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Additional Compensatory Mechanisms Altering Antisense Oligonucleotide Suppression of BCL2: Effects upon AKT1 and STAT3

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Abstract. *Antisense oligonucleotides have targeted regulatory proteins in both in vivo and in vitro prostate cancer models. We evaluated mono- and bispecific oligonucleotides which targeted and comparably suppressed B-cell lymphoma-2 BCL-2 (an apoptosis-inhibitory protein) expression in LNCaP cells. These oligonucleotides were administered with lipofectin as part of a nanoparticle delivery system. Treated cells compensated by suppressing caspase-3 (an apoptosis promoter) and enhancing expression of the androgen receptor and its co-activating p300 and IL-6 proteins. This suggests a progression to increased androgen sensitivity (in LNCaP) accompanies BCL-2 suppression and a gene activation pattern associated with more advanced prostate tumors. To further evaluate compensatory mechanisms related to tumor resistance in the present study we evaluate the expressed levels of the AKT1 oncogene and STAT3 transcription factor, finding both to be enhanced.*

Although theoretically specific, gene therapy often encounters difficulties in practice and while suitable targets are found in many regulatory pathways, their levels of expression in tumor cells are similar to those of healthy cells. Resistance to anticancer agents develops because the biochemical pathways involved are complex, and multiple compensatory mechanisms develop which restore an aggressive phenotype (1). Just as bacteria and viruses mutate to evade therapeutic agents, tumor cells are under similar selective pressure to evade chemotherapy; and the unintended consequences are only now being evaluated.

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Gene therapy has been applied for the treatment of human prostate tumors and, together with radio- (2, 3) or chemotherapy (4), antisense oligonucleotides have been administered against B-cell lymphoma-2 BCL-2 and clusterin (apoptosis inhibitors) in attempts to maintain or restore apoptosis. If such therapy is to be successful, mechanisms by which tumors compensate and become resistant (and regulatory proteins involved) must be identified. Previous studies have identified that oligonucleotide-mediated BCL-2 inhibition suppressed the expression of the apoptotic promoter caspase-3 (1) while enhancing AR (5), p300 (6), IL6 (7) and v-MYC (8) expression. This suggests that following BCL-2-suppressive therapy there is selective pressure for a more aggressive (androgen-sensitive) phenotype promoted by associated transcription factor and oncogene-driven pathways. Unexpectedly, similar studies found that side-effects produced by administering these types of oligonucleotides can also affect proteins associated with immunological targeting. The enhanced expression of prostate-specific membrane antigen (PSMA) (9) is produced only by these bispecific oligonucleotides (and not a similarly directed monospecific) directed against BCL-2. This is attributed to their intrastrand complementary base sequences which allow formation of 2X regions, able to induce interferon (an enhancer of surface antigen expression, similarly enhanced in its expression by these oligonucleotides) (10). Increased surface antigen expression could lead to better recognition and targeting by cytotoxic T-cells. The expression of secreted antigens associated with prostate cancer, namely prostate-specific antigen, prostatic acid phosphatase, and more recently prostate cancer antigen, are not increased (11).

Previous studies investigated compensatory involvement in apoptosis, androgen regulation, angiogenesis, autocrine and oncogene activities (reviewed in reference 11). We now evaluate an additional oncogene (*AKT1*) and transcription factor (STAT3) associated with metastatic progression of prostate cancer (12). For gene therapy to be successful, it must be made more specific and mechanisms of compensation identified and suppressed.

Materials and Methods

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

Base sequences. Oligonucleotides 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 (ATCC, Rockville, MD, 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 were either supplemented or replaced with fresh.

Oligonucleotide treatment prior to PCR. Fours days prior to oligonucleotide addition, when cell density approached 75% confluence, 10 ml of fresh medium was added. Cells were incubated for an additional three days then 5 ml of medium was replaced with fresh the day before oligonucleotides were added. Stock oligonucleotides (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 bispecific MR_{24} and MR_{42}.

RNA extraction. Following treatment, the media were removed, a single milliliter 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, and allowed 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 pellet formed air dried at −20˚C.

RNA quantitation. RNA was resuspended in 250 μl of DEPC-treated water, and quantitated using a Qubit florometer and Quant-iT RNA assay kit (Invitrogen, Grand Island, NY, USA). DEPC is an inhibitor of RNase activity.

RT-PCR. Extracted RNA was diluted in DEPC-treated water to 40 μg/μl. One to four milliliters of this RNA was added to 1 μl of both sense and antisense primers (forward and reverse sequences) for actin, *BCL-2, AKT1* and *STAT3*. From a kit purchased from Invitrogen, the following reactants were added for RT-PCR: 25 μl of 2X reaction mixture, 2 μl SuperScript III RT/platinum Taq mix, tracking dye, and 3 μl $MgSO_4$ (of a 5 mM stock concentration). DEPC-treated water was added to yield a final volume of 50 μl. RT-PCR was performed for 2×25 cycles using the F54 program in a Sprint PCR Thermocycler Bio-Rad, Hercules, CA, USA). As a control for RT-PCR products, 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 $MgSO_4$ was included, the difference compensated for by 3 μl of DEPC-treated water).

Primers. Actin: Forward primer sequence: 5' CAA ACA TGA TCT GGG TCA TCT TCT C 3', reverse primer sequence: 5' GCT CGT CGT CGA CAA CGG CTC; PCR product was 353 base pairs in length. *BCL-2*: Forward primer sequence: 5' GAG ACA GCC AGG AGA AAT CA 3', reverse primer sequence: 5' CCT GTG GAT GAC TGA GTA CC 3'; PCR product was 127 base pairs in length. *AKT1*: Forward primer sequence: 5' ACC TTT TCG ACG CTT AAC CT 3', reverse primer sequence: 5' TGG AGG GAA GGT TCC ATA TT 3'; PCR product was 189 base pairs in length. *STAT3*: Forward primer sequence: 5' CCT TTG ACA TGG AGT TGA CC 3', reverse primer sequence: 5' TAA AAG TGC CCA GAT TGC TC 3'; PCR product was 213 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 (1X solution: 0.089 M Tris borate and 0.002M EDTA, pH 8.3), containing 3 μl of ethidium bromide in a Fisher Biotest (St. Louis, MO, USA) electrophoresis system. Samples were run for 2 hours at a constant voltage of 70 V using a BioRad (Hercules, CA, USA) 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.

Quantitation: Gels were visualized under UV light and photographed using a Canon PowerShot ELPH 300 HS digital camera (Best Buy, Glenview, IL, USA). Photos were converted to black and white format and bands quantitated using Medical Image Processing and Visualization (Mipav) software provided by the National Institute of Health (Bethesda, MD, USA). Means and standard deviations were compared using Student *t*-tests to determine significance.

Results

BCL-2 expression. As a control for RT-PCR product production, human actin expression was tested in RNA extracted from HeLa cells (1).

LNCaP cells incubated for 24 h in the presence of 6.25 μM of oligonucleotides suppressed *BCL-2* expression, and supports the finding of comparable biological activity for both mono- and bispecific oligonucleotides measured in the *in vitro* cell growth inhibition experiments (1). When photographs of the identified product bands were scanned on agarose gels and *BCL-2* quantitated, the greatest expression was always found in untreated LNCaP cells. Those treated with oligonucleotides, whether mono- or bispecific, produced bands which clearly indicated (to the naked eye) suppression. For each oligonucleotide evaluated, the greatest amount of suppression measured approached 100% for the monospecific MR_4, and 86% and 100%, respectively, for the bispecific MR_{24} and MR_{42}. Suppression was found in both repeat PCR runs with *BCL-2* primers, as well as in repetitive agarose gel quantifications.

Figure 1. *Increased expression of AKT1 by agarose gel. AKT1 is a 189 base-pair product.*

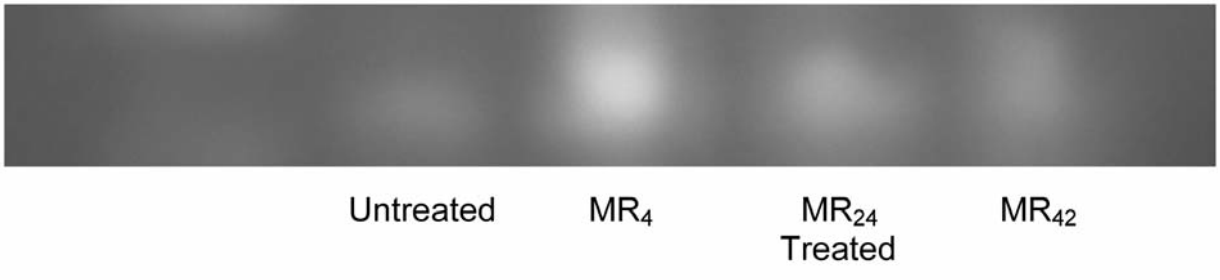

Figure 2. *Increased expression of STAT3 in agarose gel. STAT3 is a 213 base-pair product.*

AKT1. Identical amounts of extracted RNA from LNCaP cells treated with either mono- or bispecific oligonucleotides directed against BCL-2 (and EGFR in the bispecifics) were then evaluated by RT-PCR using primers directed against *AKT1*. When the background intensity was subtracted, the relative intensity of all bands corresponding to *AKT1* representing cells treated with MR_4, MR_{24} and MR_{42} compared to controls were enhanced 256.7%±105.5% (p=0.000616), 189.4%±73.6% (p=0.000425) and 182.6%±90.8% (p=0.002014). Figure 1 shows a representative gel.

STAT3 expression. Identical amounts of extracted RNA from LNCaP cells treated with either mono- or bispecific oligonucleotides directed against *BCL-2* (and *EGFR* in the bispecifics) were then evaluated by RT-PCR using primers directed against *STAT3*. When the background intensity was subtracted, the relative intensity of all bands corresponding to *STAT3* representing cells treated with MR_4, MR_{24} and MR_{42} compared to controls were increased 166.2%±88.5% (p=0.009439), 110.9%±49.7% (p=0.004279) and 37.8%±22.6% (p=0.015529). Figure 2 shows a representative gel.

Discussion

Gene therapy is often promoted as a highly specific treatment to control excessive gene expression by tumor cells (particularly of growth factors, their receptors or apoptosis inhibitors), however, it is now apparent that such therapy is not as specific as previously thought. Antisense oligonucleotides consist of nucleotide bases synthesized complimentarily in sequence to mRNA. When hybridized to mRNA, they arrest the targeted gene's mRNA expression (by translational inhibition). In clinical trials against a variety of solid tumors, the administration of first-, second- and third-generations of RNA oligonucleotides, and, more recently, siRNA derivatives, provides an effective, relatively non-toxic, inexpensive form of suppressive gene therapy. While it is understandable that genes sharing sequence homology could be inadvertently targeted when oligonucleotides are directed at common sequences, what are not expected are the non-specific effects on unrelated, untargeted genes, including those which regulate additional growth pathways, antigen expression or immune targeting.

Tumors consist of genetically unstable heterogenous cells capable of both rapid mutation and selection. Just as bacteria and viral agents develop resistance to chemotherapeutics, tumor cells react similarly to selective pressures. Hundreds of genes drive tumor cell growth and, in addition, these genes are activated by androgen bound to AR and its transcriptional cofactors (p300 and IL6). While normal cell growth and death pathways (apoptosis) are highly regulated, tumor cells develop resistance through increased production of or sensitivity to growth factors, hormones, oncogenes, or through evasion of apoptosis. This is why both growth and death pathways provide the targets for much of the gene therapy being commercially developed and mediated via antisense oligonucleotides.

Oligonucleotides produced by Oncogenex Pharmaceuticals have reached clinical trials for the treatment of prostate cancer (OGX-011), while others remain in preclinical development (OGX-225). Often administered in combination with traditional chemotherapy, these target *BCL-2*, clusterin (OGX-011 in phase II testing), heat-shock protein 27 (OGX-427), or insulin growth factor-binding proteins (OGX-225) (13). Prior to

liquidation, Genta conducted a phase III test using oligos (Genasense; oblimersen) directed against *BCL-2* for treating melanoma, chronic lymphocytic leukemia and various solid tumor types, but compensatory effects produced by this agent were not reported. Many treatments employing oligonucleotides represent efforts to restore tumor apoptosis by eliminating suppressive BCL-2 (2-4) associated with treatment resistance. Similar approaches are directed at clusterin.

Our laboratory has developed and tested bispecific oligonucleotides containing dual mRNA binding sites. We first reported that mono- and bispecific oligonucleotides directed against *BCL-2* had comparable activity when evaluated using RT-PCR (14). Subsequent experiments tested their activity against other proteins associated with growth and development, in an effort to identify compensatory changes in other pathways which could influence BCL-2 suppression. Since oligonucleotides directed against *BCL-2* are in clinical trials, it is important to identify compensatory changes. Our program has diverged into several areas of investigation in response to specific oligonucleotide-mediated *BCL-2* suppression. Investigated proteins are those associated with apoptosis, androgen regulation, oncogenes and immune targeting of surface antigens. As stated above (and reviewed in 11), compensatory mechanisms have been found in each area and this current study identifies further involvement of transcriptional factors and oncogenes.

This year (2014) the American Cancer Society estimated that in spite of early detection, screening for prostate-specific antigen and effective treatments for localized disease, in the United States there will be 29,840 deaths from prostate cancer, with 233,000 newly diagnosed cases (15). New types of treatment, including gene therapy and translational inhibition, must be developed and employed (probably in combination with traditional androgen ablation).

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