Prostate Cancer Surface Antigenicity and the Interferon Induction Hypothesis in LNCaP Cells Treated with Antisense Oligonucleotides

Marvin Rubenstein^{1,2,3*}, Courtney M.P. Hollowell² and Patrick Guinan^{1,2,3}

¹Division of Cellular Biology, Hektoen Institute for Medical Research

²The Division of Urology, Stroger Hospital of Cook County

³Rush University Medical Center, Department of Urology, University of Illinois at Chicago, Chicago, IL, 60612

Received: April 14, 2014; Accepted: June 29, 2014; Published: July 01, 2014

***Corresponding author:** Dr. Marvin Rubenstein, Chairman-Division of Cellular Biology, Hektoen Institute for Medical Research, 2240 West Ogden Avenue, 2nd floor, Chicago, IL 60612, Phone: 312-864-4621; Fax: 312-768-6010, E-mail: DrMarv@Prodigy.net

Abstract

Antisense oligonucleotides (oligos) have been administered against prostatic LNCaP cells in both *in vivo* and *in vitro* models. While most oligos consist of a single mRNA binding site targeting a single gene product, or others having sequence homology, we evaluate bispecifics directed towards two unrelated proteins. In LNCaP cells we identified bispecifics which increase the expression of prostate specific membrane antigen (PSMA) while not affecting secreted prostate specific antigen (PSA) or prostatic acid phosphatase (PAP). We then postulated that surface antigen expression is increased by bispecifics able to induce interferon (IFN- γ) through complementary intrastrand regions. To further test this hypothesis we measured the effect of oligo treatment on another secreted product, prostatic cancer antigen-3 (PCA-3).

We initially evaluated the inhibition of *in vitro* propagating LNCaP cells employing mono- and bispecific oligos directed against bcl-2 [the second bispecific binding site was against the epidermal growth factor receptor (EGFR)]. These oligos were administered with lipofectin in a nanoparticle delivery system. Employing RT-PCR, the expression of non-targeted genes products encoding PSMA, PSA, PAP, PCA-3 and IFN- γ have now been evaluated. When LNCaP prostate tumor cells were incubated with oligos and compared to lipofectin containing controls significant growth inhibition resulted.

Employing RT-PCR, the levels of mRNA encoding PSMA were unexpectedly found to be elevated following treatment with the bispecific oligos but not with a monospecific directed solely against bcl-2. No increased expression in PSA, PAP or PCA-3 following treatment with either oligo type were detected. Like PSMA, IFN- γ was significantly induced only by bispecific oligos. These data support the hypothesis that double strand forming bispecific oligos induce IFN- γ , enhancing cell surface antigen expression (PSMA) but not secretory products. Expression of tumor associated surface antigens could increase their recognition and targeting by immunologic defense mechanisms and increase the effectiveness of tumor vaccines.

Keywords: Antisense; Prostate Cancer; Therapy

Introduction

Antisense oligonucleotides (oligos) have been employed in both *in vivo* and *in vitro* prostate cancer models employing LNCaP and PC-3 cell lines. Genes targeted include protein growth factors, androgens, receptors for stimulating factors, inhibitors of apoptosis (bcl-2 and clusterin) and oncogenes. Some (developed by Oncogenex Pharmaceuticals) have entered clinical trials (OGX-011) while others are in preclinical development (OGX-225).^[1] Oligos act through a variety of mechanisms and provide a specific and relatively non-toxic method for translational arrest by degradation of annealed mRNA: oligo duplexes by RNase H^[2], protein binding and DNA triplex formation^[3,4].

Our laboratory has attempted to increase the efficacy of oligos by evaluating bispecific derivatives^[5-11] which contain more than one binding site on a single DNA strand. Bispecifics, have been evaluated against genes involved in both a single autocrine loop^[5] as well as those found in different growth regulatory pathways.^[6,9,10,11] Initial studies measured inhibition of *in vitro* growth, but more direct methods use reverse transcriptase-polymerase chain reaction (RT-PCR) to measure suppression of gene specific mRNA.

In LNCaP cells we find that monospecific oligos targeting bcl-2 and bispecifics targeting both bcl-2 and the epidermal growth factor receptor (EGFR) have comparable activities in suppressing bcl-2 expression as determined by both *in vitro* growth and bcl-2 expression measured by RT-PCR.^[11] Therefore, the presence of a second binding site did not diminish activity of the other. This finding is significant because it's naive to believe that cancer cell growth can be controlled by oligos through knockdown of a single gene product. Instead multiple genes must be down regulated, and it would be useful if more than one therapeutic activity could be combined onto a single entity.

In these experiments we evaluated the effect of bispecific oligos directed against bcl-2 and EGFR employing RT-PCR, by measuring the expression of four prostate specific gene products

not directly targeted, as well as the hypothetical role of induced IFN- γ in their expression. These (three) differentiation antigens and (non-protein) PCA-3 are surrogates for tumor growth and regression, and as biochemical markers they are early indicators for tumor recurrence (biochemical relapse). The bispecifics administered have a unique base sequence which computer models suggest could lead to intrastand binding Figure 1. Double stranded nucleic acids (poly I:C) are known to induce IFN- γ which increases the expression of some cell surface antigens (HLA antigens and receptors for tumor necrosis factor). Any increase in antigen encoding mRNA could be a rather unique result of (the hypothesized) IFN- γ induction produced by the unusual secondary structure of these oligos and support an additional theory that the prostate retains an interferon based antiviral defense system. [12]

PSMA is a trans membrane bound receptor with folate hydrolase activity. In contrast, prostate specific antigen (PSA) is a secreted kallikrein related peptidase marker associated with prostate cancer recurrence and progression. It's often employed as a screening tool and has been evaluated as a target for directed therapy. PAP was the original serum marker for prostate cancer, distinguishable from other types of acid phosphatase by formalin resistance. PSMA and PAP are considered targets for an activated immune system and have been included in prostate cancer vaccines. ALPHAVAX ARV developed by the consortium of Cytogen and Progenics targets PSMA, and Provenge developed by Dendron immunizes against PAP. Administration of therapy which inhibits growth while enhancing surface antigenicity would be of interest, as the field of immunotherapy develops. PCA-3 is being evaluated as a more specific marker for prostate cancer. Also known as DD3, this marker is a non-coding RNA which when measured in urine is considered less sensitive, but more specific than PSA when managing disease. Enhanced expression

by any surface protein would presumably aid cytotoxic (CD8+) T cell targeting and increase (anti-PSA) antibodies as reported by Lubaroff. [13]

Based on their secondary structure, these oligos have the capacity to form double stranded nucleic acids, and could act as an IFN- γ inducer in a similar manner as poly I:C. The enhanced expression of HLA and tumor necrosis factor receptors following IFN- γ exposure has long been recognized as a promoting factor for immune mediated cytotoxicity. [14,15] In these experiments we evaluated whether bispecific oligos MR₂₄ and MR₄₂ alter the expression of a more recently identified and studied prostate specific gene product (PCA-3) and determine whether and its expression pattern further supports the IFN- γ induction hypothesis.

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 μ M 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).

MR₄ (monospecific targeting bcl-2) T-C-T-C-C-A-G-C-G-T-G-C-G-C-A-T

MR₂₄ (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-A-G-C-G-T-G-C-G-C-C-A-T

MR₄₂ (bispecific targeting bcl-2/EGFR) T-C-T-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 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 was either supplemented or replaced with fresh media.

Determination of Growth

Four days prior to the addition of oligos 1 X 10⁴ LNCaP cells were added, in a total 200 μ l volume of media, to each depression of a 96 well plate and incubated at 37°C in a 5% CO₂ incubator. On the day of transfection the following solutions were prepared:

A) 1 μ l of buffer containing either oligo or a diluent was added to 50 μ l of OPTI-MEM and gently mixed. One dilution was made for each well.

B) 1 μ l of Lipofectin was diluted in 50 μ l of OPTI-MEM and mixed gently for 5 minutes at room temperature.

C) Oligo dilutions were mixed with 50 μ l of Lipofectin and gently mixed for 20 minutes at room temperature.

Figure 1: Suggested complementary binding conformation of MR₂₄ and MR₄₂.

D) 100 µl of the Lipofectin and oligos were added to 100 µl of RPMI medium and mixed.

Cells were incubated for 24-48 hrs before solutions were aspirated and re-incubated for an additional 48 hrs in 200 µl of media. Cell counts were determined following the addition of WST-1 reagent to each well, and after 2 hrs the color intensity was measured by a micro-plate 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 Students 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. 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 MR₄, or the MR₂₄ and MR₄₂ 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 pellet the 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 to 40 µg/µl in DEPC treated water. 1-4 µl of this RNA was added to 1 µl of both sense and antisense primers (forward and reverse sequences) for human actin (used as a control), PSMA, PSA, IFN-γ and PAP. 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 MgSO₄ (3 µl of a stock concentration of 5mM, 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 X 25 cycles using the F54 program in a Sprint PCR Thermocycler.

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 produced was 353 base pairs in length

PSMA [Human folate hydrolase 1 (FOLH1)]: Forward primer sequence: 5' GAG GAG CTT TGG AAC ACT GA 3'

Reverse primer sequence: 5' CCT CTG CCC ACT CAG TAG AA 3'

PCR product produced was 113 base pairs in length.

PSA [Human kallikrein-related peptidase 3 (KLK3)]: Forward primer sequence: 5' CCA GAC ACT CAC AGC AAG GA 3'

Reverse primer sequence: 5' CTG AGG GTT GTC TGG AGG AC 3'

PCR product produced was 204 base pairs in length.

PAP [Human acid phosphatase (ACP)]: Forward primer sequence: 5' TTG ACC GGA CTT TGA TGA GT 3'

Reverse primer sequence: 5' CCT GAA AGG CAG GTA TAG CA 3'

PCR product produced was 152 base pairs in length.

PCA-3 (Human prostate cancer antigen-3): Forward primer sequence: 5' GGG GAA GAG GTT TTG TGT TT 3'

Reverse primer sequence: 5' CCC TTC TGC TGT CCT ATC AA 3'

PCR product produced was 82 base pairs in length.

IFN-γ [Human interferon, gamma (IFNG)]: Forward primer sequence: 5' TCC CAT GGG TTG TGT GTT TA 3'

Reverse primer sequence: 5' AAG CAC CAG GCA TGA AAT CT 3'

PCR product produced was 198 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 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. For actin product localization, the tracking dye was included in each sample run; for all others 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 NIH.

Results

Cell Culture Experiments

LNCaP cells were incubated with MR₄, MR₂₄ and MR₄₂ and compared to lipofectin containing controls Figure 2. In an initial experiment 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 LNCaP cells were similarly incubated and compared to lipofectin containing controls. Bispecific oligos 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 MR₄ directed only towards bcl-2 in the inhibition of *in vitro* cell growth.

RT-PCR Experiments

Comparable amounts of extracted RNA from LNCaP cells treated with either mono- or bispecific oligos directed against bcl-2 (and EGFR in the bispecifics). In a series of control experiments (data not shown) to validate RNA extraction and RT-PCR procedures, the expression of human actin in HeLa cells was identified. [11] This RNA was then evaluated by RT-PCR using primers directed against PSMA, PSA, PAP, PCA-3 and IFN-γ. Product was run on agarose gels and digitally photographed. The identified product bands were cropped and scanned with Mipav software. Representative bands for PSMA, PSA, PAP, PCA-3 and IFN-γ are presented in Figures 3-7.

PSMA

When background intensity was subtracted, the intensity of the bands containing PSMA product (Table 1) were 19.3% ± 10.5, 20.9% ± 14.7, 33.7% ± 11.4 and 33.0% ± 10.8. The amount of PSMA product was significantly enhanced by the MR₂₄ (EGFR/bcl-2) and MR₄₂ (bcl-2/EGFR) bispecifics (p = 0.03 and p = 0.02 respectively). These results were pooled from both duplicate PCR runs and gels. In contrast, no difference was seen in cells treated with the MR₄ monospecific oligo. A representative band is seen on agarose gel in Figure 3.

PSA

When background intensity was subtracted, the intensity of bands containing PSA product Table 1 were 60.9% ± 11.2, 60.4% ± 8.1, 69.6% ± 4.6 and 66.1% ± 7.3 respectively for the untreated cells and those treated with monospecific MR₄ and bispecific MR₂₄ and MR₄₂. Treated cells were not significantly different from the untreated controls. A representative band is seen on agarose gel in Figure 4.

PAP

The intensity of bands containing PAP product, when compared to controls Table 1, were decreased 7.58% ± 10.6 (NS), 3.96% ± 23.1 and 17.27% ± 15.9 (p = 0.042) respectively for those treated with monospecific MR₄ and bispecifics MR₂₄ and MR₄₂. Expression of secretory PAP was therefore similar to PSA (not increased) compared to surface antigen PSMA. In cells treated with MR₄₂ the decrease may be significant. This supports the

Figure 2: Inhibition of *in vitro* growth of LNCaP cells by mono- and bi-specific oligos.

Figure 3: When background intensity was subtracted, the intensity of the bands containing PSMA product (Table 1) were 19.3% ± 10.5, 20.9% ± 14.7, 33.7% ± 11.4 and 33.0% ± 10.8 respectively for the untreated cells and those treated with monospecific MR₄ and bispecific MR₂₄ and MR₄₂.
PSMA expression in LNCaP cells treated with mono and bispecific oligos directed against BCL-2 and EGFR

Figure 4: When background intensity was subtracted, the intensity of bands containing PSA product were 60.9% ± 11.2, 60.4% ± 8.1, 69.6% ± 4.6 and 66.1% ± 7.3 respectively for the untreated cells and those treated with monospecific MR₄ and bispecific MR₂₄ and MR₄₂.
PSA expression in LNCaP cells treated with mono and bispecific oligos directed against BCL-2 and EGFR

hypothesis that double stranded oligos can act as an interferon inducer and enhance surface antigen expression which could have immunological significance if it highlights transformed cells for attack by cytotoxic T cells. A representative band is seen on agarose gel in Figure 5.

PCA-3

The intensity of bands containing PCA-3 product, when compared to controls Table 1, were not significantly altered 55.7% ± 61.3 (NS), 51.2% ± 106.5 and 68.7% ± 104.0 (NS) respectively for those treated with monospecific MR₄ and bispecifics MR₂₄ and MR₄₂. Expression of secretory PCA-3 was not increased compared to surface antigen PSMA and therefore its expression pattern resembles PSA and PAP with no increase

Table 1, further supporting the IFN- γ induction hypothesis. A representative band is seen on agarose gel in Figure 6.

IFN- γ

When background intensity was subtracted, cells treated with the bispecifics had significantly greater IFN- γ expression ($p < 0.05$) than the untreated controls and bands were visually more intense than those seen in the monospecific treated group. The intensity of bands containing IFN- γ product, when compared to controls, were increased $3.12\% \pm 10.6$ (NS), $28.5\% \pm 23.1$ ($p = 0.025$) and $19.2\% \pm 15.9$ ($p < 0.05$) respectively for those treated with monospecific MR₄ and bispecifics MR₂₄ and MR₄₂. This supports the hypothesis that double stranded oligos can act as an interferon inducer. A representative band is seen on agarose gel in Figure 7.

Discussion

The hypothesis that double stranded oligos could act as an IFN- γ inducer was first proposed following the discovery that PSMA and IFN- γ were enhanced following treatment of LNCaP cells with bispecific oligos having the capability of forming double stranded regions. The lack of similar enhancement in subsequent experiments evaluating PSA and PAP supported this hypothesis. In this study we evaluated yet another secretory product (non-protein PCA-3) and found further support for this theory.

Gene therapy is a complex process which requires both hormone and protein stimulated growth as well as the apoptotic pathway (with all their regulatory gene products) to be simultaneously regulated. For over expressed gene products there are methods to suppress their translation several of which have been clinically evaluated; for those which are diminished or lacking, methods and (viral) vehicles for their insertion must be more safely developed. Gene therapy can also be improved when combined with chemo- or immunotherapy. Translational arrest can be accomplished by antisense strategies and a cocktail of antisense oligos, could theoretically shut down many of the overexpressed genes. 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 bispecific [5] or proposed multispecific oligos [17]. While some investigators refer to “bispecific” oligos targeting genes which share a region of sequence homology and have similar biologic activity [18,19], what we define as bispecific oligos are those which target more

than one protein (unrelated in sequence), capable of binding two protein encoding mRNAs from (even) different regulatory pathways. [20]

We have now evaluated the effect of oligo mediated growth suppression on four seemingly unrelated markers of prostatic tumor progression (PSMA, PSA, PAP, PCA-3) and one induced cytokine (IFN- γ). Although LNCaP cell growth is inhibited by bispecific oligos, it appears that when activity is normalized for the amount of extracted RNA, PSA, PAP and PCA-3 expression does not increase. In contrast, the expression of PSMA and IFN- γ is enhanced by the bispecifics (targeting EGFR and bcl-2) and unchanged from controls when treated with the monospecific (targeting only bcl-2). Enhanced PSMA expression may be due to IFN- γ induction by the uniquely configured bispecific oligos and its effect on cell surface antigen expression. The recent determination that PCA-3 also remains unchanged further supports this hypothesis. Double stranded nucleic acids are known inducers of IFN- γ , similar to poly I:C and we have shown this cytokine to be induced in LNCaP cells by the bispecific oligos tested here. IFN- γ promotes the expression of HLA antigens [14] (often diminished on tumor cells) as well as increasing the number of tumor necrosis factor receptors [15], making both targets for immune destruction by cytotoxic (CD8+) T cells. When

Table 1: Changes in PSMA, PSA, PAP and PCA-3 Expression Following Treatment.

Marker	Untreated Control	Monospecific	Mean Corrected Intensity	Bispecific	Mean Corrected Intensity	Bispecific	Mean Corrected Intensity
PSMA	0	MR ₄	20.9	MR ₂₄	33.7	MR ₄₂	33.0
SD			14.7		11.4		10.8
P value vs control			NS		0.03		0.02
PSA	0	MR ₄	54.6	MR ₂₄	72.8	MR ₄₂	71.2
SD			66.1		66.3		60.9
P value vs control			NS		NS		NS
PAP	0	MR ₄	-7.6	MR ₂₄	-4.0	MR ₄₂	-17.3
SD			17.5		23.8		18.2
P value vs control			NS		NS		0.042
PCA-3	0	MR ₄	55.7	MR ₂₄	51.2	MR ₄₂	68.7
SD			61.3		106.5		104.0
P value vs control			NS		NS		NS

induced, PSMA expression on prostate tumor cells could similarly enhance tumor recognition. Therefore, in addition to a bispecific oligo's potential for growth inhibition, its effect on enhanced antigenic expression could also be clinically (immunologically) significant, particularly if combined with vaccines like Provenge. The development of ALPHAVAX ARV and Provenge, directed respectively against PSMA and PAP, would suggest the need to evaluate synergism between immune potentiating vaccines and specifically directed antisense oligos containing double stranded regions. This would appear particularly likely for ALPHAVAX ARV which targets membrane bound PSMA, and which has been deemed suitable for monoclonal targeting by oligos [21].

We predict that for antisense therapy to take the next step forward, more complex forms of antisense must be formulated and delivery mechanisms improved. Multichain (and fat soluble) oligos have already been proposed [17] which could be constructed specifically to target specific gene combinations uniquely expressed or activated in individual patients or tumor types, based on microarray analysis and expression patterns [22]. Furthermore, mechanisms for enhanced delivery and stability, some of which employ polymalic acid [23] or nanoparticle formation with polypropylin-imine dendrimers [24], have been developed. The combination of (oligo based) gene therapy, enhanced delivery and immune potentiation could provide an additional tier of therapy to biochemically relapsed prostate cancer patients.

Acknowledgments

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.

References

1. <http://www.oncogenex.com>
2. Walder RY, Walder JA. Role of RNase H in hybrid-arrested translation by antisense oligonucleotides. *Proc Natl Acad Sci USA*.1988;85(14):5011-15.
3. Felsenfeld G, Miles HT. Formation of a three-stranded polynucleotide molecule. *J Amer Chem Soc*.1957;79(8):2023-24.
4. Durland RH, Kessler DJ, Hogan M. Antiparallel triplex formation at physiological pH. In: Prospects for Antisense Nucleic Acid Therapy of Cancer and AIDS. E. Wickstrom (Ed.), New York: Wiley-Liss, 1991;219-26.
5. Rubenstein M, Tsui P, Guinan P. Bispecific antisense oligonucleotides with multiple binding sites for the treatment of prostate tumors and their applicability to combination therapy. *Methods Find Clin Pharmacol*. 2006;28(8):515-18.
6. Rubenstein M, Tsui P, Guinan P. Combination chemotherapy employing bispecific antisense oligonucleotides having binding sites directed against an autocrine regulated growth pathway and bcl-2 for the treatment of prostate tumors. *Med Oncol*. 2007;24:372-78.
7. Rubenstein, M., Tsui, P. Guinan, P. Multigene targeting of signal transduction pathways for the treatment of breast and prostate tumors: Comparisons between combination therapies employing bispecific oligonucleotides with either Rapamycin or Paclitaxel. *Med Oncol*. 2009;26:124-30.
8. Rubenstein M, Tsui P, Guinan P. Construction of a bispecific antisense oligonucleotide containing multiple binding sites for the treatment of hormone insensitive prostate tumors. *Med Hypotheses*. 2005;65:905-7.
9. Rubenstein M, Tsui P, Guinan P. Bispecific antisense oligonucleotides having binding sites directed against an autocrine regulated growth pathway and BCL-2 for the treatment of prostate tumors. *Med Oncol*. 2007;24:189-96.
10. Rubenstein M, Tsui P, Guinan P. Treatment of MCF-7 breast cancer

- cells employing mono- and bispecific antisense oligonucleotides having binding specificity towards proteins associated with autocrine regulated growth and BCL-2. *Med Oncol* 2008;25: 182-6.
11. Rubenstein M, Guinan P. Bispecific antisense oligonucleotides have activity comparable to monospecifics in inhibiting expression of BCL-2 in LNCaP cells. *In Vivo*. 2010;24:489-93.
 12. Rubenstein M, Hollowell CM, Guinan P. Does the prostate retain an endogenous antiviral defense system suggesting a past viral etiology for cancer? *Med Hypoth*. 2011;76:368-70.
 13. Lubaroff DM, Konety BR, Link BK, Timothy L Ratliff, Tammy Madsen, Richard D William. Outcomes from a phase I trial of an adenovirus/PSA vaccine for prostate cancer. *J Urol*. 2008;179-84.
 14. Basham TY, Nickoloff BJ, Merigan TC and Morhenn VB. Recombinant gamma interferon induced HLA-DR expression on cultured human keratinocytes. *J Invest Derm*. 1984;83:88-90.
 15. Msujimoto M, Yip YK, Vilcek J. Interferon-gamma enhances expression of cellular receptors for tumor necrosis factor. *J Immunol*. 1986;136:2441-44.
 16. Rubenstein M, Mirochnik Y, Chow P, Guinan P. Antisense oligonucleotide intralesional therapy of human PC-3 prostate tumors carried in athymic nude mice. *J Surg Oncol*. 1996;62:194-200.
 17. Rubenstein M, Tsui P, Guinan P. Antisense Oligonucleotides as Specific Chemotherapeutic Delivery Agents: A New Type of Bifunctional Antisense. *Medical Hypotheses and Research* 2009;5:57-61.
 18. Yamanaka K, Miyake H, Zangemeister-wittke U, et.al. Novel antisense oligonucleotides inhibiting both Bcl-2 and Bcl-xL expression induce apoptosis and enhance chemosensitivity in human androgen-independent prostate cancer cells. *Proceedings AACR*. 2004;45:2930a.
 19. Takahara K, Muramaki M, Li D, Cox ME, Gleave ME. Insulin-like growth factor-binding protein-5 (IGFBP5) supports prostate cancer cell proliferation. *J Urol*. 2009;181:184a.
 20. Rubenstein M, Tsui P, Guinan P. Bispecific antisense oligonucleotides having binding sites directed against an autocrine regulated growth pathway and bcl-2 for the treatment of prostate tumors. *Med Oncol*. 2007;24:189-96.
 21. Mirochnick, Y, Rubenstein, M, Guinan P. Targeting of biotinylated oligonucleotides to prostate tumors with antibody-based delivery vehicles. *J Drug Targeting*. 2007;15:342-50.
 22. Rubenstein M, Anderson KM, Tsui P, Guinan P. Synthesis of branched antisense oligonucleotides having multiple specificities. Treatment of hormone insensitive prostate cancer. *Med Hypoth*. 2006;67:1375-80.
 23. Lee B-S, Fujita M, Ljubimova JY, Holler E. Delivery of antisense oligonucleotides and transferrin receptor antibody in vitro and in vivo using a new multifunctional drug delivery system based on polymalic acid. *Proceedings AACR*. 2004;45:647a.
 24. Santhakumaran L, Thomas T, Thomas TJ. Nanoparticle formation in an antisense oligonucleotide by polypropyleneimine dendrimers: facilitation of cellular uptake and intracellular stability. *Proceedings AACR*. 2004;45:2938a.
 18. Yamanaka K, Miyake H, Zangemeister-wittke U, et.al. Novel antisense