdown of single gene products. Instead, multiple genes must be down-regulated. Therefore, in order to increase oligo efficacy we evaluated bispecific derivatives (3-7, 16) containing two binding sites on a single DNA strand. These bispecifics differ from those targeting genes which share sequence homology (BCL-2 and BCL-xL) (8) or the OGX-225 oligo which targets (three) structurally related insulin-like growth factor binding proteins (9). The addition of a second binding site does not appear to affect the activity of the other. Furthermore, dual binding sites can simultaneously be directed against genes involved in either a single growth-promoting autocrine loop (4) or towards those of different regulatory paths (5-7, 16), including apoptosis (directed against BCL-2).

Although oligos are increasingly entering clinical trials the effects upon non-targeted proteins are only recently being evaluated. Some of these have the potential to compensate for the original specific suppression within either the same regulatory pathway, or through others. As an example, in LNCaP cells, we found that the suppression of BCL-2 was accompanied by compensatory suppression of the apoptosis promoter caspase-3 (17) and increased expression of the androgen receptor (18). Either alteration could result in additional tumor growth, negating the tumor destructive effect (through restored apoptosis) originally intended by BCL-2 inhibition.

The purpose of this study was to further evaluate the effects of a single mono- and two bispecific oligos directed against BCL-2 on the apoptotic process employing RT-PCR to measure the expression of targeted BCL-2 and non-specific (compensatory) effects on non-targeted proteins, BAX, caspase-3 and clusterin. These proteins were chosen because they regulate apoptosis in opposing manners: BCL-2 and clusterin are inhibitory, while activated BAX and caspase-3 promote the process. If gene therapy is to be effective when directed against BCL-2, compensatory changes which compromise the effectiveness of the desired suppression must be identified. Among a mass of heterogeneous tumor cells, those which evade apoptosis are most likely to be selected and altered gene expression could include decreased expression of promoters BAX and/or caspase-3. If compensatory changes are initiated by BCL-2 suppression therapy, to re-establish apoptosis, the activity of promoters should at least be maintained (or replaced). Only the BCL-2 product was evaluated due to our previous experience in a human prostate cancer model, where a monospecific oligo (MR2) directed against EGFR suppressed in vivo cell growth in the absence of changes in the mRNA levels (10).

Materials and Methods

Oligonucleotides. Oligos (mono- and bispecific) were purchased from Eurofins MWG Operon (Huntsville, AL, USA). Each was phosphorothioated at the 5’ and 3’ positions on three terminal bases. Stock solutions were made to a final concentration of 625 µM in sterile Dulbecco phosphate buffered saline (PBS).


Cell culture. LNCaP cells (American Type Culture Collection, Manassas, VA, USA) were grown in RPMI 1640 supplemented with 10% bovine serum, 1% L-glutamine and 1% penicillin/streptomycin in a 5% CO2 incubator. On the day of transfection, the following solutions were prepared: A: One microliter of buffer containing either oligo or a diluent was added to 50 µl of OPTI-MEM (Invitrogen Inc., Carlsbad, CA, USA) and gently mixed. One dilution was made for each well. B: One microliter of lipofectin (Life Technologies Inc., Gaithersburg, MD, USA) was diluted in 50 µl of OPTI-MEM and mixed gently for 5 minutes at room temperature. C: Oligo dilutions were mixed with 50 µl of lipofectin and gently mixed for 20 minutes at room temperature. D: One hundred microliters of the lipofectin and oligo mixture was added to 100 µl of RPMI medium and mixed.

Cells were incubated for 24-48 hours before solutions were aspirated and re-incubated for an additional 48 hours in 200 µl of
media. Cell counts were determined following the addition of WST-1 reagent to each well, and after 2 hours, the color intensity was measured by a microplate reader at a wavelength of 450 nm, using a reference of 650 nm. Values obtained were determined after the subtraction of paired blank samples from the experimental wells and were multiplied by a constant to give whole integers for analysis. Microsoft Excel software was utilized to calculate means and standard deviations, and Student's t-tests were used to determine significance.

**Oligo treatment prior to PCR.** Four days prior to oligo addition, when cell density approached 75% confluence, 10 ml of fresh media was added. Cells were incubated for an additional 3 days before 5 ml of media was replaced with fresh the day before oligos were added. Stock oligos (100 μl) were added to bring the final concentration to 6.25 μM. Incubation proceeded for an additional 24 hours in the presence or absence of monospecific MR4, or the MR24 and MR42 bispecific oligos.

**RNA extraction.** Following treatment, media were removed, a single milliliter of cold (4˚C) RNAzol B was added to each 75 cm2 culture flask and the monolayer lysed by repeated passage through a pipette. All procedures were performed at 4˚C. The lysate was removed, placed in a centrifuge tube to which 0.2 ml of chloroform was added, and shaken. The mixture stayed on ice for 5 min, was spun at 12,000 × g for 15 min, and the upper aqueous volume removed and placed in a fresh tube. An equal volume of isopropanol was added, the tube shaken, and then left to stay at 4˚C for 15 min before similar centrifugation to pellet the RNA. The supernatant was removed, the pellet washed in a single milliliter of 75% ethanol, then spun for 8 min at 7500 × g. The ethanol was pipetted off and the formed pellet air dried at –20˚C.

**RNA quantitation.** RNA was resuspended in 250 μl of diethylpyrocarbonate-(DEPC, inhibitor of RNAse activity) treated water (Invitrogen), and quantitated using a Qubit fluorometer and Quant-iT RNA assay kit (Invitrogen).

**RT-PCR.** Extracted RNA was diluted to 40 μg/μl in DEPC treated water. One microliter of this RNA was added to 1 μl of both sense and antisense primers (forward and reverse sequences from Invitrogen) for human actin (used as a control) or 2 μl of combined primers for BCL-2, BAX, caspase-3 or clusterin (RealTimePrimers, Elkins Park, PA, U.S.A. (Table I). From a kit purchased from Invitrogen, the following reactants were added for RT-PCR: 25 μl of 2× reaction mixture, 2 μl SuperScript III RT/platinum Taq mix, tracking dye, and MgSO4 (3 μl of a stock concentration of 5 mM, used for BCL-2, BAX, caspase-3 and clusterin vials only). DEPC-treated water was added to yield a final volume of 50 μl. As a control for RT-PCR product production, human actin expression was tested in RNA extracted from HeLa cells which was provided in a kit purchased from Invitrogen (in the reaction mixture, no MgSO4 was included, the difference compensated for by 3 μl of DEPC treated water). RT-PCR was performed for 2×25 cycles using the F54 program in a Sprint PCR Thermocycler.

**Agarose gel electrophoresis.** 1.5% Agarose gels were prepared in a 50 ml volume of TBE buffer (1× solution: 0.089 M Tris borate and 0.002 M EDTA, pH 8.3) containing 3 μl of ethidium bromide (10 mg/ml in 1X Tris borate buffer) in a Fisher Biotest electrophoresis system. Samples were run for 2 hours at a constant voltage of 70 V using a BioRad 1000/500 power supply source. To locate the amplified PCR product, 3 μl of a molecular marker which contained a sequence of bases in 100 base pair increments (Invitrogen) as well as 2 μl of a sucrose based bromphenol blue tracking dye were run in each gel. For actin product localization, the tracking dye was included in each sample run; for all other products the tracking dye was run separately.

**Quantitation.** Gels were visualized under UV light and photographed using a Canon 800 digital camera. Photographs were converted to black and white format and bands quantitated using Mipav software provided by the National Institute of Health. Means and standard deviations were compared using Student t-tests to determine significance.

**Results**

**Cell culture experiments.** LNCaP cells were incubated with MR4, MR24 and MR42 and compared to lipofectin-containing controls (Figure 1). In an initial experiment (Experiment 1) each oligo significantly inhibited the growth of LNCaP cells: MR4 by 23.8% (p=0.0004), MR24 by 31.2% (p<0.001) and MR42 by 31.7% (p<0.001).
In a repeat experiment (Experiment 2), employing only the bispecifics, LNCaP cells were similarly incubated and compared to lipofectin-containing controls. MR24, and MR42 produced significant respective inhibitions of 49.5% \((p<0.001)\) and 56.8% \((p<0.001)\), and were at least as effective as the monospecific MR 4 directed only towards \(\text{BCL-2}\) in the inhibition of \(\text{in vivo}\) cell growth (Experiment 1).

**RT-PCR experiments.** In a series of control experiments to validate RNA extraction and RT-PCR procedures, the expression of human actin in HeLa cells was identified (19), its 353 base pair product appearing as expected between the 300 and 400 base pair markers (Figure 2).

**BCL-2 expression.** LNCaP cells incubated for 24 hours in the presence of 6.25 \(\mu\)M of oligos suppressed \(\text{BCL-2}\) expression, supporting the finding of comparable biologic activity using both mono- and bispecific oligos in the \(\text{in vivo}\) cell growth inhibition experiments. When photographs of the identified product bands were scanned on agarose gels and quantitated using Mipav software, in a series of runs, the greatest expression of \(\text{BCL-2}\) was always found in untreated LNCaP cells. Those treated with oligos, whether mono- or bispecific, produced bands which indicated obvious (to the naked eye) suppression. For each oligo evaluated, the greatest amount of suppression measured approached 100% for the monospecific MR4; and for the bispecifics MR24 and MR42, 86% and 100%, respectively. Suppression was found in both repeat PCR runs with \(\text{BCL-2}\) primers, as well as in repetitive agarose gel quantifications. Figure 3 presents a \(\text{BCL-2}\) product band in the expected 127 base pair region which in this run was inhibited 23% by treatment with the monospecific MR4, and 86% and 74%, respectively by bispecifics MR24 and MR42, as measured by Mipav software.

**BAX expression.** Comparable amounts of extracted RNA from LNCaP cells treated with either mono- or bispecific oligos directed against \(\text{BCL-2}\) (and \(\text{EGFR}\) in the bispecifics) was then evaluated by RT-PCR using primers directed against BAX. A representative band for BAX is presented in Figure 4 and appears below the marker representing 200 base pairs in the expected 168 base pair region.

When the background intensity was subtracted, the relative intensity of the bands corresponding to BAX representing cells treated with MR4, MR24 and MR42 compared to controls was \(-5.74\%\pm16.9, \ 5.54\%\pm19.2, \ \text{and} \ -15.34\%\pm32.9\) (mean\(\pm\)SD). These results were pooled from both duplicate PCR runs and gels, indicating no significant differences in BAX expression, compared to that seen with \(\text{BCL-2}\).

**Caspase-3 expression.** Comparable amounts of extracted RNA from LNCaP cells treated with either mono- or bispecific
oligos directed against BCL-2 (and EGFR in the bispecifics) were then evaluated by RT-PCR using primers directed against caspase-3. A representative band for caspase-3 is presented in Figure 5 and appears below the marker representing 300 base pairs in the expected 262 base pair region.

When the background intensity was subtracted, the relative intensity of the bands corresponding to caspase-3 representing cells treated with MR4, MR24 and MR42 compared to controls was $-35.8\% \pm 12.5 \ (p=0.0002)$, $-40.3\% \pm 16.6 \ (p=0.0006)$ and $-43.5\% \pm 26.3 \ (p=0.006)$. These results were pooled from both duplicate PCR runs and gels, and indicate similar (significant) suppression of caspase-3 activity is produced by both mono- and bispecific oligo types.

While gene therapy is often aimed at suppressing BCL-2, for re-establishment of apoptosis caspase-3 expression is essential. These experiments identify a mechanism by which tumor
variants can (again) evade apoptosis through the diminished expression of an apoptosis promoter (caspase-3). It also identifies caspase-3 as a necessary gene for expression (or replacement therapy) when oligos target BCL-2 for suppression.

Clusterin expression. Comparable amounts of extracted RNA from LNCaP cells treated with either mono- or bispecific oligos directed against BCL-2 (and EGFR in the bispecifics) was then evaluated by RT-PCR using primers directed against clusterin. A representative band for clusterin is presented in Figure 6 and appears where expected, between the markers representing 200 and 300 base pairs, as a 221 base pair product.

When the background intensity was subtracted, the relative intensity of the bands corresponding to clusterin representing cells treated with MR4, MR24 and MR42 compared to controls were 8.3%±14.5, 9.0%±17.3, and −14.1%±22.6 (mean±SD). These results were pooled from both duplicate PCR runs and gels, indicating (like BAX) there are no significant differences in clusterin expression compared to that seen with caspase-3.

Discussion

Gene therapy for cancer is a complex process because for it to work several pathways, particularly those associated with apoptosis or growth (both androgen and protein stimulated) must be simultaneously regulated (or replaced).

For gene products which are overexpressed, methods to suppress their activities have been developed, including antisense oligos which act via translational inhibition and RNAs destruction of complementary mRNA:oligo hybrids. For those proteins diminished or lacking in expression gene replacement, promotion or amplification would be necessary. As an example, replacement of the phosphatase and tensin homolog (PTEN) suppressor gene through transfection restores sensitivity to radiation treatment for prostate cancer (20).

Within a tumor mass, cells are individually heterogenous, and those which evade growth regulation or programmed cell death (apoptosis) are selected for. In addition, as their DNA becomes increasingly unstable, variants tend to accumulate additional adaptations (mutations) which further contribute to malignancy, dissemination, resistance to apoptosis, and, as a result of suppressed BCL-2 expression (18), we find the potential for increased androgen sensitivity mediated via its receptor. Selection of cells which resist apoptosis is no different than the process by which hormone sensitive prostate tumor cells, in the absence of androgen, are selected (and establish themselves) as insensitive variants. As a result of oligo-mediated BCL-2 suppression, two pathways for treatment resistance appear to become interrelated (18).

Treatment protocols administered to correct one genetic alteration can, through selective pressure, initiate compensatory changes which diminish the effectiveness of the original, and in many tumors, some of the early mutational events lead to evasion of apoptosis. This programmed process (apoptosis) clears the body of altered (transformed) or damaged cells. It is highly regulated and involves many proteins being synthesized, recognized (via receptors) or otherwise interacting with or activating each other. The efficiency of this process is regulated by many proteins, acting in multiple pathways, with individual pathways often regulated by the overall contribution and ratio of both stimulatory and inhibitory influences (21, 22). Clinically, gene therapy protocols are being administered which suppress expression of apoptotic inhibitory proteins BCL-2 and clusterin. Several include antisense oligos meant to restore apoptosis associated with chemotherapy (8) and radiation treatment (23, 24), and one is administered against prostate cancer (23).

In these experiments, we evaluated the effect of oligo-mediated growth suppression on four regulators of apoptosis: BCL-2 and clusterin (inhibitors), and BAX and caspase-3 (promoters). Based upon both inhibition of cell growth and BCL-2 expression, we conclude that bispecific antisense oligos directed against BCL-2 and EGFR, regardless of their tandem orientation, are at least as effective as the mono-specific type directed solely towards BCL-2. The addition of a second
mRNA binding site (directed towards EGFR) to these oligos does not prevent activity at the site specific for BCL-2.

Therapeutic action produced by both mono- and bispecific oligos diminished BCL-2 activity as determined by RT-PCR. Inadvertently, it was compensated by diminished caspase-3 activity (a non-targeted promoter of apoptosis); while BAX and clusterin (neither targeted) activity remained unaffected. As a result of reduced caspase-3 activity, the apoptosis pathway can either become or remain suppressed even in the absence of BCL-2.

This study suggests that compensatory changes in the regulation of apoptosis may not be widespread or be limited to some apoptosis promoters (such as caspase-3), since the expression of the non-targeted apoptosis inhibitor clusterin is not affected. If clusterin expression had been increased, the apoptotic process could be further inhibited. These experiments (summarized in Table II) identify a mechanism by which tumor variants are selected which (again) evade apoptosis through the diminished expression of apoptosis promoters. Maintaining caspase-3 expression is essential if BCL-2 is suppressed and we identify it as a necessary gene for expression (or replacement therapy) when oligos target BCL-2.

For gene therapy to be successful, effects on untargeted genes must be identified. Should additional proteins (inhibitors of apoptosis) be indicated for suppression, bispecific oligos or even a proposed multifunctional/branched derivative could be developed (25). Additional studies are underway to identify altered expressions in other proteins associated with apoptosis.

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Table II. Summary of gene expression results directed against BCL-2.

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<th>OLIGOS</th>
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<th>Bispecific</th>
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<td>Inhibitor of apoptosis</td>
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<td>Non-targeted BAX</td>
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<td>Promoter of apoptosis</td>
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<td>Inhibitor of apoptosis</td>
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