Multiple Pathways Compensate for Antisense Suppression of Bcl-2 in LNCaP Cells Contributing to Tumor Resistance

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Abstract

Antisense oligonucleotides (oligos) have been employed against in vivo and in vitro prostate cancer models targeting growth regulatory proteins. While most oligos target growth factors or their receptors, others are directed against apoptosis inhibitors and mediators of androgen action. Those which suppress Bcl-2 activity have even reached clinical trials in prostate cancer patients. We previously evaluated a set of oligos which targeted and comparably suppressed the expression of the apoptosis inhibitor protein Bcl-2. LNCaP cells adapted to this restoration of apoptosis with suppression of caspase-3 (another apoptosis promoter). In this continuing study we evaluated additional proteins associated with tumor progression and found the expression of the Androgen Receptor (AR), its p300 and IL-6 co-activators and the v-myc oncogene are enhanced.

This suggests that oligo treatment directed against Bcl-2 can be evaded through compensatory changes in apoptosis, androgen sensitivity and oncogene activity. We conclude that therapeutic suppression of Bcl-2 can lead to other types of tumor resistance, promoting transformation of the tumor to a more aggressive (androgen driven) phenotype.

Keywords: Antisense; Bcl-2; Androgen receptor; p300; Prostate cancer; Therapy

Introduction

Gene therapy has now reached clinical trials for the treatment of human prostate tumors with antisense oligonucleotides (oligos) targeting Bcl-2 and clusterin, in efforts to restore apoptosis following radio- [1] or chemotherapy [2]. If such therapy is to be successful, it is important to examine mechanisms by which tumors compensate and become resistant.

We recently reported [3] that oligo mediated inhibition of Bcl-2 suppressed the expression of the apoptotic promoter caspase-3. We now evaluate effects upon the androgen receptor (AR), its transcriptionsal co-activators (p300 and interleukin IL-6), and the v-myc oncogene. Increased androgen sensitivity could drive the tumor to become more androgen sensitive and aggressive, while v-myc is linked to chromosomal alterations at 8q24, thought to be involved in the initiation of cancer in prostate intraepithelial cells at the earliest stages of disease [4]. For gene therapy to be successful it must be more specific and the mechanisms of compensation identified and suppressed.

Effective therapeutics target unique characteristics of etiologic agents including bacterial cell walls and ribosomes or viral encoded proteolytic enzymes. The development of tumor resistance is less specific or distinct since cancer cells are not substantially different from non-cancerous (differentiated cells). They use the same biochemical pathways and, unless virally induced, most are (even antigenically) similar to normal cells. The effectiveness of chemotherapy capitalizes on the fact that within a tumor mass a greater proportion of cells are in the process of replicating. Therefore, anticancer drugs frequently target some aspect of DNA synthesis. In prostate and breast cancers, growth factor (either hormone or protein targeted) deprivation provides another type of therapy. In an extension of this approach, transcriptional activity initiated by DNA hormone response elements recognized by androgen (AR) or estrogen (ER) receptors or co-activators (p300 and IL-6), which are more prevalent in advanced, hormone insensitive disease [5], could also be targeted. However, for most chemotherapy tumor cell specificity is relative, often lacking, and most agents administered (like Taxol) have significant toxicity towards other replicating cells.

Gene therapy is based on a similar premise and while effective protocols employ either translational suppression (oligo mediated) or replacement (of inactivated, mutated or deleted suppressor genes like PTEN) technology [6] both tumor and normal cells express the same genes. Targets for gene therapy are found in many pathways and it is likely that hundreds (or thousands) of genes ultimately become involved in the process of malignant transformation. Although tumors can express an overall altered pattern of gene expression, the levels of many growth regulatory genes are often similar to those of normal cells. Resistance develops because the biochemical pathways involved are complex and highly regulated by many stimulatory and inhibitory factors, each altered by therapy; therefore it has been suggested that tumors can alter their dependence upon single influences by reliance upon others through compensation [3].

Tumors are essentially heterogeneous masses of rapidly growing and selectively adapted cells whose sole purpose is to survive, replicate and, while doing so, evade therapeutic interventions. The best example is the emergence of hormone insensitive prostate cancer cells following androgen deprivation therapy, resulting in the increased expression of the autocrine loop consisting of transforming growth factor-alpha (TGF-a) and its binding site, the epidermal growth factor receptor (EGFR) in prostate and breast tumors [7].

As bacteria and viruses mutate to evade antibiotic and antiviral agents, tumor cells are under similar selective pressure to evade chemotherapy. Although newly developed forms of gene therapy provide specific ways to inhibit uncontrolled growth or promote (re-
establish) apoptosis, the unintended consequences of intervention are poorly understood, and some may compensate for the originally intended effect. Now that antisense mediated protocols are in clinical trials, these studies are increasingly relevant.

**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).

\[
\text{MR}_{54} \quad \text{(monospecific targeting Bcl-2)} \quad T-C-T-C-C-C-A-G-C-G-T-G-C-G-C-A-T
\]

\[
\text{MR}_{54} \quad \text{(bispecific targeting EGFR/Bcl-2)} \quad G-A-G-G-G-T-C-G-C-A-T-C-G-C-T-G-C-T-C-T-C-G-C-T-G-C-G-C-A-T
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**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 the fresh one.

**Oligo treatment prior to PCR**

When cell density in the culture flasks approached 75% confluence (4 days prior to oligo addition), 10 ml of additional fresh media was added. Cells were incubated for another 3 days before 5 ml of media was replaced with fresh the day before oligos were added. 100 μl of stock oligos were added (in RPMI 1640 media) 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 MR₅₄ and MR₃₄ 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 12,000 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 form the pellet RNA. The supernatant was removed, the pellet washed in a single ml 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 DEPC treated H₂O, and quantitated using a Qubit fluorometer and Quant-it RNA assay kit (Invitrogen). DEPC is an inhibitor of RNase activity.

**RT-PCR**

Extracted RNA was diluted in DEPC treated water to 40 μg/μl. 1-4 μl of this RNA was added to 10 μl of both sense and antisense primers (forward and reverse sequences) for bcl-2, Ark, p300, IL-6 and v-myc. 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 MgSO₄ (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 X 25 cycles using the FS4 program in a Sprint PCR Thermocycler. 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).

**Primers**

**Actin:**

Forward primer sequence: 5’ CAA ACA TGA TCT GGG TCA TCT TCT C 3’

Reverse primer sequence: 5’ GCT CGT CGT CGA AGA AAT CA 3’

**Bcl-2:**

Forward primer sequence: 5’ GAG ACA GCC AGG AGA AAT CA 3’

Reverse primer sequence: 5’ TGT CTC TGC TCA GGC TCA AA 3’

**p300:**

Forward primer sequence: 5’ CGG AAG CTG AAG AAA CTT GG TCT C 3’

Reverse primer sequence: 5’ GCT CGT CGT CGA CAA CGG CTC TCT C 3’

**Caspase-3:**

Forward primer sequence: 5’ CCC CTG GAT CTA CCA GCA TA 3’

Reverse primer sequence: 5’ TGT CTC TGC TCA GGC TCA AA 3’

**Androgen receptor (AR):**

Forward primer sequence: 5’ CGG AAG CTG AAG AAA CTG GG TCT C 3’

Reverse primer sequence: 5’ ATG GCT TCC AGG ACA TTC AG 3’

**IL-6:**

Forward primer sequence: 5’ ATG CAA TAA CCA CCC CTG AC 3’

Reverse primer sequence: 5’ GAG GTG CCC ATG CTA CAT TT TCT C 3’
PCR product produced was 167 base pairs in length.

v-myc:
Forward primer sequence: 5’ CGA CGA GAC CTT CAT CAA AA 3’
Reverse primer sequence: 5’ TGC GGT GTA GAG GGT AG 3’
PCR product produced was 248 base pairs in length.

Detection and quantitation of product

Agarose gel electrophoresis: 1.5% agarose gel was prepared in a 50 ml volume of TBE buffer (1X solution: 0.089 M Tris borate and 0.002 M EDTA, pH 8.3), containing 3 μl of ethidium bromide in a Fisher Biotech electrophoresis system. Samples were run for 2 hours at a constant voltage of 70 using a BioRad 1000/500 power supply source. To locate the amplified PCR product, 3 μl of a molecular marker (Inviditrogen) 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 ELPH 300 HS PowerShot digital camera. Photos 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

Bcl-2 expression

As a control for RT-PCR product production, human actin expression was tested in RNA extracted from Hela cells. Figure 1 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 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 [8]. 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% for the mono-specific MR4; and for the bispecifics MR24 and MR42, 86% and 100%, respectively.

Caspase-3 expression

Comparably 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 3 and appears below the marker representing 300 base pairs in the expected 262 base pair region.

When 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 were -35.8% ± 12.5 (P=0.0002), -40.3% ± 16.6 (P=0.0006) and -43.5% ± 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 each oligo type.

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 for tumors to select variants
Androgen receptor (AR) 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 AR. When background intensity was subtracted, the relative intensity of all bands corresponding to AR representing cells treated with MR4, MR24, and MR42 compared to controls were enhanced 31.2% ± 26.0 (P = 0.015), 58.5% ± 51.4 (P = 0.019) and 53.1% ± 45.9 (P = 0.019). These results were pooled from both duplicate PCR runs and multiple gels (a total of six gels were evaluated), and indicate similar (significant) enhancement of AR activity is produced by each oligo type. A representative band is depicted in Figure 4.

p300 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 p300. When background intensity was subtracted, the relative intensity of all bands corresponding to p300 representing cells treated with MR4, MR24, and MR42 compared to controls were increased 82.9% ± 5.9 (P = 0.006), 93.0% ± 87.3 (P = 0.007). These results were pooled from both duplicate PCR runs and multiple gels (a total of six gels were evaluated), and indicate similar (significant) enhancement of p300 activity is produced by each oligo type. A representative band is depicted in Figure 5.

IL-6 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 IL-6. When background intensity was subtracted, the relative intensity of all bands corresponding to IL-6 representing cells treated with MR4, MR24, and MR42 compared to controls were increased 236.9% ± 154.4 (P = 0.0015), 219.3% ± 170.4 (P = 0.0005) and 139.2% ± 88.8 (P = 0.0015). These results were pooled from both duplicate PCR runs and multiple gels (a total of seven gels were evaluated), and indicate similar (significant) enhancement of IL-6 activity is produced by each oligo type. A representative band is depicted in Figure 6.

v-myc 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 v-myc. When background intensity was subtracted, the relative intensity of all bands corresponding to v-myc representing cells treated with MR4, MR24, and MR42 compared to controls were increased 170.1% ± 105.6 (P = 0.0027), 201.4% ± 101.1 (P = 0.0006) and 18.4% ± 32.3 (P = 0.19). These results were pooled from both duplicate PCR runs and multiple gels (a total of six gels were evaluated), and indicate significant enhancement of v-myc activity produced by oligos which target Bcl-2 at the S’ position. A representative band is depicted in Figure 7.

Discussion

This year (2012), the American Cancer Society (ACS) estimates 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 28,170 deaths from prostate cancer with 241,740 newly diagnosed cases [8]. New types of treatment, including gene therapy and translational inhibition, must be developed and employed (probably in combination with traditional androgen ablation).

The androgen receptor (AR) (also known as NR3C4; nuclear receptor subfamily 3, member 4) and its co-activators play a principle role in male sexual development, prostate function, cancer progression and various treatment strategies. Following the cytoplasmic binding of the AR to testosterone or its metabolite, dihydrotestosterone (DHT), it undergoes a conformational change which is accompanied by dissociation of heat shock proteins and translocation into the cell nucleus. The AR dimerizes, binds to hormone response elements of the DNA, and acts as a transcription factor to enhance the synthesis of growth stimulating proteins, such as insulin-like growth factor [9,10].

Gene therapy could target transcriptional activity initiated by DNA hormone response elements recognized by androgen (AR) receptors or its various co-activators (including p300 and IL-6),
more prevalent in advanced, hormone insensitive disease [5]. These factors are essential for cell growth and some (p300) govern cyclin expression which regulates the transition between the G1, S, G2 and M phases of mitosis [11]. Together, these factors (IL-6 with p300/CREB binding protein) also play a role in the androgen independent expression of prostate specific antigen (PSA) [12]. Treatment of prostate cancer cells with siRNA directed against p300 reduces cancer cell growth [13], and eliminates the ability of IL-6 to induce PSA [13]. IL-6 is increased in the blood of patients with advanced and metastatic disease [14], and anti-IL-6 therapy employing tocilizumab and ALD518 is now in clinical trials [15,16].

Innovative protocols to disrupt androgen driven tumor progression have also employed antisense oligos directed against the enzyme for conversion of testosterone to dihydrotestosterone (5-alpha reductase), heat shock proteins, p300 and the AR itself. Although LNCaP cells express an AR which is mutated in the binding domain the Eder [17] and Rubenstein groups [18] have separately demonstrated growth inhibition in this in vitro model employing oligos.

Oligos (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 oligos target Bcl-2, clusterin (OGX-011 in Phase II testing), heat shock proteins 27 (OGX-427) or insulin growth factor binding proteins (OGX-225) [19]. Many represent efforts to restore tumor apoptosis by eliminating suppressive Bcl-2 [1,2] associated with treatment resistance. Similar approaches are directed at clusterin. For (tumor suppressor) genes which are either diminished or lacking (tumor suppressor) genes which are either diminished or lacking or have developed (proposed) enhanced expression of undesired proteins, the oligo approach can again be applied, employing other bispecific or even (proposed) multispecific forms [24] which simultaneously targets many proteins.

**References**

4. Rubenstein M, Guinan P (2010) Bispecific antisense oligonucleotides have enhanced expression of v-myc is produced by a single bispecific (MR2 and not MR4) is unknown, and does not fit any of the expression patterns previously found [3,8,21,22], where both were either similar to the monospecific or enhanced. Enhancement limited to these bispecifics has been more fully investigated and it’s thought to be related to intrastand base pairing, rather than a mechanism of compensation. This double strand acts as an interferon inducer [21] and promotes enhanced prostate specific membrane antigen (PSMA) expression [22]. One reason could be related to the 5’ location of the Bcl-2 directed activity in both the mono- and affected bispecific oligo. Perhaps steric hindrance or another aspect of intrastand base pair binding makes the MR4 oligo less active. Currently, Genta is conducting a phase 3 test using oligos (Gensense; oblimersen) directed against Bcl-2 for treating melanoma, chronic lymphocytic leukemia and various solid tumors [23], but compensatory effects produced by this agent have not (yet) been reported.

Tumors are resilient in their efforts to overcome (even newly developed) therapeutics and become resistant. If gene therapy is to be effective, we must understand how primary effects evoke compensatory changes. It would also be significant to see whether these changes are replicated in *in vivo* models. If these lead to enhanced expression of undesired proteins, the oligo approach can again be applied, employing other bispecific or even (proposed) multispecific forms [24] which simultaneously targets many proteins.


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23. www.genta.com


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