in vivo *29*: 687-694 (2015)

Suppression of BCL2 by Antisense Oligonucleotides and Compensation by Non-Targeted Genes May Enhance Tumor Proliferation

MARVIN RUBENSTEIN[1,2,3,4], COURTNEY M. P. HOLLOWELL[2] and PATRICK GUINAN[1,2,4,5]

[1]Division of Cellular Biology, Hektoen Institute for Medical Research, Chicago, IL, U.S.A.;
[2]Division of Urology, Stroger Hospital of Cook County, Chicago, IL, U.S.A.;
Departments of [3]Biochemistry and [4]Urology, Rush University Medical Center, Chicago, IL, U.S.A.;
[5]Department of Urology, University of Illinois at Chicago, Chicago, IL, U.S.A.

Abstract. *Antisense oligonucleotides have been used to target regulatory proteins in both in vivo and in vitro models of prostate cancer. Our previous studies showed that oligonucleotide-treated LNCaP prostate cancer cells compensate for diminished expression of B-cell chronic lymphocytic leukemia/lymphoma 2 (BCL2), an apoptosis inhibitor, by suppressing the expression of caspase-3 (an apoptosis promoter) while enhancing that of serine/threonine protein kinase (AKT1) (another apoptosis inhibitor). In addition, we found an enhanced expression of the androgen receptor (AR), its p300 and interleukin-6 (IL6) co-activators, polymerase transcription mediator (MED12), and growth-regulating signal transducer (STAT3). The net result was an altered pattern of gene expression often associated with more aggressive and proliferative tumors. To further evaluate adaptive compensatory mechanisms related to tumor resistance, aggression and proliferation, herein we evaluated the level of expression of a proliferation antigen (KI-67) and mitosis-regulating cyclins (B1 and D1). Compared to the relative levels of compensation detailed above, we found the expression of KI-67 to be statistically the most enhanced non-targeted protein yet identified in compensation for suppression of BCL2. Expression of cyclin D1 was also significantly enhanced, although to a much lesser extent. As a result, we propose that oligonucleotide-mediated treatment could be more effective when directed towards KI-67 and BCL2. This could be accomplished by dual monospecific targeting KI-67 and BCL2, or with a bispecific (or proposed multispecific) oligonucleotide simultaneously targeting both.*

Correspondence to: Dr. Marvin Rubenstein, Chairman-Division of Cellular Biology, Hektoen Institute for Medical Research, 2240 West Ogden Avenue, 2nd floor, Chicago, IL 60612, U.S.A. Tel: +1 3128644621, Fax: +1 3127686010, email: DrMarv@Prodigy.net

Key Words: Antisense, BCL2, prostate cancer, gene therapy, compensation, KI-67.

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 activity, the actual transcription of most genes regulating tumor growth is similar to that of normal cells. Resistance develops because the biochemical pathways involved are complex, frequently redundant and regulated to differing degrees by combinations of both stimulatory and inhibitory factors (as in apoptosis). We previously reported that non-targeted genes are directly affected by what was believed to be specific therapy, and that some are susceptible to altered expression to an extent having the potential to reverse the effects of the originally intended treatment. This process of compensation (1) may drive tumors to become more aggressive, which in the LNCaP prostate cancer model appears to lead to greater sensitivity (than usual) to androgens and therefore potentially more aggressive tumor. 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 based on suppression of gene translation (including antisense oligonucleotide-mediated gene therapy). While oligonucleotide-based therapy is already entering the clinical environment, the unintended compensatory consequences of intervention are poorly understood, and could contribute to the emergence and selection of more aggressive cancer cells.

Gene therapy has been clinically employed for the treatment of human prostate tumors and together with radio- (2, 3) or chemotherapy (4), antisense oligonucleotides have been administered against inhibitors of apoptosis [particularly B-cell chronic lymphocytic leukemia /lymphoma 2 (b-cell lymphoma2 [BCL2]) and clusterin] in attempts to increase

this often tumor-suppressed but desirable activity. In addition to oligonucleotide-mediated suppression of BCL2, Genentech has developed another type of inhibitor involving a small bioavailable molecule [venetoclax (GDC-0199/ABT-199)] currently in phase I and II clinical trials for the treatment of a variety of tumor types (trial registration number: NCT02055820).

In a number of previous studies (summarized in Table I), we found that LNCaP cells treated with antisense oligonucleotide directed against BCL2 (administered in a nanoparticle suspension of lipofectin) compensated by suppressing expression of caspase-3 (an apoptosis promoter (1)), and enhancing serine/threonine protein kinase (AKT1) (an apoptosis inhibitor) (5), androgen receptor (6) (AR), and AR co-activators p300 (7) and interleukin-6 (IL6) (8). In addition, expression of programmed death (PD1), its ligand PDL1 (immune checkpoint blockage markers) and FAS-ligand, which activate apoptosis through signal transduction, were also enhanced (9), as was that of suppressor protein p53 (10), oncogene *v-myc* (11), polymerase transcription mediator (MED12) (12) and signal transducer (STAT3) (5, 12). This suggests that at least in this LNCaP model, therapeutic approaches to restoring apoptosis (including the use of antisense oligonucleotides to suppress BCL2) can lead to altered expression of non-targeted genes and regulatory changes not only involving apoptosis, but also androgen sensitivity, suppressor/oncogene transcriptional activity and immune responsiveness. Many of the androgen-related alterations are similar to the expression patterns associated with more advanced prostate tumors, while regulation of apoptosis involved both mitochondrial and signal transducing pathways. As noted, enhanced expression of PD1, PDL1 and FAS-ligand and activation of apoptosis (9) presumable would lead to further inhibition of T-cell activity. Compensatory effects identified with these proteins are important since the PD1/PDL1 pathway is now recognized as a target for monoclonal antibody-directed immunotherapy used to treat various types of solid tumors, particularly melanoma and lung tumors.

The present study evaluated the expression of the proliferative antigen KI-67 along with two regulators of mitosis, cyclins B1 and D1. The expectation was that as a result of the numerous compensatory changes noted above, some of which suggest emergence of a more aggressive tumor phenotype, increased proliferation (mitosis) should follow. Expression of KI-67 could be greatly enhanced (13), and if so could provide an additional therapeutic target for antisense oligonucleotides, when similarly targeting BCL2. The biological significance for studying KI-67 is that it is a marker that is exclusively associated with proliferation, and its expression is considered a prognostic indicator for some tumor types (*e.g.* breast). It is expressed throughout all phases of cell division (G_1, S, G_2, and mitosis) but absent while cells rest (G_0). Others have employed antisense oligonucleotides against KI-67 and demonstrated inhibition of both *in vitro* and *in vivo* growth (13) in various models, in addition to inducing apoptosis and reversing the tumorigenicity of breast cancer cells (14). We also evaluated the expression of cyclins B1 and D1 which regulate transition through various checkpoints of the mitotic cycle. Cyclin B1 interacts with and complexes with CDK1. Together they are involved in the early events of mitosis (chromosome condensation, nuclear envelope breakdown, and spindle pole assembly). Cyclin D1 dimerizes with CDK4/6 while regulating the transition from G_1 to S phase. For gene therapy to ultimately be successful it must be made more specific and mechanisms of compensation must be identified and possibly targeted for additional suppression. This could include the use of mono-specific gene regulatory (oligonucleotides) agents employed simultaneously, but could also include either bispecific or even (proposed) multispecific oligonucleotides (15), which simultaneously target BCL2, KI-67 or these cyclins.

We employed reverse transcriptase-polymerase chain reaction (RT-PCR) in these experiments to determine alterations in gene expression. Although more sophisticated techniques are available, we found this method to be both sensitive enough to identify those genes involved with compensation, and able to identify non-targeted genes (such as KI-67, and the cyclins) that are particularly affected and could provide combination targets for BCL2-suppressive therapy.

Materials and Methods

Oligonucleotides. Mono- or bispecific oligonucleotides 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's phosphate buffered saline.

Each oligonucleotide contained at least one CAT sequence and targeted the area adjacent to the mRNA AUG initiation codon for the respective targeted protein (*EGFR* or *BCL2*): MR_4 (monospecific targeting *BCL2*): T-C-T-C-C-C-A-G-C-G-T-G-C-G-C-C-A-T; MR_{24} (bispecific targeting *EGFR*/*BCL2*): 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 *BCL2*/*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 (GIBCO: Grand Island, NY, 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 3 days before 5 ml of medium was replaced with fresh the day before oligonucleotides were added.

Table I. *A summary of previous studies with relative protein expression changes and statistical significance noted.*

Protein	Expression after treatment with			Role	Special category Conformation-dependent
	MR_2 (monospecific)	MR_{24} (bispecific)	MR_{42} (bispecific)		
Apoptosis					
Caspase-3	−35.80%	−40.30%	−43.50%	Promoter of apoptosis	
	0.000203	0.000628	0.006013		
AKT1	256.70%	189.40%	182.60%	Oncogene/inhibitor	
	0.000616	0.000425	0.002014	of apoptosis	
FAS-ligand	88.60%	66.70%	75.80%	Initiator of apoptosis	
	0.00033	0.015039	0.001784		
PD1	149.30%	320.70%	193.90%	Programmed death initiator,	
	0.018476	0.034078	0.005988	immune checkpoint inhibitor	
PDL1	33.30%	51.00%	28.50%	Programmed death ligand,	
	0.006079	0.014394	0.010793	immune checkpoint inhibitor	
Androgen sensitivity					
AR	31.20%	58.50%	53.10%	Androgen-binding receptor	
	0.14869	0.019349	0.018608		
p300	82.90%	93.00%	105.40%	Androgen receptor co-activator	
	0.006297	0.044429	0.007257		
IL6	236.90%	219.30%	139.20%	Androgen receptor co-activator/	
	0.001585	0.005231	0.001537	Cytokine	
Differentiation antigen					
PSA	No change	No change	No change	Prostate-specific antigen	No identified base pair complementarity in oligonucleotides
PSMA	No change	74.50%	71.10%	Prostate-specific	Bispecific oligonucleotides
	0.03	0.02		membrane antigen	have intrastrand base complementarity
PAP	No change	No change	No change	Prostatic acid phosphatase	No identified base pair complementarity in oligonucleotides
PCA-3	No change	No change	No change RNA molecule secreted in urine	Prostate cancer antigen-3	No identified base pair complementarity in oligonucleotides
CD44	No change	No change	No change	Stem cell marker	No identified base pair complementarity in oligonucleotides
IFNγ	No change	61.30%	24.50%	Inducible cytokine involved in	Bispecific oligonucleotides
	0.005831	0.002701		cell surface antigen expression	have intrastrand base complementarity
Growth/proliferative marker					
KI-67	363.00%	427.15	355.70%	Proliferation marker	
	0.000229	0.001137	0.002017		
Cyclin D1	51.20%	73.30%	33.7	Regulates cell cycle	
	0.021419	0.032361	0.048313		
STAT3	166.20%	110.90%	37.80%	Signal transducer regulating growth	
	0.009439	0.004279	0.015529		
MED12	138.10%	181.30%	No change	Polymerase transcription mediator	
	0.018239	0.022919	NS	of growth	
Onco-/suppressor gene					
v-myc	170.10%	201.40%	No change	Oncogene	
	0.002741343	0.000641946	NS		
p53	47.50%	86.50%	58.00%	Suppressor gene	
	0.021376	0.00013	0.007671		

IL6: Interleukin-6; CD44: cluster of differentiation 44; IFNγ: interferon gamma; STAT3: growth-regulating signal transducer; MED12: mediator complex subunit 12.

One hundred microliters of stock oligonucleotides were added to bring the final concentration to 6.25 μM. Incubation proceeded for an additional 24 h in the presence or absence of monospecific MR_4, or the MR_{24} and MR_{42} bispecifics in a nanoparticle suspension with lipofectin.

RNA extraction. Following treatment, the medium was 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, 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 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 diethylpolycarbonate (DEPC)-treated water, and quantitated using a Qubit fluorimeter 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. then 1-4 μl of this RNA was added to1 μl of both sense and antisense primers (forward and reverse sequences, see below) for actin, BCL2, KI-67 and cyclins B1 and D1. The same procedure was followed to obtain the data listed in Table I, which summarizes our findings obtained from evaluating many non-targeted genes. Only those which demonstrated compensation are listed; those which were either poorly expressed or not affected are not listed. 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 (Fisher, St. Louis, MO, USA). 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 $MgSO_4$ was included, the difference compensated for by 3 μl of DEPC-treated water).

Primers. Primer sequences were obtained from the National Center Biotechnology Information (NCBI) website: http:// www.ncbi.nlm.nih.gov/nuccore/NM_031966. They were designed to amplify 100-300 bp fragments and anneal between 58-60˚C. Sequence: Actin: Forward: 5'-CAA ACA TGA TCT GGG TCA TCT TCT C-3', reverse: 5'-GCT CGT CGT CGA CAA CGG CTC-3', PCR product was 353 base pairs in length; BCL2: forward: 5'-GAG ACA GCC AGG AGA AAT CA-3', reverse: 5'-CCT GTG GAT GAC TGA GTA CC-3', PCR product was 127 base pairs in length; KI-67: forward: 5'-TTG GAG AAT GAC TCG TGA GC-3': reverse: 5'-CGA AGC TTT CAA TGA CAG GA-3', PCR product was 218 base pairs in length; cyclin B1: forward: 5' ATA CCT ACT GGG TCG GGA AG-3', reverse: 5'-TCA CCA ATT TCT GGA GGG GGG TA-3', PCR product was 234 base pairs in length; cyclin D1: forward: 5'-TTC AAA TGT GTG CAG AAG GA-3', reverse: 5'-GGG ATG GTC TCC TTC ATC TT-3', PCR product was 221 base pairs in length.

Agarose gel electrophoresis. Agarose gels (1.5%) 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 h at a constant voltage of 70 V using a BioRad 1000/500 power supply (Fisher). 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 quantified 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

KI-67 expression. Identical amounts of extracted RNA from LNCaP cells treated with either mono- or bispecific oligonucleotides directed against *BCL2* (and *EGFR* in the bispecifics) were then evaluated by RT-PCR using primers directed against KI-67. When the background intensity was subtracted, the relative intensity of all bands corresponding to KI-67 representing cells treated with MR4, MR24 and MR42 compared to controls were increased 363.0±158.9% (p=0.000229), 427.1±232.3% (p=0.001137) and 355.7±210.5% (p=0.002017) respectively. These results were pooled from replicate gels and the increase in expression was the greatest seen compared to all our previous experiments (Table I) (1,5-7,8-12,15-18). A representative gel is shown in Figure 1.

Cyclin B1 expression. Expression of cyclin B1 was not detected. Whereas the actin-positive control was.

Cyclin D1 expression. Identical amounts of extracted RNA from LNCaP cells treated with either mono- or bi-specific oligonucleotides directed against BCL2 (and EGFR in the bispecifics) were then evaluated by RT-PCR using primers directed against cyclin D1. When the background intensity was subtracted, the relative intensity of all bands corresponding to cyclin D1-representing cells treated with MR4, MR24 and MR42 compared to controls were increased 51.2±33.2% (p=0.021419), 73.3±39.5% (p=0.032361) and 33.7±27.3% (p=0.048313) respectively. A representative gel is shown in Figure 2.

Discussion

Gene therapy is often promoted as a highly specific and deliverable treatment to control aberrant gene expression by tumor cells, particularly when growth factors, their receptors or apoptosis inhibitors are excessively produced. However, it

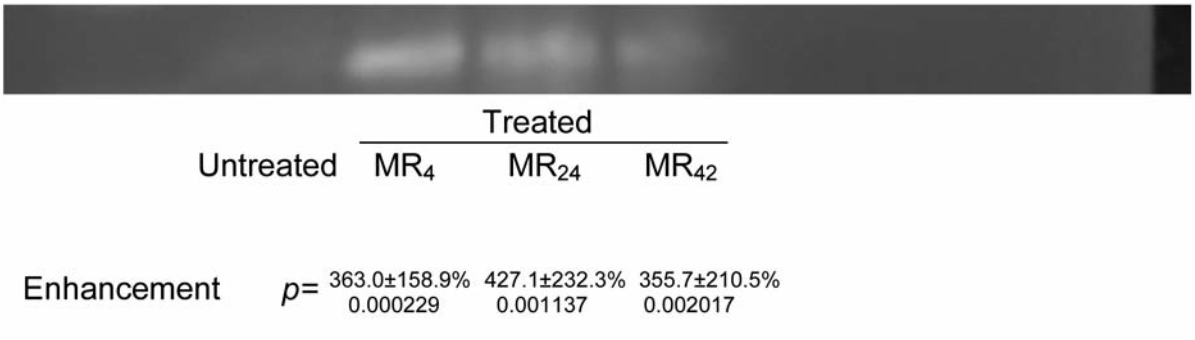

Figure 1. *KI-67 expression is greatly enhanced by oligonucleotide treatment as indicated in a representative agarose gel.*

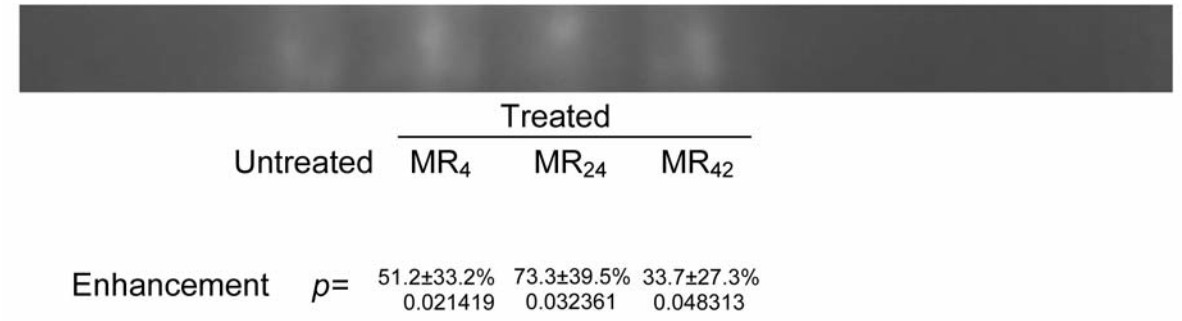

Figure 2. *Cyclin D1 expression is greatly enhanced by oligonucleotide treatment as indicated in a representative agarose gel.*

is now apparent that gene therapy is not as specific as thought previously. Antisense oligonucleotides consist of nucleotide bases synthesized complimentarily in sequence to mRNA. When hybridized to mRNA, they produce a translational arrest of the targeted gene's mRNA expression. 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. These include the phosphorothioated oligonucleotides used in these evaluations and other formulation including 2'-methoxyethyl-RNA, morpholinos, siRNA, miRNA *etc*. Modifications to the oligonucleotide backbone and base structure are used to prevent nuclease degradation, increase systemic half- life or enhance distribution and delivery. Some of these derivatives have been evaluated clinically, but all are directed against single-gene transcriptional (mRNA) products. In contrast, the oligonucleotides discussed herein included both mono- and bispecific forms, each having a base sequence complementary to and directed against mRNA encoding the apoptosis inhibitor BCL2, (bispecifics included an additional site directed against EGFR). We evaluated bispecific oligonucleotides 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 (16), therefore administration of a single oligonucleotide having two mRNA targets could have an additional suppressive benefit. Furthermore, we have shown that both mono- and bispecific oligonucleotides have comparable activity in suppressing BCL2 (16).

While it is understandable that genes which share sequence homology would also be susceptible to antisense oligonucleotides when directed at common sequences, what is not expected are the effects on non-targeted genes, many of which control additional growth-regulatory pathways. We have also shown that certain complementary stretches of base sequences within the oligonucleotide could also produce unanticipated effects on the expression of cell surface antigens (and differentiation proteins). In an early evaluation of bispecifics, we reported the enhanced expression of prostate-specific membrane antigen (17) when oligonucleotides were directed against BCL2. The unique capacity to produce such changes by these bispecfics (and not a similarly directed monospecific) is attributable to an unusual double-strand conformation present in bispecifics and induction of interferon (an enhancer of surface antigen expression) (18). Such

expression could enable better recognition and targeting of cancer cells by cytotoxic T-cells (18).

Tumors are a mass of genetically unstable heterogeneous cells capable of both rapid mutation and selection. Just as bacteria and viral agents develop resistance to chemotherapeutics, tumors cells have a similar capability. In prostate cancer it is thought that hundreds of genes (including those listed here) drive tumor cells to grow, in addition to the effects of androgen acting through the AR (as a transcriptional factor), AR coactivating proteins (p300, IL4, IL6), regulators of apoptosis (BCL2, clusterin, AKT1), transcriptional factors (MED12, STAT3) and various autocrine loops (involving transforming growth factor-alpha, its EGFR-binding site, insulin-like growth factor and its receptor).

Initial evaluation of protein expression associated with compensation regulating the traditional mode of apoptosis (mitochondrial mediated) focused on BCL2, BAX, bcl2-associated death promoter (BAD), clusterin *etc*. However, more recent work evaluated proteins associated with tumor cell destruction, *via* apoptosis, mediated by a secondary route for activation, involving direct signal transduction. This is a process of initiating apoptosis through binding of activating proteins (ligands) to cell surface receptors. When ligands bind to these receptors they activate a destructive cascade of protein interactions which lead to cell death. These receptors are structurally similar to the tumor necrosis factor receptor (CD95) and also regulate the immune system's cytotoxic T-cell response. As mediators of the immune system, their expression can have unanticipated effects on certain types of therapy since these proteins, particularly PD1 and PDL1, are now being targeted by monoclonal antibodies to treat patients *via* immune checkpoint blockade, particularly those with melanoma, mesothelioma and lung cancer (19). Our studies found that expression of PD1, its ligand PDL1 and FAS-ligand were all significantly enhanced following BCL2 suppression and therefore we include immunoregulation as an additional pathway for compensatory-based resistance to control of apoptosis (9).

Oligonucleotides (produced by Oncogenex Pharmaceuticals) have reached clinical trials for the treatment of prostate cancer (OGX-011), while others remain in pre-clinical development (OGX-225). Often administered in combination with traditional chemotherapy, these oligonucleotides target BCL2, clusterin (OGX-011 in phase II testing), heat-shock protein 27 (OGX-427) or insulin growth factor-binding proteins (OGX-225) (20). Genta conducted a phase III test using oligonucleotides (Genasense; oblimersen) directed against BCL2 for treating melanoma, chronic lymphocytic leukemia and various solid tumors (21), but compensatory effects produced by this agent were not reported. Many represent efforts to restore tumor apoptosis by eliminating suppressive BCL2 (2-4) associated with treatment resistance. Similar approaches are directed at clusterin, but compensatory mechanisms activated by these oligonucleotides have not been evaluated. Since derivatives of antisense oligonucleotides (siRNA, miRNA) continue to be developed and tested, and while some directed against BCL2 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 were 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

These results demonstrate that one of the greatest changes in expression resulting from suppression of BCL2 is compensation involving the enhanced expression of KI-67. The statistically significant change we report herein was the greatest found out of all of our previous studies (Table I). This finding validates our previously reported results by identifying their cumulative effect upon proliferation, but also suggests KI-67 as a target for additional oligonucleotide- based therapy, particularly when similar treatment is directed towards BCL2. Such therapy could be delivered by simultaneous administration of two targeted monospecifics, a bispecific oligonucleotide targeting both KI-67 and BCL2, or a proposed (15) multispecific branched derivative. So many growth regulatory pathways are altered in response to BCL2 suppression that it is not surprising that these would culminate in increased proliferation, and KI-67 expression. Based on the relatively modest increases seen in cyclin D1 activity, it is unlikely these changes should be similarly addressed.

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 Pritzker Traubert Family Foundation, the Janet Rubenstein Memorial Foundation, the Sternfeld Family Foundation, and the Herbert C. Wenske Foundation.

References

1 Rubenstein M, Hollowell CMP and Guinan P: Inhibition of BCL2 by antisense oligonucleotides is followed by a compensatory suppression of caspase-3 in LNCaP cells. Eur J Clin Med Oncol *3*: 1-6, 2011.

2 Mu Z, Hachem P and Pollack A: Antisense BCL2 sensitizes prostate cancer cells to radiation. The Prostate *65*: 331-340, 2005.

3 Yip KW, Mocanu JD, Au PY, Sleep GT, Busson D, Yeh P, Gilbert WC, O'Sullivan R, Gullane B, Bastianutto C and Liu FF: Combination BCL2 antisense and radiation therapy for nasopharyngeal cancer. Clin Cancer Res *11*: 8131-8144, 2005.

4 Yamanaka K, Miyake H, Zangemeister-wittke U, Jansen B and Gleave M: Novel bispecific antisense oligonucleotides inhibiting both BCL2 and BCL-xL expression induce apoptosis and enhance chemosensitivity in human androgen- independent prostate cancer cells. Proceedings AACR *45*: (online) Abstract #2930, 2004.
5 Rubenstein M, Hollowell CMP and Guinan P: Additional compensatory mechanisms altering antisense oligonucleotide suppression of BCL2: Effects upon AKT1 and STAT3. In Vivo *28*: 867-870, 2014.
6 Rubenstein M, Hollowell CMP and Guinan P: In LNCaP cells enhanced expression of the androgen receptor compensates for BCL2 suppression by antisense oligonucleotides. Ther Adv Urology *3*: 73-79, 2011.
7 Rubenstein M, Hollowell CMP and Guinan P: In LNCaP cells enhanced expression of both androgen receptor and co-stimulatory protein p300 compensate for antisense oligonucleotide suppression of BCL2 Ther Adv Urology *3*: 243-250, 2011.
8 Rubenstein M, Hollowell CMP and Guinan P: Increased expression of the androgen receptor with p300 and IL-6 coactivators compensate for oligonucleotide suppression of BCL2. No increased CREBBP or IL-4 expression. Ther Adv Urology *5*: 85-93, 2013.
9 Rubenstein M, Hollowell CMP and Guinan P: Following inhibition of BCL2 by antisense oligonucleotides compensatory suppression of apoptosis involves the direct signal transduction pathway of LNCaP cell. Online J Apoptosis *4*: 1-10, 2015.
10 Rubenstein M, Hollowell CMP and Guinan P: Oligonucleotide suppression of BCL2 in LNCaP cells is compensated by increased androgen sensitivity, p53 and oncogene activity, and suppressed caspase-3. Med Oncol *30*: 599, 2013.
11 Rubenstein M, Hollowell CMP and Guinan P: No compensation in VEGF expression follows antisense suppression of BCL2 activity. In Vivo *26*: 937-940, 2012.
12 Rubenstein M, Hollowell CMP and Guinan P: In LNCaP inhibition of BCL2 by nucleotides results in compensatory changes involving apoptosis, transcription and immunoregulation. Enliven: J Genet Mol Cell Biol *1(1)*: 001, 2014.
13 Kausch I, Lingnau A, Endl E, Sellmann K, Deinert I, Ratliff TL, Jocham D, Sczakiel G, Gerdes J and Böhle A: Antisense treatment against Ki-67 mRNA inhibits proliferation and tumor growth *in vitro* and *in vivo*. Int J Cancer *105*: 710-716, 2003.
14 Wang R, Luo D, Ma X, Yang W, Chen R, Liu Y, Meng L, Zhou J, Xu G, Lu YP, Wang S and Ma D: Antisense Ki-67 cDNA transfection reverses the tumorigenicity and induces apoptosis in human breast cancer cells. Cancer Invest *26*: 830-835, 2008.
15 Rubenstein M, Anderson KM, Tsui P and Guinan P: Synthesis of branched antisense oligonucleotides having multiple specificities: Treatment of hormone-insensitive prostate cancer. Med Hypoth *67*: 1375-1380, 2006.
16 Rubenstein M and Guinan P: Bispecific antisense oligonucleotides have activity comparable to monospecifics in inhibiting expression of BCL2 in LNCaP cells. In Vivo *24*: 489-493, 2010.
17 Rubenstein M, Hollowell CMP and Guinan P: Increased prostate-specific membrane antigen expression in LNCaP Cells following treatment with bispecific antisense oligonucleotides directed against BCL2 and EGFR. Med Oncol *27*: 1212-1218, 2010.
18 Rubenstein M, Hollowell CMP and Guinan P: Bispecific oligonucleotides may induce interferon in LNCaP cells enhancing surface antigen expression: Effect of intra-strand base pair complementarity. In Vivo *25*: 61-67, 2011.
19 http://www.washingtonpost.com/business/studies-merck-drug-keytruda-effective- against-3-cancers/2015/04/19/01d94cfc-e6b4-11e4-8581-633c536add4b_story.html
20 Chi KN, Siu LL, Hirte H, Hotte SJ, Know J, Kollmansberger C, Gleave M, Guns E, Powers J, Walsh W, Tu D and Eisenhauer E: A phase I study of OGX-011, a 2'-methoxyethyl phosphorothioate antisense to clusterin, in combination with docetaxel in patients with advanced cancer. Clin Cancer Res *14*: 833, 2008.
21 Kling J: Safety signal dampens reception for mipomersen antisense. Nat Biotechnol *28*: 295-297, 2010.
22 Siegal RL, Miller KD and Jemal A: Cancer Statistics, 2015. CA Cancer J Clin *65*: 5-29, 2015.

Received August 25, 2015
Revised October 5, 2015
Accepted October 8, 2015