Following BCL-2 Suppression by Antisense Oligonucleotide Compensation by Non-Targeted Genes Does Not Involve TGF-β1

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Abstract
Antisense oligonucleotides (oligos) have targeted regulatory proteins in both in vivo and in vitro cancer models including those of the prostate. In efforts to identify compensatory alterations in non-targeted gene expression this study evaluated mono- and bispecific oligos which targeted and equally suppressed the expression of bcl-2 (an apoptosis inhibitory protein). Previous evaluations have shown that treated LNCaP cells compensate by suppressing caspase-3 (an apoptosis promoter) and enhancing AKT-1 (an apoptosis inhibitor), androgen receptor (AR), co-activating p300 and IL-6 expression. In addition immune checkpoint markers PD-1, its ligand PD-L1 and fas-ligand, which activate apoptosis through signal transduction, are also enhanced, along with suppressor protein p53 (unexpectedly), oncogene v-myc, polymerase transcription mediator MED-12 and signal transducer STAT-3. This suggests that, in LNCaP, therapeutic approaches to restore apoptosis (including the use of antisense oligos) which suppress bcl-2 can lead to altered expression in non-targeted genes and regulatory changes not only involving apoptosis, but also androgen sensitivity, suppressor/oncogene activity and immune responsiveness. Alterations in apoptosis involved both mitochondrial and signal transducing pathways and the altered patterns of androgen co-activating proteins are similar to expression patterns associated with more advanced prostate tumors.

To further evaluate adaptive, compensatory mechanisms related to tumor resistance we now evaluated the level of transforming growth factor-beta 1 (TGF-β1) expression, which depending upon culture conditions has been shown to both promote and inhibit tumor growth. We find expression of this growth factor is unaffected by oligo mediated suppressive bcl-2 therapy and like caspase-3 (whose activity may require either replacement or maintenance for optimal efficacy) may require additional therapeutic manipulation to suppress epithelial tumor growth.

Keywords: Antisense; bcl-2; TGF-beta; Prostate cancer; Therapy

Introduction
Gene therapy is in theory specific, but encounters difficulties in practice. While suitable targets are found in many pathways, and tumors express altered patterns of gene expression, the actual activity of most regulatory genes is similar to normal cells. Resistance develops because the biochemical pathways involved are complex and regulated by combinations of both stimulatory and inhibitory factors; many affected by therapy. Evaluation of non-targeted genes, following what was believed to be specific gene therapy suggests that tumors

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frequently alter their dependence on the initially targeted gene products, by relying upon others through the process of compensation [1]. Just as bacteria and viruses mutate to evade antibiotic and antiviral agents, tumor cells are under similar selective pressure to evade therapy, whether chemically induced (chemotherapy) or that based on suppression of gene translation (including oligo mediated gene therapy). While oligo based therapy is already entering the clinical environment, the unintended consequences of intervention is poorly understood [1].

Gene therapy has been clinically employed for the treatment of human prostate tumors and together with radio [2,3] or chemotherapy [4] antisense oligos has been administered against inhibitors of apoptosis, particularly bcl-2 and clusterin, in attempts to restore that process mediated by the mitochondrial pathway. Additional, targets could also include those involved with apoptotic activation mediated by signal transduction and initiated via cell surface receptors (fas/fas-ligand and PD-1/PD-L1). If such therapy is to be successful, it is important to examine mechanisms by which tumors compensate and become resistant [1].

We previously reported that LNCaP cells treated with antisense oligos directed against bcl-2 compensated by suppressing caspase-3 [1] (an apoptosis promoter), and enhancing AKT-1 (an inhibitor of apoptosis) [5], androgen receptor [6] (AR), co-activating p300 [7] and IL-6 expression [8]. In addition immune checkpoint markers PD-1, its ligand PD-L1 and fas-ligand, which activate apoptosis were also enhanced [9], as were suppressor protein p53 [10] (unexpectedly), oncogene v-myc [11], polymerase transcription mediator MED-12 [12] and signal transducer STAT-3 [5,12]. This suggests that, in LNCaP, therapeutic approaches to restore apoptosis (including the use of antisense oligos) which suppress bcl-2 can lead to altered expression in non-targeted genes and regulatory changes not only involving apoptosis, but also androgen sensitivity, suppressor/oncogene activity and immune responsiveness. Alterations in apoptosis involved both mitochondrial and signal transducing pathways and the altered patterns of androgen co-activation proteins are similar to expression patterns associated with more advanced prostate tumors. Recently we demonstrated that cell surface proteins which regulate immune checkpoint blockade (PD-1 and PD-L1 and fas-ligand) and activate apoptosis via signal transduction were also enhanced [9], Presumably leading to further inhibition of T-cell activity. Unexpectedly altered gene expression, as a result of oligo therapy, also affects other cell surface (and differentiation) proteins. In an early evaluation of bispecifics we reported the enhanced expression of prostate specific membrane antigen (PSMA) [13] when oligos were directed against bcl-2. The unique capacity to produce such changes by these bispecifics (and not a similarly directed monospecific) is attributable to an unusual double strand conformation present in bispecifics and interferon induction (an enhancer of surface antigen expression) [14]. Such expression could enable better recognition and targeting by cytotoxic T cells [14].

As summarized above, previous studies evaluated effects upon apoptosis, androgen regulation, and angiogenesis, autocrine, and oncogene/suppressor protein activities. We now evaluated transforming growth factor-beta 1 (TGF-β1), an autocrine protein associated with both tumor suppressing and tumor promoting activity [15]. The loss of TGF-β1 signaling is thought to promote metastasis [16] and tumorigenity [17] in prostate cancer. However, in the presence of serum, it also can act in a growth inhibitory manner against these cells in culture [17]. Its effects upon tumor cell migration and invasion are mediated by PGE2 through activation of the P13K/AKT/mTOR pathway [15]. For gene therapy to ultimately be successful it must be made more specific or mechanisms of compensation must be identified and subsequently suppressed.

Methods

Oligonucleotides

Oligos (mono or bispecific) were purchased from Eurofins MWG Operon (Huntsville, AL). 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 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).

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**MR₁ (monospecific targeting bcl-2)**
T-C-T-C-C-A-G-C-G-T-G-C-G-C-A

**MR₄ (bispecific targeting EGFR/bcl-2)**

**MR₂₄ (bispecific targeting bcl-2/EGFR)**

**Cell Culture**

LNCaP cells were grown in RPMI 1640 supplemented with 10% bovine serum, 1% L-glutamine and 1% penicillin/streptomycin in a 5% CO₂ incubator. Log phase cells were harvested using EDTA/trypsin and equally distributed into 75 cm² flasks (Corning, NY). At intervals media were either supplemented or replaced with fresh ones.

**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 media the day before oligos were added. 100 μl of stock oligos 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 MR₁, or the MR₂₄ and MR₂₄ bispecifics.

**RNA Extraction**

Following treatment, media was removed, a single ml 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 12,000g 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 ml of 75% ethanol, then spun for 8 min at 7500g. The ethanol was pipetted off and the formed pellet air dried at -20°C.

**RNA Quantitation**

RNA was resuspended in 250 μl of DEPC treated H₂O, and quantitated using a Qubit fluorimeter and Quant-iT RNA assay kit (Invitrogen).

**RT-PCR**

Extracted RNA was diluted in DEPC treated water to 40 μg/μl. 1-4 μl of this RNA was added to 1 μl of both sense and antisense primers (forward and reverse sequences) for β-actin, bcl-2 and TGF-β1. From a kit purchased from Invitrogen the following reactants were added for RT-PCR: 25 μl of 2X reaction mixture, 2 μl Super Script III RT / platinum Taq mix, tracking dye, and 3 μl MgSO₄ (of a 5mM stock concentration). DEPC treated water was added to yield a final volume of 50 μl. RT-PCR was performed for 2 X 25 cycles using the F54 program in a Sprint PCR Thermocycler. As a control for RT-PCR product production, human actin expression (β-actin) 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.

**Primers**

**β - Actin**
Forward primer sequence: 5’ CAA ACA TGA TCT GGG TCA TCT C 3’
Reverse primer sequence: 5’ GCT CGT CGT CGA CAA CGG CTC
PCR product produced 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 produced was 127 base pairs in length.

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TGF-β1
Forward primer sequence: 5’ CGT GGA GCT GTA CCA GAA ATA 3’
Reverse primer sequence: 5’ TCC GGT GAC ATA AAA AGA TAA 3’
PCR product produced was 106 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 electrophoresis system. Samples were run for 2 hours at a constant voltage of 70 mV 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 bromophenol blue tracking dye were run in each gel.

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 illumination intensity was quantitated using Medical Image Processing and Visualization (Mipav) software provided by the National Institute of Health. Means and standard deviations were compared using Student t-tests to determine statistical significance.

RESULTS

Bcl-2 Expression
As a control (data not shown) for RT-PCR product production, human actin expression was tested in RNA extracted from HeLa cells (Figure 1).

![Figure 1: Expression of β-Actin on Agarose Gel.](image)

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LNCaP cells incubated for 24 hours in the presence of 6.25 μM of oligos suppressed Bcl-2 expression, and support the finding of comparable biologic activity in both mono- and bispecific oligos measured in the in vitro cell growth inhibition experiments [1]. 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. For each oligo evaluated, the greatest amount of suppression measured approached 100% ± SEM% for the mono-specific MR; and for the bispecifics MR₂₄ and MR₄₂, 86 ± SEM% and 100 ± SEM%, respectively. Suppression was found in both repeat PCR runs with bcl-2 primers, as well as in repetitive agarose gel quantifications [18].

TGF-β1 Expression

Identical 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 TGF-β. When background intensity was subtracted, the relative intensity of all bands corresponding to TGF-β representing cells treated with MR₄, MR₂₄ and MR₄₂ compared to controls were respectively increased 36.1% ± 62.4 (P = 0.151851), 48.4% ± 80.7 (P = 0.138169) and 92.4% ± 146.8 (P = 0.121822). These results were pooled from seven duplicate gels and, although values are slightly elevated for each treatment group, indicate no significant changes in TGF-β expression. A representative gel is found in Figure 2.

![Figure 2: TGF-β Expression is unchanged by Oligo Treatment as Indicated in a Representative Agarose Gel.](image)

Discussion

It’s now apparent that some forms of gene therapy, namely antisense oligos is not as specific as previously thought. Now in clinical trials against a variety of solid tumors, this method is an effective, relatively non-toxic and inexpensive form of therapy and various types of antisense RNA have been constructed for this purpose (phosphorothioated oligos used in these evaluations, 2’-MOE-RNA, morpholinos, siRNA, miRNA etc.). Some of these derivative oligos have been evaluated clinically, but all are directed against single gene products. In contrast, the oligos discussed in this paper included both mono and bispecific forms each having a base sequence complementary to and directed against mRNA encoding the apoptosis inhibitor bcl-2 and bispecifics included an additional site directed against epidermal growth factor receptor [EGFR]). We evaluate bispecific oligos because it would be naïve to believe targeting a single mRNA would be sufficient to produce a clinical response in most tumors, and activity at one site does not affect binding at a second [18], therefore administration of a single oligo having two targets could have an additional suppressive benefit. Furthermore we have shown that both types of oligos have comparable activity suppressing bcl-2 [18].

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While it’s understandable that genes which share sequence homology would also be susceptible to antisense oligos, when directed at common sequences, what is not expected are the non-specific effects on non-targeted genes, many of which control additional growth regulatory pathways [1].

Antisense oligos 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 oligos target bcl-2, clusterin (OGX-011 in Phase II testing), heat shock protein 27 (OGX-427) or insulin growth factor binding proteins (OGX-225) [19]. Genta conducted a phase 3 test using oligos (Genasense; oblimersen) directed against bcl-2 for treating melanoma, chronic lymphocytic leukaemia and various solid tumors [20], but compensatory effects produced by this agent were not reported. Many represent efforts to restore tumor apoptosis by eliminating suppressive bcl-2 [2-4] associated with treatment resistance. Since derivatives of antisense oligos (siRNA, miRNA) continue to be developed and tested, and while some directed against bcl-2 are in clinical trials, it is important to identify compensatory changes that result.

This year (2015) the American Cancer Society (ACS) estimated that in spite of early detection, screening for prostate specific antigen (PSA) and effective treatments for localized disease, in the United States there will be an estimated 27,540 deaths from prostate cancer with 220,800 newly diagnosed cases (22). New types of treatment, including gene therapy and translational inhibition must be developed and employed (probably in combination with traditional androgen ablation).

Conclusion

Although previous studies, which evaluated effects upon proteins involved with apoptosis, androgen regulation, angiogenesis, autocrine and oncogene/suppressor activities, found that many have their expression altered in compensation to bcl-2 suppression, it appears that TGF-β is not involved (at least in the LNCaP model system).

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