in vivo *24*: 489-494 (2010)

Bispecific Antisense Oligonucleotides have Activity Comparable to Monospecifics in Inhibiting Expression of BCL-2 in LNCaP Cells

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Abstract. Antisense oligonucleotides (oligos) have been employed against prostate cancer models in both in vivo and in vitro systems. Most oligos employed by investigators include only a single mRNA-binding site, and target only a single gene. However, some target multiple genes which share sequence homology. Recently, our lab has developed bispecific oligos, which target two different genes not related by sequence homology, and which are able to regulate activity in either the same or in different biological pathways. To date, the effectiveness of bispecific oligos has been evaluated solely by in vitro cell growth inhibition studies. This study evaluates the suppression of targeted mRNA by both mono- and bispecific oligos directed towards the apoptosis regulatory protein BCL-2. The bispecifics used contain binding sites for both the epidermal growth factor receptor (EGFR) and BCL-2 mRNA and differ only in the 5' to 3' tandem orientation. LNCaP cells incubated for 24 hours in the presence of 6.25 μM of oligos suppress the expression of BCL-2 mRNA and support the finding that there is comparable biologic activity produced by both mono- and bispecific oligos in in vitro cell inhibition experiments. For each type of oligo (mono- or bispecific) evaluated, the greatest amount of BCL-2 mRNA suppression approached 100% as produced by the monospecific MR_4 (directed only against BCL-2) and for the bispecifics $MR2_4$ and MR_{42}, 86% and 100%, respectively. Suppression was found in duplicate PCR runs employing BCL-2 primers, as well as in multiple agarose gel quantifications. Based upon both inhibition of in vitro growth and bCL-2 expression measured by PCR, we conclude that bispecific antisense oligos directed against both EGFR and BCL-2 mRNAs are at least as effective as a monospecific oligo directed solely towards BCL-2. Therefore the addition of a second mRNA-binding site to these oligos does not prevent activity at the initial site specific for BCL-2.

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Key Words: Antisense, prostate cancer, therapy.

Antisense oligonucleotides (oligos) have been employed against prostate cancer models in both *in vivo* and *in vitro* systems. Genes targeted have included protein growth factors, receptors for these factors and androgens, inhibitors of apoptosis (BCL-2), oncogenes, and more recently chaperon proteins. Antisense oligos are usually administered either directly employing various means of transfection, or they can be inserted using retroviral vectors (1). Oligos provide a specific and relatively non-toxic method for translational arrest. However, it is probably naïve to believe that inhibiting the expression of a single gene is likely to affect a cure, regulate growth, limit metastatic dissemination, or restore apoptosis and androgen dependency. Therefore, for this method of gene therapy to be truly effective, multiple genes (which are overexpressed) must be suppressed; and those which are suppressed, or mutated must be replaced.

Antisense oligos act through a variety of mechanisms which include degradation of annealed mRNA:oligo duplexes by RNase H (2), protein binding and DNA triplex formation (3, 4). Most studies of oligo activity reported have been quantitated indirectly by inhibition of *in vitro* growth, however, more direct methods can use polymerase chain reaction (PCR) to measure suppression of specific mRNA.

Our laboratory has attempted to increase the efficacy of oligo activity through combination therapy, and various chemotherapeutic agents have been administered either in combination (5-7), or in sequence (8, 9), with oligos. More recently, we suggested and are currently engaged in the evaluation of newly described bispecific oligos (5-7, 10-12).

These derivatives contain more than one binding site on a single linear DNA strand and are able to target two genes, which can be unrelated in base sequence or biochemical pathway of their activity. This approach is distinctly different from those using oligos which target two or more genes sharing sequence homology (13) and the OGX-225 oligo which targets three insulin-like growth factor-binding proteins (14).

Bispecifics have been evaluated against genes involved in both a single autocrine loop (5), as well as those found in several growth regulatory paths (6, 7, 11, 12), but until now, results have been evaluated solely by inhibition of *in vitro* cell growth. This is the first report involving bispecific oligos which reduce the mRNA of a targeted gene product (BCL-2), utilizing PCR. The bispecifics employed are directed against both the epidermal growth factor receptor (EGFR) and BCL-2. Only the BCL-2 product was evaluated due to our previous experience in a human prostate cancer model, where a monospecific oligo (MR_2) directed against EGFR suppressed *in vitro* cell growth in the absence of changes in the mRNA levels (15).

Materials and Methods

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

Base sequences. Each oligo contained at least one CAT sequence and targeted the area adjacent to the mRNA AUG initiation codon for the respective targeted protein (EGFR or BCL-2).
MR_4 (monospecific targeting BCL-2) T-C-T-C-C-C-A-G-C-G-T-G-C-G-C-C-A-T; MR_{24} (bispecific targeting EGFR/BCL-2) G-A-G-G-G-T-C-G-C-A-T-C-G-C-T-G-C-T-C- T-C-T-C-C-C-A-G-C-G-T-G-C-G-C-C-A-T MR_{42} (bispecific targeting BCL-2/EGFR) T-C-T-C-C-C-A-G-C-G-T-G-C-G-C-C-A-T-G-A-G-G-G-T-C-G-C-A-T-C-G-C-T-G-C-T-C

Cell culture. LNCaP cells (American Type Culture Collection [ATCC], Manassas, VA, USA) were grown in RPMI-1640 supplemented with 10% bovine serum, 1% L-glutamine and 1% penicillin/streptomycin in a 5% CO_2 incubator. Log-phase cells were harvested using EDTA/trypsin and equally distributed into 75 cm^2 flasks (Corning, NY, USA). At intervals media was either supplemented or replaced with fresh.

Determination of growth. Four days prior to the addition of oligos, 1×10^4 LNCaP cells were added in a total 200 μl volume of media to each depression of a 96-well plate and incubated at 37˚C in a 5% CO_2 incubator. On the day of transfection the following solutions were prepared: A) 1 μl 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) 1 μl 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) 100 μl 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. Fours days prior to oligo addition, when cell density approached 75% confluence, 10 ml of fresh media were 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 MR_4, or the MR_{24} and MR_{42} bispecifics.

RNA extraction. Following treatment, media were removed, 1 ml of cold (4˚C) RNAzol B was added to each 75 cm^2 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, then allowed to stay at 4˚C for 15 min before similar centrifugation to pellet the RNA. The supernatant was removed, the pellet washed in 1 ml of 75% ethanol, then spun for 8 min at 7,500×*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) treated water (Invitrogen), and quantitated using a Qubit florometer and Quant-iT RNA assay kit (Invitrogen). DEPC is an inhibitor of RNase activity.

RT-PCR. Extracted RNA was diluted to 40 μg/μl in DEPC treated water and 1-4 μl of this RNA was added to1 μl of both sense and antisense primers (forward and reverse sequences) for either BCL-2 or human actin (used as a control). 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 $MgSO_4$ (3 μl of a stock concentration of 5 mM, used for BCL-2 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. RT-PCR was performed for 2×25 cycles using the F54 program in a Sprint PCR Thermocycler

Primers used.
Actin: Antisense primer sequence: 5' CAA ACA TGA TCT GGG TCA TCT TCT C 3', sense primer sequence: 5' GCT CGT CGT CGA CAA CGG CTC. The PCR product produced was 353 base pairs in length.

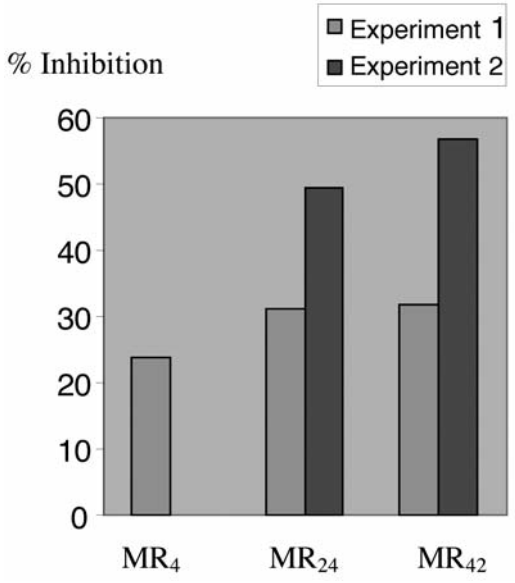

Figure 1. *Inhibition of in vitro growth of LNCaP cells by mono- and bispecific oligonucleotides. Values represent means from replicate samples.*

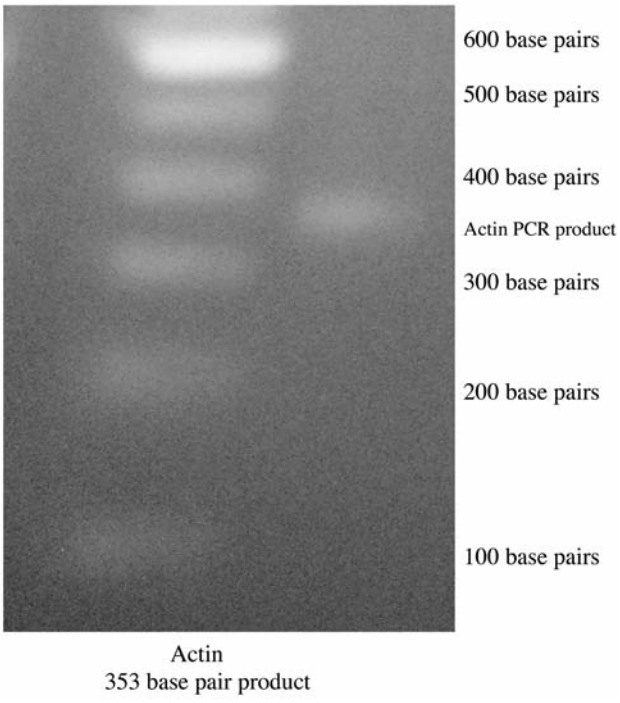

Figure 2. *Agarose gel showing actin control PCR product migrating within the base pair region expected.*

Bcl-2: Antisense primer sequence: 5' GAG ACA GCC AGG AGA AAT CA 3', sense primer sequence: 5' CCT GTG GAT GAC TGA GTA CC 3'. The PCR product produced was 127 base pairs in length.

Detection and quantitation of product.
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 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 (Invitrogen) 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 the BCL-2 product the tracking dye was run separately.
Quantitation: Gels were visualized under UV light and photographed using a Canon 800 digital camera. Photos were converted to black and white format and bands quantitated using Mipav software provided by the National Institutes of Health (NIH).

Results

Cell culture experiments. LNCaP cells were incubated with MR_4, MR_{24} and MR_{42} and compared to lipofectin-containing controls (Figure 1). In an initial experiment, each oligo significantly inhibited the growth of LNCaP cells: MR_4 by 23.8% (p=0.0004), MR_{24} by 31.2% (p<0.001), and MR_{42} by 31.7% (p<0.001).

In a repeat experiment LNCaP cells were similarly incubated and compared to lipofectin containing controls. MR_{24}, and MR_{42} produced respective significant inhibitions of 49.5% (p<0.001) and 56.8% (p<0.001).

Bispecific oligos MR_{24} and MR_{42} which targeted both EGFR and BCL-2 were at least as effective as the monospecific MR_4 directed only towards BCL-2 in the inhibition of *in vitro* cell growth.

RT-PCR 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 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. Figure 2 provides an example of one such band (actin) suitable for scanning and quantitation. The molecular weight markers shown in the left column are (in 100 base pair increments) 600 and 100 base pairs (from top to bottom). The band visualized between molecular markers of 300 and 400 base pairs is the expected 353 base pair human actin PCR product.

LNCaP cells incubated for 24 hours in the presence of 6.25 µM of oligos demonstrate a suppression of BCL-2 expression, and support the finding of comparable biologic activity of both mono- and bispecific oligos seen in *in vitro* cell inhibition experiments. For each oligo evaluated, the greatest amount of suppression measured approached 100% for both the mono-specific MR_4 and for the bispecifics MR_{24} and MR_{42}, 86% and 100%, respectively. Suppression was found in both repeat PCR runs with *BCL-2* primers, as well

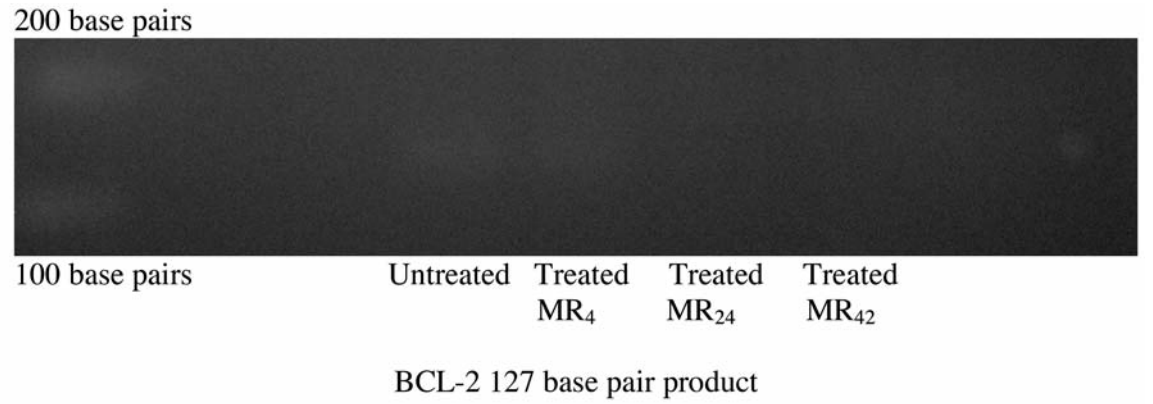

Figure 3. *Agarose gel showing BCL-2 product suppressed by both mono- and bispecific oligo treatment. PCR product is within the expected base pair region.*

as in repetitive agarose gel quantifications. Figure 3 presents a BCL-2 product band in the expected 127 base pair region which was inhibited 23% by treatment with the monospecific MR_4, and 86% and 74%, respectively by bispecifics MR_{24} and MR_{42}, as measured by Mipav software.

Based upon both inhibition of cell growth and BCL-2 expression, we conclude that bispecific antisense oligos directed against EGFR and BCL-2, regardless of their tandem orientation, are at least as effective as the monospecific 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 initial site specific for BCL-2.

Discussion

Genetics-based therapies for cancer are still in the conceptual phase, and although we have identified many pathways which regulate cell growth, the genes involved, and have ways to measure their expression from a clinical perspective, the resulting protocols are largely ineffective. Therefore, it is apparent that for gene therapy to progress, it is necessary that at least several pathways (and their regulatory genes) be simultaneously regulated. For gene products which are overexpressed, methods to suppress their activities have been developed and several of these are clinically useful; for those which are diminished or lacking, methods for their replacement must be more safely developed.

Translational arrest can be accomplished by several antisense strategies (1-4). In the simplest form, this type of therapy provides a strand of nucleic acid whose base sequence is complementary to a portion of encoding mRNA. Binding to this section prevents the mRNA from being translated, and also promotes nuclease degradation. A cocktail of antisense oligos could thus theoretically shut down many of the overexpressed genes, and improve regulation of cell growth. Rather than employ a pool of separate oligos, each targeting a different gene (16) it would be desirable if several activities could be regulated by a single oligo. Such oligos have previously been reported by others (13). However, these oligos target genes which share a region of sequence homology, and such genes often have similar biologic activity, and, if they encode growth factors or binding proteins interact with similar targets (14). What we define as true bispecific oligos target more than one protein (unrelated in sequence), and may even bind mRNA encoding proteins from unrelated pathways.

We have evaluated the activity of bispecific oligos which regulate proteins within the same autocrine loop (5), as well as those which regulate proteins involved in distinctly different biological paths (6, 7, 11, 12). In addition, we have evaluated them in both the presence and absence of traditional chemotherapeutics (diethylstilbestrol [DES], Taxol, Cytoxan, mitoxantrone and platinates) (6, 8, 9), or immune regulatory agents (rapamycin) (7). Our studies demonstrate that in *in vitro* culture, bispecific oligos are at least comparable to monospecific oligos which target only one of the proteins employed on the bispecific. In addition, we find that oligo activity can be significantly enhanced by the combination therapy described above.

In this study, we specifically evaluated the expression of one protein (BCL-2) targeted by both mono- and bispecific oligos. Using RT-PCR we found that the suppressive effect of oligos directed against BCL-2 was similar in both mono- and bispecfic oligos. This conclusion is important because when regulating excessive gene expression, it would be best that more than one protein be regulated by a single administered agent. These findings could also be important in regulating expression of microRNA (miRNA). This is a rapidly evolving concept where naturally occurring miRNA has been found to either promote cancer cell growth, or to prevent it (suppressive miRNA). Regulation of (cancer cell) growth promoting miRNA (oncogenic) could be similarly targeted by

complementary sequenced oligos, and since there are over 500 identified miRNAs, and many are overexpressed in cancer, multispecific approaches would again have to be considered.

We predict that for antisense therapy to take the next step forward, more complex forms of antisense must be formulated and delivery mechanisms enhanced. Multichain (and fat soluble) oligos have already been proposed which could be constructed specifically to target specific gene combinations particularly overexpressed or activated in an individual patient or tumor type based on microarray analysis (17). Furthermore, mechanisms for enhanced delivery and stability, some of which employ polymalic acid (18) or nanoparticle formation with polypropylin-imine dendrimers (19), have also been evaluated.

Our conclusions are modest and indicate comparable suppressive activity by mono- and bispecific oligos targeting BCL-2 as determined by both inhibition of *in vitro* growth as well as the expression of the targeted protein. This implies that multiple targeting techniques can be developed which do not compromise the activities of additional binding sites on the same molecules (oligos).

Acknowledgements

The Cellular Biology Laboratory at the Hektoen Institute is supported, in part, by the Blum Kovler Foundation, the Cancer Federation, Safeway/Dominicks Campaign for Breast Cancer Awareness, Lawn Manor Beth Jacob Hebrew Congregation, the Max Goldenberg Foundation, the Sternfeld Family Foundation, and the Herbert C. Wenske Foundation.

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Received January 22, 2010
Revised April 20, 2010
Accepted April 23, 2010