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MECHANISTIC STUDIES OF GUANINE-RICH OLIGONUCLEOTIDES

By

Allicia C. Girvan
B. S., Southwest Baptist University, 2000

A Dissertation Submitted to the Faculty of
the Graduate School of
the University of Louisville
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the Requirements for the Degree of

Doctor of Philosophy

Department of Biochemistry and Molecular Biology
University of Louisville
Louisville, Kentucky

May 2006

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A Dissertation Approved on

April 28, 2006

By the following Dissertation Committee:

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Dissertation Director

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DEDICATION

To my loving husband

Sonny

Thank you for your unconditional love
and encouragement everyday

ACKNOWLEDGEMENTS

I hold such admiration and respect for my mentor Dr. Paula Bates. Her guidance and training has provided me with the foundation in which I will use throughout my entire career. She has shown me how to be successful in both career and life, for which I am grateful. I also would like to thank my committee members: Dr. John Trent, Dr. Tom Geoghegan, Dr. Russ Prough, and Dr. John Eaton for all their guidance and flexibility enabling me to finish. I want to extend my sincere and immense thanks to my past and present members of the lab who I not only call coworkers but also good friends: Sheila Thomas, Millicent Gornek-Winner, Lavona Casson, Simone Jueliger, Virna Dapic, Xiaohua Xu, and Yun Teng. I would like to express gratitude to friends I have had the pleasure of meeting in the department: Brian, Amy, George, Kristy, Avedis, Alvin, Tariq, Jim, Shahenda and Krista. I will be forever grateful to my parents, Gary and Karen, for their encouragement and sacrifice throughout my entire life to enable my success. I would like to express my gratitude to my sons, Caleb and Joshua, who could turn a bad day into a great one with one smile. I whole-heartedly thank my loving husband, Sonny, for all of his love and support through these years. He has endured my stress with nothing but encouragement, understanding, and motivation to persevere.

ABSTRACT

MECHANISTIC STUDIES OF GUANINE-RICH OLIGONUCLEOTIDES

Allicia C. Girvan

May 2006

Guanine-rich oligonucleotides (GROs) are being developed as a novel anticancer agents. GROs exhibit potent antiproliferative properties against several malignant cell lines and in established *in vivo* tumor models. In a recent Phase I clinical trial for patients with advanced cancer, one of these GROs was found to have promising signs of clinical activity with no serious side effects. It has been shown that the activity of GROs requires the formation of a G-quartet structure, and their activity correlates with its ability to bind to a specific complex of proteins. One important GRO-binding protein that has been previously identified is nucleolin, a multifunctional protein that is expressed at high levels in cancer cells. In this report, we investigate the novel mechanism of GRO activity by identifying the proteins that bind to an active GRO with use of mass spectrometry. Fourteen nuclear proteins and three cytoplasmic proteins were identified as associating with the active GRO, all of which vary in cellular functions described in the present study. The identified GRO-associated proteins include a protein known as NEMO (NF-κB essential modulator). NF-κB transcription regulates the expression of genes involved in cellular processes such as cell growth, differentiation, and apoptosis. NF-κB signaling is often deregulated in cancer cells, and NEMO is a regulatory component of the cascade.

We demonstrate here that GROs bind to NEMO and nucleolin inside the cell, inhibit NF-κB transcription, and prevent phosphorylation of the NF-κB signaling cascade in response to TNFα. To further investigate the mechanism of GROs, we utilized global expression technology to determine the effects of GROs on the entire cell. Microarray gene expression analysis revealed the alteration of 63 genes after 2 hours of treatment with GRO, and 238 genes after 18 hours of treatment with GRO revealing multiple biological systems affected. The data presented in this report reveals important features of the effects of GROs on the cell which gives insight into the antiproliferative mechanism of the novel anticancer agent.

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CHAPTER I

BACKGROUND AND INTRODUCTION

OLIGONUCLEOTIDE THERAPEUTICS

Oligonucleotides are short nucleic acid chains of DNA or RNA up to 100 nucleotides long. Although synthesized chemically, oligonucleotides are identical to naturally occurring nucleic acid sequences with a sugar-phosphate backbone and either purine or pyrimidine base attached to the sugar ring. Because of their structural similarity, oligonucleotides mimic the interactions of nucleic acids found in nature. They have the potential to recognize unique DNA or RNA by sequence specific hybridization or bind to proteins by recognizing a specific sequence or shape (reviewed in [1]).

Therapeutic strategies based on the use of oligonucleotides have become very attractive due to their apparent high target specificity and few toxic side effects [1, 2]. Oligonucleotide therapy is now being developed as treatment in a broad range of diseases such as cancer, genetic disorders, coronary disease, inflammatory disease, and viral infections including HIV, influenza, and hepatitis [1-3]. There are several different strategies utilizing oligonucleotides in therapy such as triplex formation, antisense, siRNA, and aptamer oligonucleotides.

Triplex forming oligonucleotides

Triplex forming oligonucleotides (TFOs) are molecules that bind in a sequence-specific manner to duplex DNA creating a third strand. TFOs bind in the major grove of duplex DNA on polypurine/polypyrimidine tracts. Upon binding, TFOs can alter gene expression by physically blocking the target site and inhibiting transcription initiation or transcription elongation (Figure 1A). Another mechanism by which TFOs modify gene expression is by site-directed mutagenesis. TFOs can either deliver a mutagen-causing agent, or cause mutagenesis within the binding site in the absence of a mutagen (Figure 1B). TFOs can also function by stimulating recombination in the target sequence by inducing DNA repair in tandem homologous regions or by introducing a homologous donor to the target sequence (Figure 1C) [4-6].

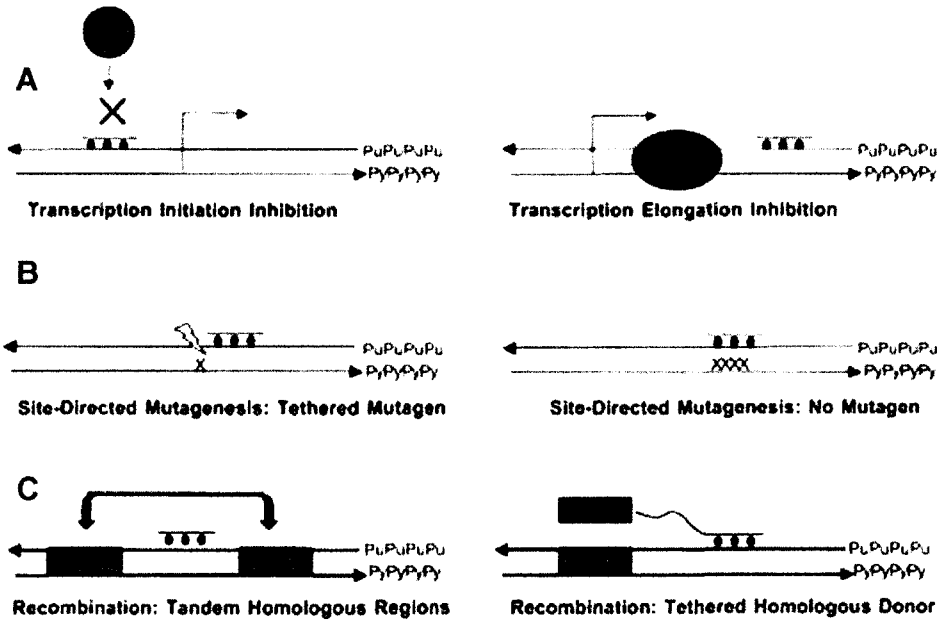
Antisense oligonucleotides

Antisense oligonucleotides (ASOs) refer to oligonucleotides designed specifically to bind to target mRNA and inhibit the expression of the target gene. Once the ASO hybridizes to the complementary mRNA strand, gene expression can be modified by preventing the formation of ribosomal complexes, thus inhibiting the translation of the target protein (Figure 2). ASOs can also function by binding to the target mRNA, and activating RNase H which leads to the degradation of the target mRNA, and inhibiting the expression of the gene of interest (Figure 2) [7-9].

Figure 1: Mechanisms of Triplex forming oligonucleotides.

(A) Blocking of transcription by interfering with transcription factors or elongation;

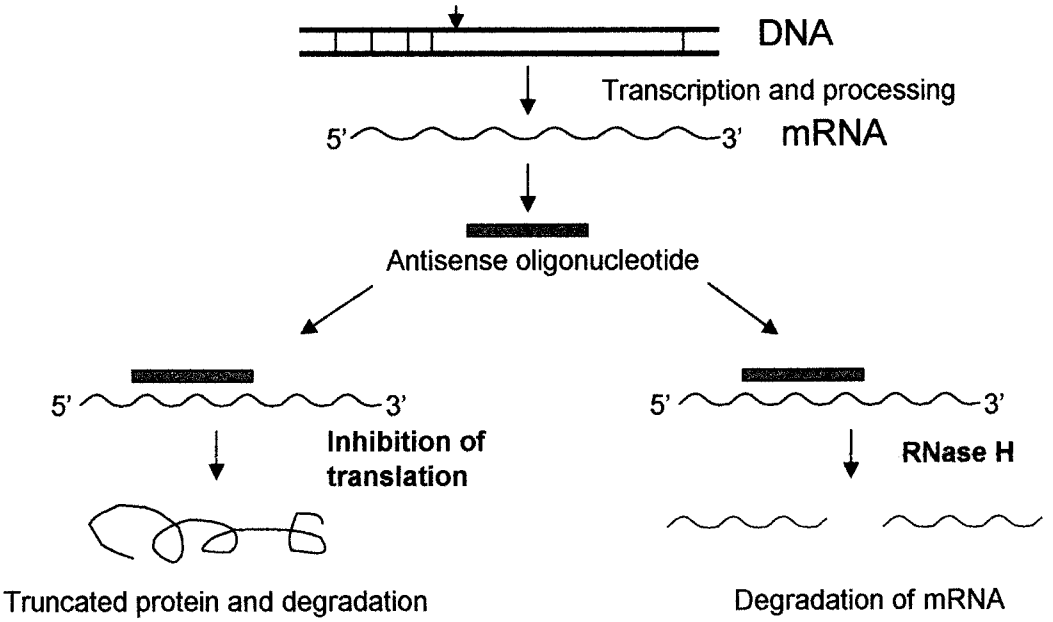
(B) TFO directed site-specific mutagenesis; (C) Triplex-induced recombination [4].



Reproduced from [4].

Figure 2: Mechanisms of antisense oligonucleotides.

Antisense oligonucleotides inhibit translation by either blocking ribosome formation or activating RNase H [7].



Modified from [7].

Small interfering RNA

Small interfering RNA (siRNA) refers to small double-stranded RNA nucleotides designed to lead to the degradation of complementary mRNA resulting in down regulation of the gene of interest. The mechanism of siRNA begins with the production of double-stranded RNA complementary to the target mRNA. This double-stranded RNA is then cleaved by the enzyme Dicer into smaller siRNA fragments that are then unwound by a helicase. One strand of siRNA is then incorporated into RNA-induced silencing complex (RISC). Subsequently, RISC is guided to the target mRNA for cleavage, resulting in reduced translation of the target mRNA (Figure 3) [7].

Aptamer oligonucleotides

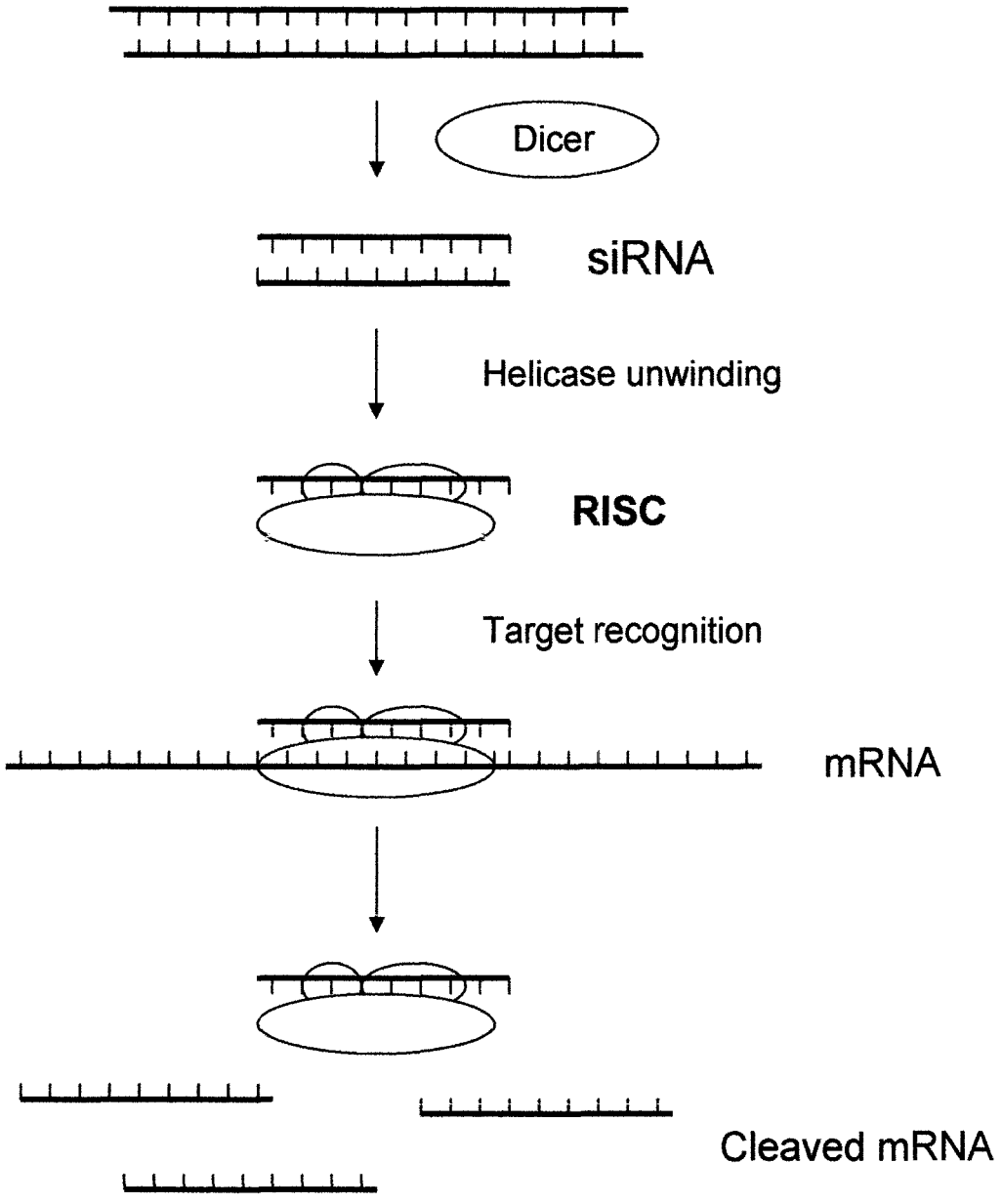
Aptamers are RNA or DNA oligonucleotides that can bind with high specificity to a wide range of target molecules including amino acids, antibiotics, proteins, and nucleic acids. Aptamers also bind to their target molecules with very high affinity due to their ability to fold upon binding to the target molecule. This allows the incorporation of the nucleic acid into the structure of the molecule such as a protein. Upon binding to the target, apatmers subsequently modulate is activity [10-13].

GUANINE-RICH OLIGONUCLETIDES AND G-QUARTETS

Nucleic acids rich in guanine are capable of forming four-stranded structures that are stabilized by G-quartets. G-quartets contain four coplanar guanines stabilized by Hoogsteen hydrogen bonds with a monovalent cation in the center (Figure 4). These G-

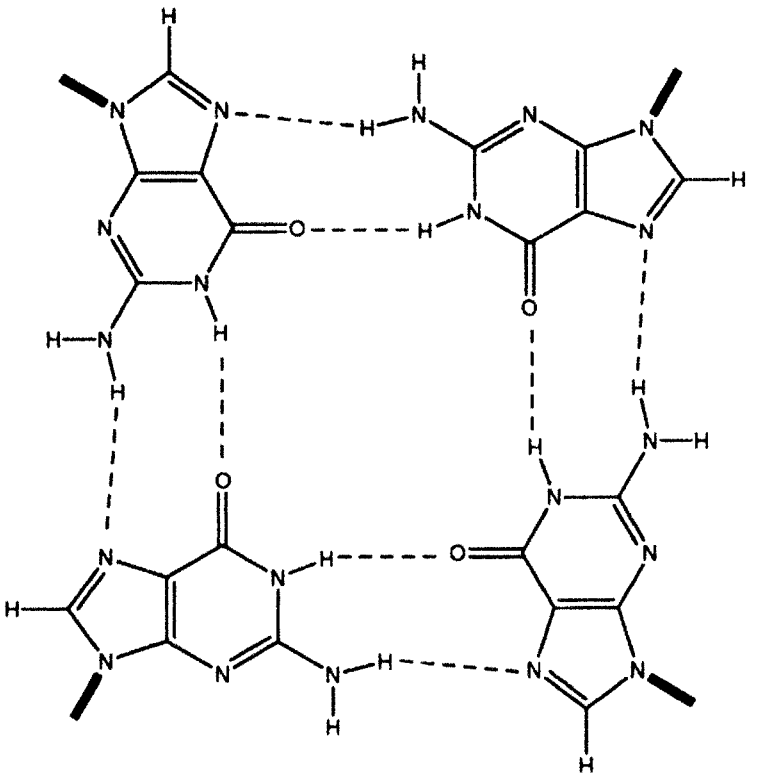
Figure 3: Mechanism of small interfering RNA.

Double stranded RNA is cleaved by Dicer into siRNAs. Helicases unwind the siRNA and then the siRNA is incorporated into RISC, which guides the siRNA to its target mRNA for cleavage [7].



Modified from [7].

Figure 4: Schematic diagram of the G-quartet showing four guanines linked by Hoogsteen hydrogen bonding.



Reproduced from [15].

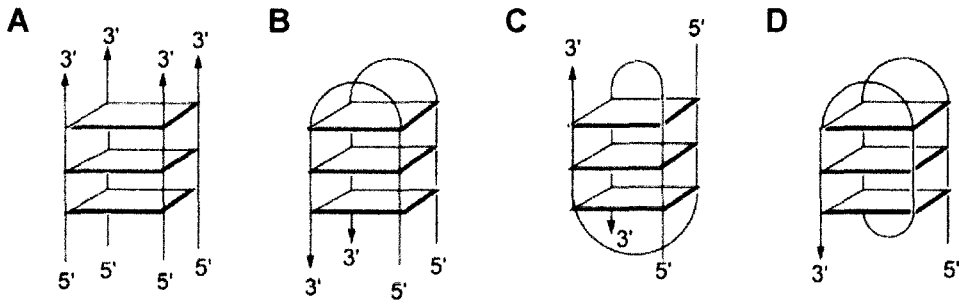
quartets are capable of hydrophobic stacking that increases the stability of the structure with increasing number of stacks. Based on the number of single-stranded oligonucleotides involved in formation of the G-quartets, there are several different strand configurations possible as seen in Figure 5. G-quartets can contain four single-stranded oligonucleotides that form a tetraplex structure (Figure 5A). Two strand dimerization can occur with hairpin loops along the edge (Figure 5B) or diagonally (Figure 5C). A monomer structure can also occur with the single oligonucleotide strand folding upon itself (Figure 5D) [14, 15].

It is now widely believed that G-quartets are involved in regulating diverse biological functions. Sequences capable of forming the G-quartet motif have been identified in telomeres [16], which are specialized sequences at the ends of chromosomes. The enzyme telomerase adds the telomeric repeat sequence onto chromosome ends. It has been shown that 85-90% of all human cancers are positive for telomerase, and most somatic cells appear to lack detectable levels of the enzyme [17]. Recently, there has been intense interest in telomeres and G-quartets as a novel target for cancer therapy [18].

Other sequences capable of forming G-quartets have been identified *in vitro*. There has been particular interest in sequences such as the *c-myc* promoter [19], the HIF-1α promoter [20], the *c-kit* promoter [21], the fragile X repeat sequence [22], and the Cystatin B promoter, which has been implicated in epilepsy [23]. Guanine-rich strands have also been reported in the human insulin gene [24], immunoglobulin switch regions [25], and HIV1 RNA [26].

Figure 5: Schematic representing different G-quartet motifs.

(A) Four single-stranded oligonucleotides forming a tetraplex; (B) a dimer of oligonucleotides with edgewise loops; (C) a dimer of oligonucleotides with diagonal loops; and (D) a monomer.



Reproduced from [14].

G-quartet motifs most likely bind to specific proteins by the aptamer mechanism, which involves shape-specific recognition of the protein. A number of proteins that interact specifically with G-quartets have been previously identified. Nucleolin has been reported to bind to G-quartet sequences in immunoglobin switch regions [25], telomeres [27], ribosomal genes [28], and the c-myc promoter [29], although its function in G-quartet binding is not yet understood. Other proteins capable of binding to G-quartets include helicases implicated Bloom's [30] and Werner's [31] syndromes, the Saccharomyces cerevisiae protein CdC13p [32], and the zinc finger protein Gq1 [33].

PROPERTIES OF GUANINE-RICH OLIGONUCLEOTIDES

Guanine-rich oligonucleotides (GROs) are a class of G-quartet forming oligonucleotides that exhibit potent growth inhibitory effects against many malignant cells lines [34-37]. Figure 6 (A-C) shows the antiproliferative activity of GROs against breast cancer cells in culture (for all GRO sequences see Table 1). The active GRO (GRO29A) almost completely inhibits the growth of hormone-dependent (MCF7) and hormone-independent (MDA-MB-231) breast cancer cell lines, whereas the control oligonucleotide (GRO15B) has little effect. This growth inhibition is also seen in an *in vivo* model with mice. Figure 6D shows that AGRO100 (a truncated version of GRO29A) can completely inhibit the growth of human DU145 prostate tumor xenografts in nude mice, whereas the control oligonucleotide shows little effect

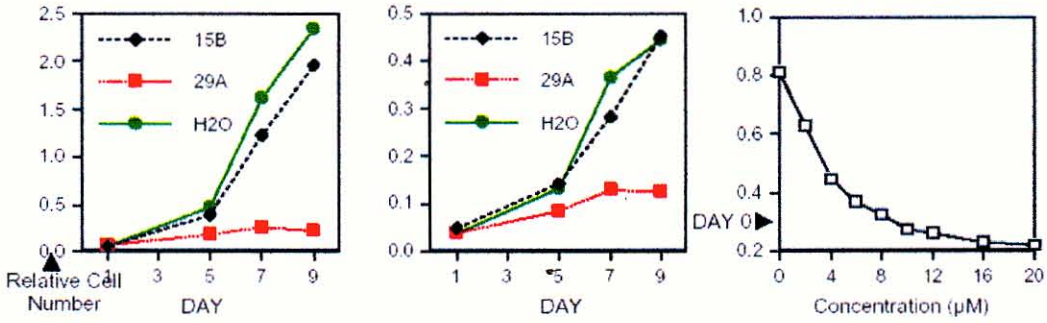
These results lead to a Phase I clinical trial in patients with advanced cancer. Seventeen patients received AGRO100 and results indicated that AGRO100 had little

Table 1: Sequences and antiproliferative activities of GROs.

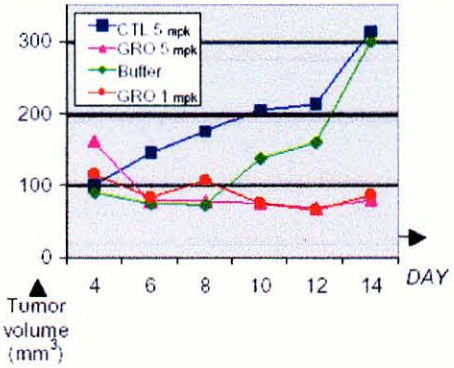
GRO	Sequence	Antiproliferative activity
GRO29A	5' -TTTGGTGGTGGTGGTTGTGGTGGTGGTGG	active
GRO15B	5' -TTGGGGGGGGTGGGT	inactive
AGRO100	5' -GGTGGTGGTGGTTGTGGTGGTGGTGG	active
CRO26	5' -CCTCCTCCTCCTTCTCCTCCTCCTCC	inactive
TEL	5' -TTAGGGTTAGGGTTAGGGTTAGG	inactive

Figure 6: Antitumor activity of GROs.

(A and B) GRO29A inhibits the growth of MDA-MB-231 and MCF7 breast cancer cells in culture (cells were treated as described in [31]); (C) dose-dependent antiproliferative effects of GRO29A (single-dose) against MDA-MB-231; (D) activity of AGRO100 against established humor tumor xenografts (DU145) in nude mice at dose equivalent to 1 or 5 mg/kg (mpk). Tumor volume was measured using calipers and values shown are the mean for 6 animals. Error bars are not shown but differences were statistically significant for 5 mpk ($p=.044$; $p=.082$ for 1 mpk).



C Effect of AGRO100 on tumor growth *in vivo*



toxicity and no serious side effects [38]. An analysis of the efficacy of the drug revealed seven patients showed disease stabilization lasting between 2 and 15 months. Additionally, one patient exhibited almost complete remission of disease with his tumor regressing from 19 cm to less than 1 cm, and the regression continued for more than 16 months [39]. These results demonstrate AGRO100 as a promising novel anti-cancer agent.

The antiproliferative activity of GROs apparently has a novel mechanism of action that is completely different from conventional chemotherapeutic drugs. Upon treatment with GRO, cancer cells exhibit S phase cell cycle arrest, DNA replication inhibition, and cell death [35]. From the sequence of active GROs, it is clear that they are not working by a hybridization-dependent mechanism such as triplex, antisense, or siRNA. Instead, it is thought that they function as aptamers by binding to specific proteins via shape-specific recognition [34-37]. A structural analysis of GROs revealed that the antiproliferative activity of GROs requires the formation of the G-quartet motif. The analysis also revealed that the growth inhibitory activity of GROs positively correlates with their ability to bind to a complex of proteins [37], one of which has been previously identified as nucleolin [34].

Nucleolin is a multifunctional 110-kDa phosphoprotein that has been implicated in a variety of biological processes such as cell growth and proliferation [40-42], ribosome biogenesis [40, 43], DNA replication [44], cytokinesis and nuclear division [45], and apoptosis [46, 47]. Nucleolin is generally considered a predominantly nucleolar

protein, however, there have been numerous reports demonstrating that nucleolin can also be present in the nucleoplasm, cytoplasm, and on the cell surface [34, 48-53]. Futhermore, the redistribution of nucleolin has been shown in response to stimuli such as heat shock [44, 54], T-cell activation [55], mitosis [56], treatment with cyclin-dependent kinase inhibitor [57], and viral infection [58-60]. It is now widely believed that nucleolin can act as a shuttle protein transporting viral and cellular proteins between the cytoplasm and nucleus [60-62]. There is also evidence suggesting a possible role for nucleolin in cancer etiology. Cancer prognostic markers known as AgNORs (silver-stained nucleolar organizer regions) have been shown to contain nucleolin in large amounts [63, 64], and it has been shown there are increased levels of nucleoin in rapidly proliferating cells such as cancer cells [65]. It has also been previously observed in our group that nucleolin is present on the surface of several malignant cell lines while absent on the surface of normal cell lines (unpublished observations).

It is our hypothesis that active GROs exert growth inhibitory effects on malignant cells by binding and modulating specific proteins that affect relevant pathways. Nucleolin is a known GRO binding protein that has previously been seen on the surface of cancer cells but primarily absent on the plasma membrane of nonmalignant cells. GROs could bind to nucleolin and inhibit the function of nucleolin, and/or GROs could be internalized by surface nucleolin and allow GROs to bind and modify the function of proteins other than nucleolin. **The purpose of this project is therefore to elucidate the precise molecular mechanism of GRO antiproliferative activity against cancer cells by identifying the relevant proteins that interact with GROs and elucidating the**

pathways affected by them. We propose that by investigating molecular changes in GRO binding proteins and incorporating global expression data, the affected biological pathways can be identified. Knowledge of specific cellular proteins and pathways affected by GROs may lead to new molecular targets in the treatment of cancer, design of small molecule inhibitors, and better prediction of therapeutic response of cancer. The specific aims of this dissertation have been:

Specific Aim 1: To identify GRO binding proteins and confirm binding by diverse methodology.

Specific Aim 2: To investigate changes in levels, localization, and activity in GRO treated cells.

Specific Aim 3: To confirm the relevant biochemical pathways involved in the mechanism of GRO antiproliferative activity using proteomic and microarray analysis.

Rationale

GROs inhibit the proliferation of cancer cells *in vitro* and *in vivo* by a mechanism that is completely different from conventional chemotherapeutic drugs or traditional oligonucleotide strategies. One important GRO-binding protein has been identified as nucleolin, which has many cellular functions. GROs could directly inhibit the function of nucleolin, however, it is difficult to determine which function because of the numerous

actions of nucleolin. If nucleolin is the primary target, we would anticipate that the identified GRO binding proteins may interact with nucleolin and be involved in nucleolin-regulated processes such as such as ribosome biogenesis or apoptosis. Alternatively, nucleolin on the surface of the cell could bind to the GRO and transport the G-quartet inside the cell. The GRO could then bind to other intracellular targets and affect a number of cellular pathways. We would then expect the identified GRO binding proteins to be involved in functions unrelated to nucleolin.

CHAPTER II

IDENTIFICATION AND ANALYSIS OF PROTEINS THAT BIND TO GUANINE-RICH OLIGONUCLEOTIDES

INTRODUCTION

Cancer is a major public health problem and is estimated to cause more than half a million deaths annually in the United States [66]. Prevention, screening and treatment regimens have improved over the last decade; however toxicity from treatment still remains a problem. This has lead to considerable interest in the development of novel tumor-specific agents such as oligonucleotide therapy. Oligonucleotide therapy is attractive due to the expectation that they will produce a response that is tumor-specific and therefore, effective without toxicity to normal cells [67]. The strategies utilized by oligonucleotide therapy include antigene (also known as triplex forming oligonucleotides), antisense, siRNA, and aptamer recognition [7, 10].

We have previously reported a novel class of phosphodiester guanine-rich oligonucleotides (GROs) that exhibit antiproliferative effects against several cancer cell lines [34-37]. Upon treatment with GROs, cells undergo inhibition of DNA replication, S phase cell cycle arrest, and cell death [35]. The antiproliferative GROs were also shown to form a G-quartet, a stable secondary structure consisting of four coplanar guanines joined by Hoogsteen hydrogen bonding. Although all active GROs adopted the G-quartet

motif, its secondary structure alone could not predict the antiproliferative activity of GRO. However, the biological activity of GRO could be determined by its ability to bind to a complex of proteins, one of which has been identified as nucleolin [34, 36].

Nucleolin is primarily thought of as a major nucleolar protein expressed by proliferating cells, where it is directly involved in the regulation of ribosome biogenesis and maturation [41]. There have been numerous reports, however, implicating nucleolin in several different biological functions such as cell growth and proliferation [40-42], ribosome biogenesis [40, 43], DNA replication [44], cytokinesis and nuclear division [45], and apoptosis [46, 47]. Nucleolin can also function as a cell surface receptor, where it acts as a shuttling protein between cytoplasm and nucleus [60-62]. Nucleolin may also play a role in cancer progression due to its critical involvement in cell proliferation [42] and increased expression in rapidly proliferating cells such as cancer cells [65], which may be implicated in the mechanism of GROs.

GROs may have significant therapeutic potential as anticancer agents with much less toxicity than conventional cytotoxic drugs. There are important aspects known about the function of GROs, such as cell cycle arrest and nucleolin binding. However, the precise molecular mechanism of GROs is not known; a powerful tool in deciphering the mechanism of GROs is the identification of GRO-associated proteins. Here we report that the binding of GROs to a complex of proteins posivitely correlates with the antiproliferative activity of GRO. To further elucidate the mechanism of GROs, the

proteins that bind to GROs *in vitro* have been analyzed and identified by mass spectrometry.

MATERIALS AND METHODS

Oligonucleotides

All oligonucleotides have a phosphodiester backbone, were purchased in the desalted form from Integrated DNA Technologies Inc. and used without further purification. Oligonucleotides were resuspended in water, sterilized by filtration through a 0.2 μm filter, then diluted with sterile water to give stock solutions of 400-500 μM that were stored in aliquots at -20 °C. Sequences of oligonucleotides used were as follows: GRO29A, 5'-d(TTTGGTGGTGGTGGTTGTGGTGGTGGTGG)-3'; GRO15B, 5'-d(TTGGGGGGGGTGGGT)-3'; AGRO100, 5'-d(GGTGGTGGTGGTTGTGGTGGTGGTGG)-3'; GRO15A, 5'-d(GTTGTTTGGGGTGGT)-3'; TEL, 5'-d(TTAGGGTTAGGGTTAGGGTTAGG)-3'. For the experiment shown in Figure 7, the following biotin (Bt) linked versions of GRO29 and 15B were used: 5'-Bt-d(TTTGGTGGTGGTGGTTGTGGTGGTGGTGG)-3' and 5'-Bt-d(TTGGGGGGGGTGGGT)-3'.

Electrophoretic Mobility Shift Assay (EMSA)

TEL oligonucleotide was radiolabeled with ^{32}P using T4 kinase. At the indicated concentrations, labeled oligonucleotide was preincubated alone or in the presence of of an unlabeled competitor oligonucleotide for 30 min at 37°C. HeLa nuclear extracts (bandshift grade: Promega, Inc.) were added, and samples were incubated for an additional 30 min at 37°C. Preincubation and binding reactions were carried out in buffer

A(20 mM Tris·HCL, pH 7.4, 140 mM KCl, 1 mM dithiothreitol, 0.2 mM phenylmethanesulfonyl fluoride, and 8% v/v glycerol). Electrophoresis was carried out using 5% polyacrylamide gels in TBE buffer (90mM Tris-borate and 2 mM EDTA).

Identification of candidate GRO-binding proteins

Extracts were prepared from HeLa cells as previously described [47]. Samples (250 μg) were incubated with a biotin-linked active GRO (GRO29A) or biotin-linked inactive control oligonucleotide (GRO15B). The oligonucleotide-associated proteins were then captured by streptavidin precipitation (Streptavidin Magnashere Paramagnetic Particles, Promega) and electrophoresed on SDS-polyacrylamide (8%) gels. Silver staining was used to identify bands present in the GRO29A-precipitated lane but absent in the control GRO15B lane. These bands were excised, subjected to trypsin digestion, and analyzed by MALDI-TOF mass spectrometry analysis as described [19].

RESULTS

Activity of GRO correlates with protein binding

Because GROs have no anticipated antisense interactions, and they are capable of forming G-quartet structures, we hypothesized that the growth inhibitory effects of GROs results from the binding to (and subsequent modulation of) certain cellular proteins. Electrophoretic mobility shift assay (EMSA) analysis of radiolabeled GRO and nuclear extracts revealed a GRO-protein complex that correlates with the antiproliferative activity of GRO. Nuclear extracts were incubated with unlabeled active GRO (29A and 26B), inactive GRO (15B), or a GRO with intermediate activity (15A) that competed with ^{32}P-

labeled TEL, which represents the human telomere sequence that is a G-quartet forming oligonucleotide. The absence of the indicated complex correlates with the antiproliferative activity of each GRO (See Figure 7).

Identification of GRO binding proteins

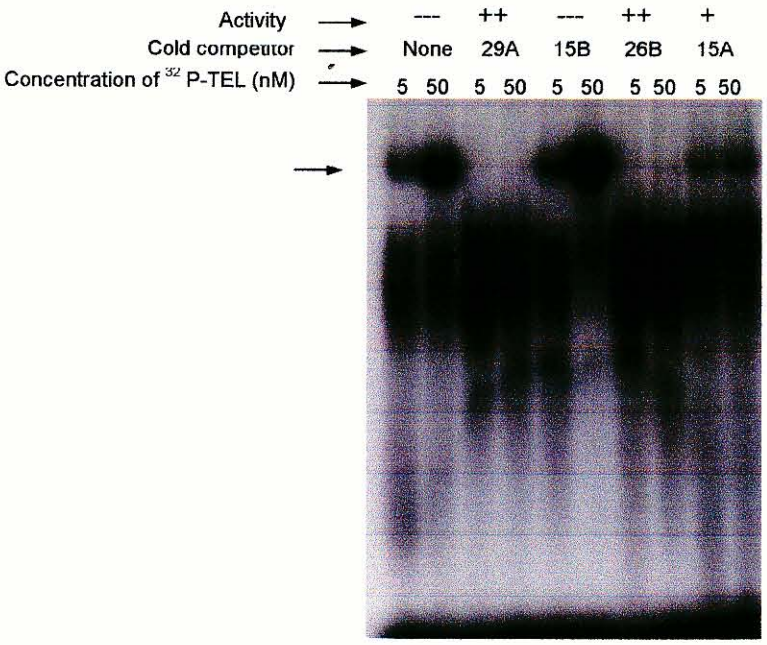
To identify the proteins that bind to active GROs, precipitation with biotinylated oligonucleotides was used to isolate the proteins in nuclear and cytoplasmic extracts that bind to active GRO29A and inactive GRO15B. Running the samples on an 8% SDS polyacrylamide gel revealed several proteins that bind to the active GRO29A but not to the inactive GRO15B (see Figure 8). The bands visible in the GRO29A sample but not present in the GRO15B sample were excised and subjected to proteomic analysis using MALDI-TOF mass spectrometry to identify the proteins that bind to GRO29A (See Table 2). One of the GRO-binding proteins was identified as nucleolin, a previously identified GRO binding protein that is implicated in GRO antiproliferative activity [34], thus validating the technique. The additional 14 proteins identified to associate with the active GRO are summarized below:

Polypyrimidine tract-binding protein (PTB)-associated splicing factor (PSF)

PSF is a 100 kDa polypeptide that was first identified and characterized in a complex with PTB. The polypyrimidine tract is a region found in most introns where several factors bind and is important for characterizing the 3' splice site. PSF binds to polypyrimidine tracts and is an essential factor required during the second catalytic step in splicing [68]. Additional examples exhibiting the role of PSF in pre-mRNA splicing

Figure 7: Activity of GROs correlate with protein binding.

EMSA analysis of HeLa nuclear extracts and ^{32}P-labeled TEL (representing human telomere sequence) showing the complex formed by the nuclear proteins and TEL as indicated by the arrow. Unlabeled GROs compete for the complex that correlates with the activity of GRO.


Activity

++

++
+
Cold competitor
None
29A
15B
26B
15A
Concentration of 32 P-TEL (nM)
5 50 5 50 5 50 5 50 5 50

Figure 8: Identification of GRO binding proteins.

Nuclear and S-100c extracts (100 μg) from HeLa cells were incubated with biotinylated active GRO (29A) or biotinylated inactive GRO (15B). The bound proteins were then captured by streptavidin and run on an 8% SDS polyacrylamide gel. Silver staining revealed several bands present with the 29A but absent with the control 15B as indicated by the arrows. These bands were excised and identified by MALDI-TOF mass spectrometry analysis (See Table 2).

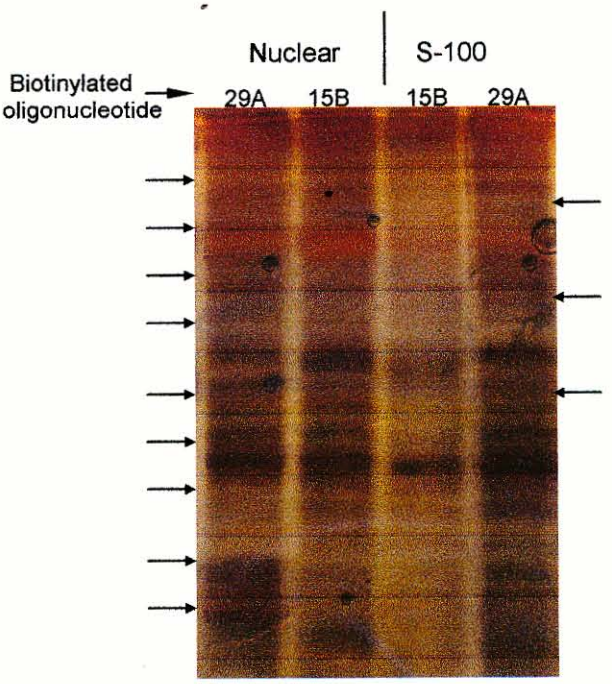

Nuclear
S-100
Biotinylated oligonucleotide
29A
15B
15B
29A

Table 2: Nuclear and cytoplasmic proteins identified by mass spectrometry that precipitated with the active GRO.

Table 2: Specific GRO29A binding proteins	
Nuclear binding proteins	Cytoplasmic binding proteins
nucleolin	HSP 90β
PSF	β Tubulin
P54nrb	actin
HnRNP C	
Transcription factor IIIA	
alpha synuclein	
complexin I	
immunoglobulin heavy chain	
HnRNP A1	
cytochrome oxidase VIC	
NF-kB essential modulator	
HnRNP A2/B1	
RAD50 repair protein	
ribosomal protein L19	

include: PSF is a component of the spliceosome C complex [69], PSF physically interacts with U1A in a complex that functions in splicing and polyadenylation [70], and PSF has been shown to bind to RNA polymerase II [71]. It has also been shown that PSF is involved in binding and nuclear retention of defective RNAs [72] [reviewed in [73]].

PSF also demonstrates activity involved in DNA unwinding and DNA pairing. PSF co-purifies with topoisomerase I and partially co-localizes with topoisomerase I in interphase cells. *In vitro* studies have shown that the addition of recombinant PSF to purified topoisomerase I increases the specific activity of the enzyme [74]. PSF has also been shown to exhibit DNA reannealing activity by forming a DNA duplex from two homologous DNA strands [75].

In regards to tumorigenesis, PSF may play a role in proliferation. Transformed cells show elevated levels of PSF compared to non-immortalized cells. Additionally, proliferating cells exhibit two-fold higher activity of PSF than quiescent cells, suggesting a possible role for PSF in proliferation [76].

P54nrb

P54nrb was originally purified as a heterodimer with PSF [77], and it is highly homologous to the C-terminus of PSF [78]. In fact, many reports have indicated p54nrb and PSF copurify and share many functions within the cell. One of which is pre-mRNA processing. P54nrb forms a complex with PSF that interacts with U1A and functions in splicing and polyadenylation [70]. Like PSF, p54nrb has been shown to bind to RNA

polymerase II [71]. P54nrb also shares with PSF the function of binding and nuclear retention of defective RNAs [72]. Another common function of p54nrb and PSF is DNA unwinding; p54nrb copurifies with PSF and topoisomerase I and exhibits functional activity on the enzyme [74].

Heterogeneous nuclear ribonucleoproteins (hnRNPs) A1, A2/B1, and C

HnRNPs were first described as a family of proteins bound to RNA polymerase II transcripts to form hnRNP particles [79], and there are now 19 hnRNP members identified in the family [80]. All hnRNPs contain an RNA binding domain usually located at the N-terminus which is involved in sequence-specific binding of DNA or RNA. Another structural motif found in hnRNPs is known as the RGG motif, which includes Arg-Gly-Gly tripeptide repeats. The RGG domain has been implicated in protein-protein interactions, RNA binding, transcriptional activation, and nuclear localization [81].

The hnRNPs are involved in several cellular roles, one of which is DNA regulation. HnRNP A2/B1 has been implicated in DNA repair as a negative regulator by binding and inhibiting DNA-PK, a multi protein complex which controls DNA repair [82]. HnRNP C has also been identified as a protein that responds to double strand breaks of chromosomal DNA damaged by gamma irradiation [83]. In addition, there is evidence supporting that hnRNP A1 and hnRNP A2/B1 are involved in telomere biogenesis. *In vitro* experiments revealed that hnRNP A1 and hnRNP A2/B1 bind to

human telomeric DNA sequences [84, 85]; furthermore, cells treated with siRNA to reduce expression of hnRNP A1 and hnRNP A2/B1 have shorter telomere tails [86].

HnRNPs have also been implicated in mRNA splicing. HnRNP A1, hnRNP A2/B1, and hnRNP C have proposed functions in splicing [87], with the most evidence supporting the role of hnRNP A1 as a negative regulator in mRNA exon splicing. Site-specific cross-linking and immunoprecipitation identified hnRNP A1 as a protein bound to the c-Src exon. Moreover, addition of hnRNP A1 to an *in vitro* system decreased c-Src splicing [88].

Nuclear-cytoplasmic shuttling is another cellular function associated with hnRNPs. HnRNP A1 and hnRNP C have been implicated in a proposed model of nuclear export; hnRNP C contains a nuclear retention signal, while hnRNP A1 contains a nuclear export signal. It is proposed that pre-mRNA is retained within the nucleus by hnRNP A1 with hnRNP C also bound to the mRNA. Prior to nuclear export, hnRNP C dissociates resulting in the strand being exported out of the nucleus by hnRNP A1. Once in the cytoplasm, hnRNP A1 dissociates from the mRNA strand and hnRNP A1 is imported back into the nucleus [89].

HnRNPs have also been implicated in tumor development and progression. Expressions of hnRNP A1 and hnRNP A2/B1 have been shown to vary at different stages of the cell cycle, peaking at S phase in squamous carcinoma cells; but more importantly, cell proliferation decreased upon treatment with siRNA decreasing expression of hnRNP

A1 and hnRNP A2/B1 [90]. Additionally, expression levels of many hnRNPs are altered in different cancer tissues. Lung cancer tissue has increased levels of hnRNP A1, hnRNP A2/B1, and hnRNP C [80]; there is also evidence suggesting hnRNP A2/B1 could be useful in detection of preclinical lung cancer [91]. Increased levels of hnRNP A2/B1 are also seen in cancer tissues of the breast, pancreas, stomach and esophagus [80].

Transcription factor IIIA (TFIIIA)

TFIIIA is a zinc metalloprotein that contains nine consecutive zinc finger domains that function in sequence-specific binding of DNA and RNA [92]. TFIIIA has two functions in eukaryotic cells, the first being a transcription factor that regulates the expression of the 5 S ribosomal RNA gene. TFIIIA first binds to the internal control region (ICR) on the gene, which subsequently initiates an ordered assembly of factors that form a functional transcription complex [93]. TFIIIA also functions as a 5 S RNA transcript chaperone that moves the transcript from the nucleus to the cytoplasm [94].

α-synuclelin

α-synuclein is a 140 amino acid neuronal protein that has been implicated in several neurodegenerative diseases. It was first discovered by a point mututaion linking the protein to a rare familial form of Parkinson's disease (PD) [95]. Subsequently, other mutations in the α-synuclein gene have been identified in familial PD [96, 97]. Cellular inclusions called Lewy bodies are found in several neurodegenerative diseases such as PD and cortical Lewy body dementia. It has been shown that α-synuclein is the primary

protein component of Lewy bodies, and α-synuclein has been implicated in the etiology of these diseases [reviewed in [98]].

Complexin I

Complexins constitute a small family of neuronal proteins that include two closely related isoforms known as complexin I and complexin II. The complexins play a critical role in regulating the Ca^{2+}-dependent neurotransmitter release [99]. In gene knockout studies in mice, down regulation of complexin I resulted in reduced transmitter release caused by decreased sensitivity to Ca^{2+} in synaptic secretion process [100]. Additionally, it has been observed that complexin I mRNA levels are decreased in the hippocampal subfields of patients with schizophrenia or biopolar disorder [101].

Immunoglobulin heavy chain variable region

The basic subunit of an antibody molecule is a pair of identical Ig heavy chains (IgH) and a pair of identical Ig light chains (IgL). The N-terminal portion of the heavy chain and light chain has a variable and unique amino acid sequence that is known as the variable region; this variable region is involved in specific antigen binding. Variable regions of antigen receptors, like IgH, are assembled by a process known as VDJ recombination (variable, diversity, and joining gene segment recombination) [reviewed in [102]]. Analysis of the VDJ recombination on IgH genes has been shown to be a useful marker in diagnosing the pathology of several lymphomas [reviewed in [103]].

Cytochrome *c* oxidase subunit VIc

Cytochrome *c* oxidase is a complex protein that transfers electrons from cytochrome *c* to oxygen in the last step of the mitochondrial respiratory chain. There are 13 individual protein subunits composing cytochrome *c* oxidase: I, II, III, IV, Va, VIa, VIb, VIc, VIIa, VIIb, VIIc, and VIII. Subunits I, II, and III are encoded by mitochondrial DNA, and comprise the catalytic core. The remaining subunits are synthesized from cellular nuclear DNA [reviewed in [104]]. Although more work is needed to elucidate the precise role of subunit VIc, there is evidence suggesting its presence is crucial for the activity of the enzyme. Null mutants for subunit VIc show a complete loss of cellular respiration [105, 106]; subunit VIc is considered essential for stability of the complex [107] and critical is the ordered sequence of assembly events [108].

NF-κB essential modulator (NEMO)

NF-κB refers to a family of transcription factors that regulates the expression genes controlling the immune and stress response, inflammatory reaction, cell adhesion, and apoptosis [reviewed in [109]]. The classical activation of NF-κB is a tightly controlled process where the active NF-κB complex is a homo- or heterodimer composed of proteins from the NF-κB/Rel family. In its inactive state, NF-κB resides in the cytoplasm bound to a complex with its inhibitor protein, IκB. When an extracellular signal, such TNF-α or IL-1, binds to the appropriate cell surface receptor, the signal is transferred by adaptor proteins. The IKK complex (consisting of IKKα, IKKβ, and NEMO/IKKγ) then becomes activated and is able to phosphorylate IκB, which then disassociates from the NF-κB dimer. The unbound NF-κB dimer is then free to move

into the nucleus and drive transcription of many genes [110]. NF-κB signaling is frequently deregulated in cancer encoding a variety of genes expressing pro-proliferation and anti-apoptotic proteins, and the down-regulation of NF-κB has become an attractive strategy for developing new cancer treatments [111].

RAD50 repair protein

Rad50 is a key protein component of the MRE11/RAD50/NBS1 (MRN) complex which is a central player in cellular response to DNA double-strand breaks. The MRN complex is involved in a variety of processes such a homologous recombination, non-homologous end joining, telomere maintenance, and DNA damage checkpoint activation. RAD50 and MRE11 form the core of the MRN complex, with RAD50 functioning in DNA binding activity. The MRN complex has three primary functions, the first being DNA damage recognition. The MRN complex binds to the damaged DNA and activates the ATM kinase protein. The second is function is ATP-driven nuclease activity. The MRN complex creates a clean DNA end that can be further processed in homologous recombination or replication fork rescue. The third function is involves linking DNA ends with other sister chromatids; this function is important in cell processes such as meiotic recombination or T-loop and D-loop formation in telomeres [complete review in [112]].

Ribosomal protein L19

Ribosomes are complex macromolecules that consist of rRNA and ribosomal proteins, with rRNA comprising the catalytic center and ribosomal proteins at the

periphery of the structure. Biochemical and structural evidence shows that ribosomal proteins are involved in every translation process with the exception of peptide transferase at the catalytic center. Specifically, ribosomal proteins play a role in the exit of the polypeptide from the ribosome. The tunnel exit is a very important location in which the nascent chain can interact with external factors; the exit area is encircled by several ribosomal proteins, including ribosomal protein L19 [113, 114]. Additionally, increased levels of ribosomal protein L19 have been shown in human breast tumors that overexpress the erbB-2 gene [115].

Heat shock protein 90β (HSP90β)

HSP90 is an abundant molecular chaperone that assists in the correct folding and maturation of cellular proteins. There are two isoforms with 85% identity, HSP90α and HSP90β, which exhibit no significant difference in properties. HSP90 exists as a homodimer bound by the C-terminal domain; the N-terminal domain contains the binding site for ATP. The chaperone activity of HSP90 depends on the conformational cycling between opening and closing a molecular clamp, which is dependent upon ATPase activity [116]. HSP90 is implicated in a variety of post-translational functions, such as translocation of proteins across membranes, assembly/disassembly of transcriptional complexes, and regulation of signaling molecules [117]. Because of its multifunctionality, HSP90 has several substrates including the IKK complex [118], SV40 large T-antigen [119], and actin [120].

There has been intense interest in inhibitors of HSP90 as anticancer therapy. There is increased expression of HSP90 in several human cancers; specifically, overexpression of HSP90 in breast cancer correlates with poor prognosis [121]. Both cytotoxic and cytostatic anticancer activities have been reported for HSP90 inhibitors in cell culture models and animal tumor models, and inhibitors of HSP90 are now entering clinical trials with great expectations [reviewed in [117]].

β-tubulin

Microtubules are the dynamic pipe-like proteins which are the primary component of the cytoskeleton. They are primarily composed of α- and β-tubulin heterodimers that aggregate in a head to tail arrangement to form long tubes that constitute protofilaments, and protofilaments form the pipe-like microtubule structure. Microtubules are involved several processes, one of which is to form the mitotic spindles required for the movement of chromosomes to the poles of the new cell during cell division. Another function of microtubules is to give cells both shape and organized structure. Microtubules also provide the tracts for transport of vesicles, organelles, and mitochondria. These critical cellular functions make microtubules a target for anticancer drugs. There are already several successful anticancer drugs that target tubulin including taxanes, colchicines, and vinca alkaloids [complete review in [122]].

Actin

Actin is a major protein in eukaryotic cells and an essential component of the cytoskeleton. Actin filaments provide the basic infrastructure in functions such as

maintaining cell morphology, adhesion, motility, exocytosis, endocytosis, and cell division [123]. It has become evident that actin filaments are regulated by signal transduction pathways of the GTPase Ras superfamily (Rac, Rho, and Cdc42) [124]. There is accumulating evidence that actin remodeling is involved in the growth and motility of malignant cells. Because of this, there is an interest in targeting actin polymerization as an anticancer agent with focus on actin binding proteins and the GTPase Ras signaling pathway [reviewed in [125]].

DISCUSSION

We have previously described the potent antiproliferative effects of G-rich oligonucleotides in several malignant cell lines [34-37] with a novel mechanism unrelated to antisense or antigene activity. Although the precise molecular mechanism is unknown, we have demonstrated that the growth inhibitory effects of these oligonucleotides correlate with their ability to bind to a specific cellular protein complex, as seen in Figure 7 [34, 36]. Because of this significant relationship between antiproliferative function and protein binding, we investigated the proteins that bind to GROs to give insight into the precise mechanism of GROs. Several proteins were identified by mass spectrometry to bind to a functionally active GRO as seen in Table 2; however, these GRO-protein associations should be confirmed by other methods such as Western blot analysis to ensure to the authenticity of the interaction.

One important protein identified as a GRO-binding protein is nucleolin, and nucleolin has been previously identified as the primary target in the antiproliferatve

activity of GROs [34]. Therefore, there are two possible scenarios that exist; either GROs bind to the specific proteins or protein complexes directly, or GROs primarily bind to nucleolin which precipitates the additional proteins identified. The latter seems more plausible due to extreme multifunctional nature of nucleolin. Review of the functions of the identified GRO-binding proteins revealed several cellular processes in which nucleolin has been implicated such as rRNA maturation, ribosome assembly, DNA repair, telomere maintenance, mRNA binding, and nucleocytoplasmic transport [41]. Additionally, several of the GRO-binding proteins identified have previously been shown to associate with nucleolin: hnRNP A1 [126], hnRNP C [127], HSP90 [126], and actin [51].

It is our hypothesis that active GROs exert growth inhibitory effects on malignant cells by binding and modulating specific proteins that affect relevant pathways. Although more work is needed to elucidate the precise molecular mechanism, the identification of GRO-binding proteins gives insight into some of the possible cellular processes affected by GROs.

CHAPTER III

AGRO100 INHIBITS ACTIVATION OF NUCLEAR FACTOR-κB (NF-κB) BY FORMING A COMPLEX WITH NEMO AND NUCLEOLIN

INTRODUCTION

Many common types of cancer, especially in their advanced stages, do not respond well to traditional chemotherapic agents or may acquire resistance to therapy. Novel agents that work by mechanisms that are different from existing therapies, particularly those that are able to target multiple pathways important for cancer cell survival, may therefore be useful in the treatment of advanced cancer. We have reported previously that certain guanosine-rich phosphodiester oligodeoxynucleotides, termed GROs, have antiproliferative activity against a variety of cancer cell lines [34-37]. Furthermore, an active GRO (named GRO29A) can cause cell cycle arrest and induction of cell death in human cancer cell lines, but not in non-malignant human cells [35]. Following pre-clinical *in vivo* studies that demonstrated that it had no detectable toxicity in normal tissues [38], a truncated version of GRO29A known as AGRO100 (recently renamed AS1411), has been tested in a Phase I clinical trial of patients with advanced cancer. The results of this trial were recently presented [38] and indicated that AGRO100 was well tolerated (no toxicity was observed) and had promising clinical activity.

In previous *in vitro* studies, we have examined a series of G-rich sequences in order to determine a structure-activity relationship and to gain insight into their novel mechanism [34, 36, 37]. We found that active GROs can have diverse G-rich sequences, but all could adopt folded conformations that are stabilized as G-quartets. However, formation of a stable G-quadruplex was not sufficient for activity. In addition, there was a very good correlation between the biological activity of GROs and their abilities to form a specific complex containing a protein that was identified as nucleolin [34, 36, 37]. There is strong evidence from UV crosslinking and southwestern blotting [34] that GROs bind directly to nucleolin rather than interact through an intermediary protein, and therefore we have proposed that nucleolin is the primary target of GROs such as AGRO100.

Despite their probable target having been identified, the detailed mechanism of action for GROs is not yet fully understood. In part, this is because little is known about the role of nucleolin in cancer biology. Nucleolin protein is expressed at high levels in rapidly proliferating cells such as cancer cells [65], and nucleolin is a major component of a set of cancer prognostic markers (AgNORs) whose levels are elevated in cancer cells compared to normal or pre-malignant cells [63, 64]. Although it is best known as a nucleolar protein with a role in ribosome biogenesis, nucleolin is in fact a remarkably multifunctional protein that can also be present in the nucleoplasm, cytoplasm and on the cell surface [40-42, 50-52]. In addition to its multiple functions in ribosome biogenesis [40-42], nucleolin is thought to play a role in numerous cellular processes, including apoptosis [47], signal transduction [47, 48, 52, 91, 128], DNA replication [44, 54],

mRNA stability [127, 129, 130], protein trafficking [50, 131], stress response [132], and telomerase function [133]. It has been reported by several research groups that nucleolin binds to G-quadruplex-forming DNA sequences, such as the human telomere [85, 134], immunoglobulin switch regions [25] and ribosomal DNA [28], but the significance of these interactions *in vivo* has not yet been determined.

Recently, we identified NEMO (NFκB essential modulator, also known as IKKγ and IKKAP1) as a potential GRO-associated protein following mass spectrometry analysis of proteins that were precipitated when an active biotin-linked GRO was incubated with cancer cell extracts (see Chapter II). It was selected for further investigation because of its important role in NF-κB signaling, a pathway which is often deregulated in cancer cells [110, 135, 136], and which has been reported previously to be inhibited by polyguanosine sequences [137] via an uncharacterized mechanism. "NF-κB" refers to a family of proteins that function as dimeric transcription factors to affect the expression of genes involved in immune and inflammatory responses, cell growth, differentiation and apoptosis [136, 138, 139]. In non-stimulated cells, NFκB is normally bound to an inhibitor called IκBα, which sequesters it in the cytoplasm in an inactive state. Upon ligation of an appropriate cell surface receptor (for example, by TNFα), the IκB kinase (IKK) complex consisting of NEMO, IKKα, and IKKβ becomes activated. This IKK complex then phosphorylates IκBα, leading to its ubiquitination and flagging it for degradation by the 26S proteasome. Upon degradation of IκBα, NF-κB is released and translocates to the nucleus to activate gene expression. Targets of NF-κB include many anti-apoptotic and pro-proliferation genes and this pathway is constitutively

activated in many human cancers [110, 135, 136]. For this reason, NF-κB and the proteins that control its activation have become interesting targets for cancer drug discovery [136, 139].

The purpose of the present study was to further investigate the significance of our preliminary observation that NEMO associates with an active G-rich oligonucleotide. In particular, our aims were to verify that AGRO100 was associated with NEMO in treated cancer cells, to determine the biological consequences of this association and to investigate the possible role of nucleolin in mediating it.

MATERIALS AND METHODS

Oligonucleotides

The oligodeoxynucleotides used in the present study were AGRO100, an antiproliferative GRO whose sequence is 5'-d(GGTGGTGGTGGTTGTGGTGGTGGTGG)-3', and CRO26, an inactive control oligonucleotide whose sequence is 5'-d(CCTCCTCCTCCTTCTCCTCCTCCTCC)-3'. All oligonucleotides had a phosphodiester backbone and, unless otherwise stated, were purchased in the desalted form from Integrated DNA Technologies Inc. (Coralville, IA) and used without further purification. Oligonucleotides were resuspended in water, sterilized by filtration through a 0.2 μm filter, then diluted with sterile water to give stock solutions of 400-500 μM that were stored in aliquots at -20 °C. For experiments shown in Figure 10, the following biotin (Bt) linked versions of AGRO100 and CRO26 were used: 5'-Bt-d(TTTGGTGGTGGTGGTTGTGGTGGTGGTGG)-3', purchased from MWG Biotech,

and 5'-Bt-d(TTTCCTCCTCCTCCTTCTCCTCCTCCTCC)-X-3', where X is a propylamine modification, synthesized on a Beckman 1000M instrument as previously described [35].

Cell culture and treatment

Cell lines used were HeLa (human cervical cancer), DU145 (human prostate cancer), A549 (human non-small cell lung cancer), MCF-7 (human breast cancer) and Hs27 (non-malignant human skin fibroblasts), which were obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells were grown in a standard incubator in DMEM (Gibco) supplemented with 10% heat-inactivated (65°C for 20 min) fetal bovine serum (Gibco) or charcoal-stripped fetal bovine serum (Biomeda, for MCF-7 only), 100 units/ml penicillin and 100 units/ml streptomycin. For the cell proliferation assays (Figure 9), cells were plate at low density (1000 cells per well or 2000 cells per well for MCF7 and Hs27) in 96-well plates and incubated 18 h at 37°C to allow adherence. They were treated by addition of AGRO100 directly to the culture medium to give the final concentration indicated in the figure. Cells were incubated for a further 5 days in the presence of oligonucleotide and then proliferation was determined using the MTT assay, as previously described [34]. For all other experiments, unless otherwise stated, cells were plated in 6-well plates at about 50% confluence and incubated 18 h to allow adherence, then treated by addition of oligonucleotide directly to the culture medium. Where indicated, TNFα (R & D Systems, Inc.) was added to the culture medium to a final concentration of 7.5 ng/ml.

Capture of biotinylated GRO-protein complexes from HeLa cells

Cells were grown in 6-well plates as described and treated by addition of biotin-linked oligonucleotide at 3 μM final concentration. After incubation for 2 h at 37°C, cells were lysed under mild conditions and proteins associated with biotinylated oligonucleotides were captured by streptavidin precipitation, as described previously [34]. Captured proteins were electrophoresed on SDS-polyacrylamide (8%) gels and transferred to PVDF membranes. Membranes were blocked with 5% nonfat dried milk in PBS plus 0.05% Tween20 (PBST) then incubated with primary antibody in PBST (1 μg/ml anti-nucleolin, sc-8031, Santa Cruz or 1 μg/ml anti-NEMO, sc-8032, Santa Cruz). Following incubation with secondary antibody (0.5 μg/ml anti-mouse HRP-conjugated, Santa Cruz) bands were visualized by chemiluminescence (ECL, Amersham Biosciences).

IKK kinase assay

HeLa cells were plated in 6-well plates as described. After 1 h incubation with oligonucleotide, TNFα was added for a further 30 min. Cell lysates were prepared using 0.1 M Tris.HCl pH 8.0, 1% Triton X-100, 1% deoxycholate, 0.5% SDS, 2 mM phenylmethylsulfonyl flouride (PMSF). The lysates (10 μg) were then assessed for IKK kinase activity as previously described [140], utilizing 2.5 μg of IκBα-GST fusion protein (Santa Cruz) as the substrate. The gels were dried and analyzed by autoradiography.

Detection of phosphorylated IκBα

HeLa cells were treated exactly as described for the IKK kinase assay. Lysates were prepared by addition of lysis buffer (62.5 mM Tris.HCl pH 6.8, 2% SDS, 10% glycerol, 50 mM dithiothreitol), electrophoresed on 8% polyacrylamide-SDS gels and transferred to PVDF membrane. Western blot analysis was then performed using anti-phospho-IκBα antibody (1 μg/ml, Cell Signaling Technology). To visualize the total protein loaded, the membrane was stained with 3 drops of Higgins India Ink 4415 in PBS/Tween 20 for 2 hours and washed with PBS/Tween 20.

Transient transfection and measurement of luciferase activity

Cells were plated in 24-well plates at a density of 2 x 10^4 cells per well and were transiently transfected with an NF-κB reporter plasmid and an internal control plasmid (described below) using Superfect reagent (Qiagen), according to the manufacturer's protocol. At 24 h post-transfection, oligonucleotide was added directly to the medium for the final concentration indicated. After 1h incubation, recombinant TNFα was added to the medium for 6 h (where indicated) and cell lysates were prepared using reporter lysis buffer (Promega). Lysates were added to the substrate reagents supplied in the Dual-Luciferase reporter system (Promega) and luciferase activity was measured using a luminometer (Zylux corporation). The NF-κB reporter plasmid contained tandem repeats of the κB sequence upstream of the minimal simian virus 40 (SV40) promoter and firefly luciferase gene in pLuc-MCS vector (Stratagene) and was a kind gift from Dr. Fajan Yang. As an internal control to account for variations in transfection efficiency, pRLnull (Promega), which expresses *Renilla* luciferase by basal transcription, was used (a gift

from Dr. Robert Mitchell). Luciferase activity is expressed as the activity of firefly luciferase divided by *Renilla* luciferase.

Immunoprecipitation of nucleolin

HeLa cells were plated as described. After 1 h incubation with oligonucleotide, TNFα was added for a further 2 h and then S-100 extracts were then prepared [47]. The extracts (100 μg) were incubated with 10 μg of anti-nucleolin antibody (sc-8031, Santa Cruz) for 2 h at 4°C in RIPA buffer (150 mM NaCl, 10 mM Tris.Hcl pH 7.5, 0.1% SDS, 1% Triton X-100, 1% Deoxycholate, 5 mM EDTA, 1mM PMSF, 2 μg/ml leupeptin). Goat anti-mouse coated magnetic beads (Magnabind, Pierce) were then added to the samples and incubated for 1 h at 4°C. The beads were captured and unbound sample was removed by washing three times with RIPA buffer. The precipitated proteins were eluted from the beads by the addition of SDS sample loading buffer (4% SDS, 2% glycerol, 5% β-mercaptoethanol, 0.75 M Tris.HCl, pH 8.8) and heating at 65°C for 15 min. Western blot analysis, as described above, was then utilized to detect the presence of NEMO and nucleolin.

RESULTS

Antiproliferative activity of AGRO100

In previous work [34], we have shown that a G-rich oligonucleotide named GRO29A can inhibit proliferation in prostate, breast and cervical cancer cells. Subsequently, we determined that the 3'-amino modification and the three 5'- thymidines of GRO29A are not necessary for nuclease resistance or biological activity [35, 37]. Therefore, AGRO100, the oligonucleotide selected for clinical development, lacks these

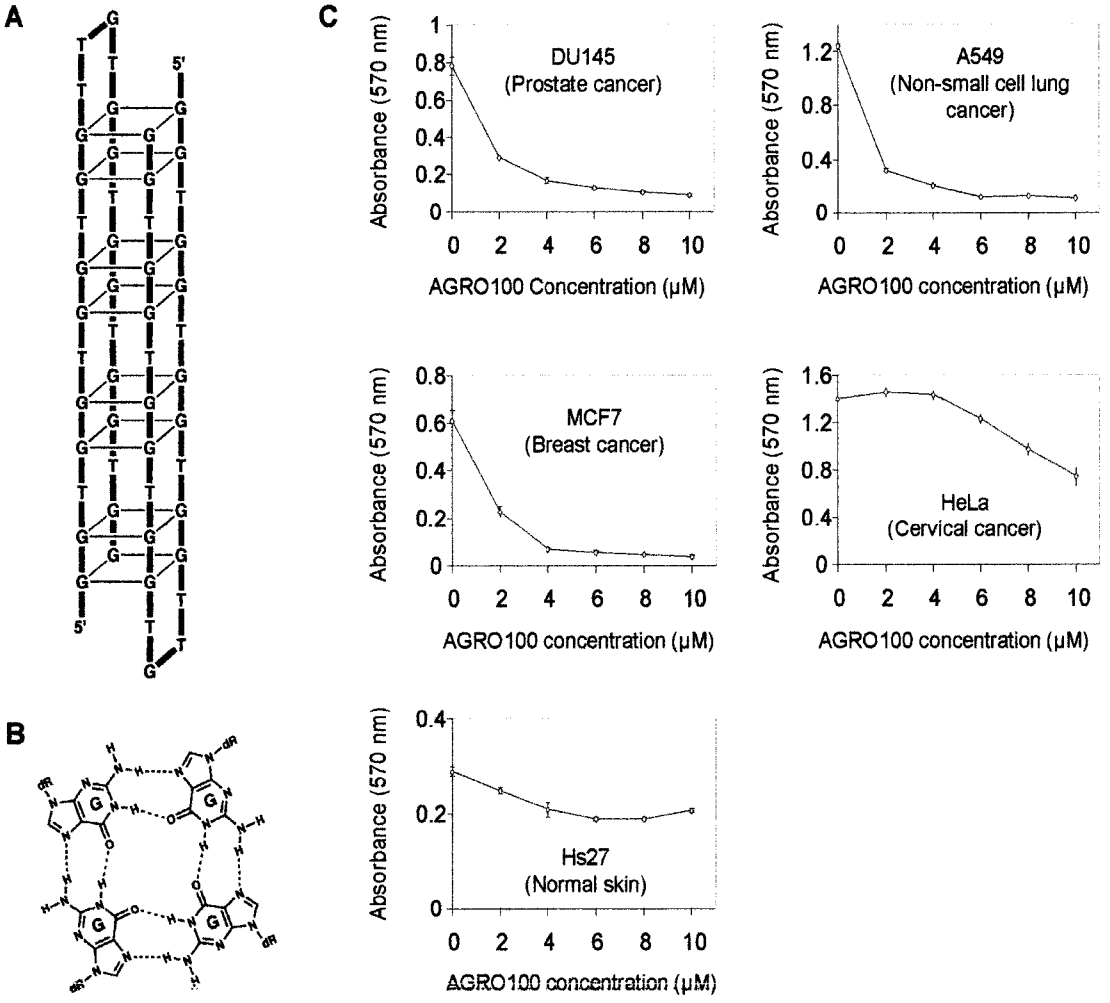
features and is an unmodified 26-mer oligodeoxynucleotide. Thermal denaturation experiments, carried out as previously described [34], indicate that AGRO100 forms a stable structure that melts at 76°C (Bates, unpublished observations). As shown schematically in Figure 9, this structure is predicted [37] to be a bimolecular quadruplex that is stabilized by the formation of eight G-quartets. To first verify the antiproliferative activity in the cell lines of interest, AGRO100 was added directly to the culture medium and cells were incubated in the presence of the oligonucleotide for 5 days. This was followed by determination of relative cell number using a colorimetric MTT assay. As expected, based on our previous studies of GRO29A [34-37], micromolar concentrations of AGRO100 strongly inhibited the proliferation of a variety of human cancer cells, but had a lesser effect on Hs27 cells, which are non-malignant human skin fibroblasts (Figure 9C).

NEMO is an AGRO100-associated protein

In preliminary experiments designed to identify candidate proteins that may play a role in GRO activity, we incubated biotin-linked G-rich oligonucleotides with concentrated HeLa cell extracts *in vitro*. This was followed by streptavidin precipitation, electrophoresis of captured proteins and mass spectrometry analysis of bands that were precipitated by an active GRO but not a control oligonucleotide. Several specific GRO-associated proteins were identified by this approach, including nucleolin and NEMO (see Chapter II).

Figure 9: Structure and activity of AGRO100.

(A) Schematic diagram of the structure proposed [37] for AGRO100, which comprises two strands of AGRO100 folded in antiparallel orientation to form a bimolecular quadruplex containing eight G-quartets; (B) G-quartet showing the hydrogen bonding arrangement of the four planar guanosines; (C) Antiproliferative activity of AGRO100 in various human cell lines. Cells were incubated for 5 days following a single treatment with the indicated concentration of AGRO100 (which was added directly to the medium) and proliferation was measured using an MTT assay. The identity of the cell line used and its origin are indicated in each graph. The ordinate indicates absorbance at 570 nm, which is directly proportional to the number of viable cells in the sample. Data points represent the mean of triplicate samples with standard error bars.


A
B
C
Absorbance (570 nm)
DU145
(Prostate cancer)
AGRO100 Concentration (μM)
A549
(Non-small cell lung cancer)
AGRO100 concentration (μM)
MCF7
(Breast cancer)
AGRO100 concentration (μM)
HeLa
(Cervical cancer)
AGRO100 concentration (μM)
Hs27
(Normal skin)
AGRO100 concentration (μM)

To confirm that complex formation between NEMO and AGRO100 occurs in treated cancer cells, we incubated HeLa cervical carcinoma cells with biotin-linked oligonucleotides, then lysed the cells under mild conditions and precipitated with streptavidin. Western blot analysis of the streptavidin-precipitated proteins (Figure 10) revealed that NEMO was precipitated from cells treated with biotin-linked AGRO100 but not from untreated cells or those treated with a biotin-linked C-rich oligonucleotide as a control. As expected, nucleolin was also specifically precipitated by biotin-linked AGRO100 (Figure 10). These experiments have been carried out at least three times, with reproducible results.

Inhibition of IKK activity in AGRO100-treated cells

Because NEMO is known to be essential for function of the IKK complex in the classical pathway of NF-κB signaling [138], we next investigated the effect of AGRO100 treatment on IKK activity. First, we performed an IKK kinase assay, which assesses the capability of cell lysates to phosphorylate the IKK substrate, IκBα, in the presence of radiolabeled ATP. HeLa cells were treated for 1 h with AGRO100 or CRO26 and then stimulated with TNFα for 30 min. As seen in Figure 11A, there was substantial induction of IKK activity upon stimulation with TNFα in the absence of oligonucleotide.

Figure 10: NEMO and nucleolin are associated with AGRO100.

HeLa cells were treated with biotin-linked AGRO100 (AGRO) or biotin-linked control oligonucleotide (CRO) or were untreated (-). After 2 h, cells were lysed under mild conditions and added to streptavidin-coated magnetic beads for precipitation of oligonucleotide-associated proteins. The precipitated proteins were then subjected to western blot analysis to detect the presence of either nucleolin or NEMO. Non-precipitated lysate (5 μg) was used for comparison, left lane.

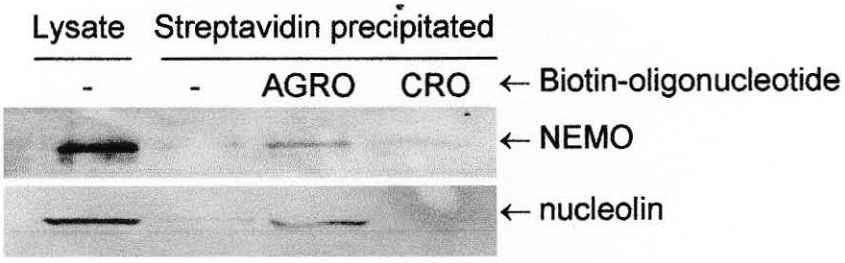
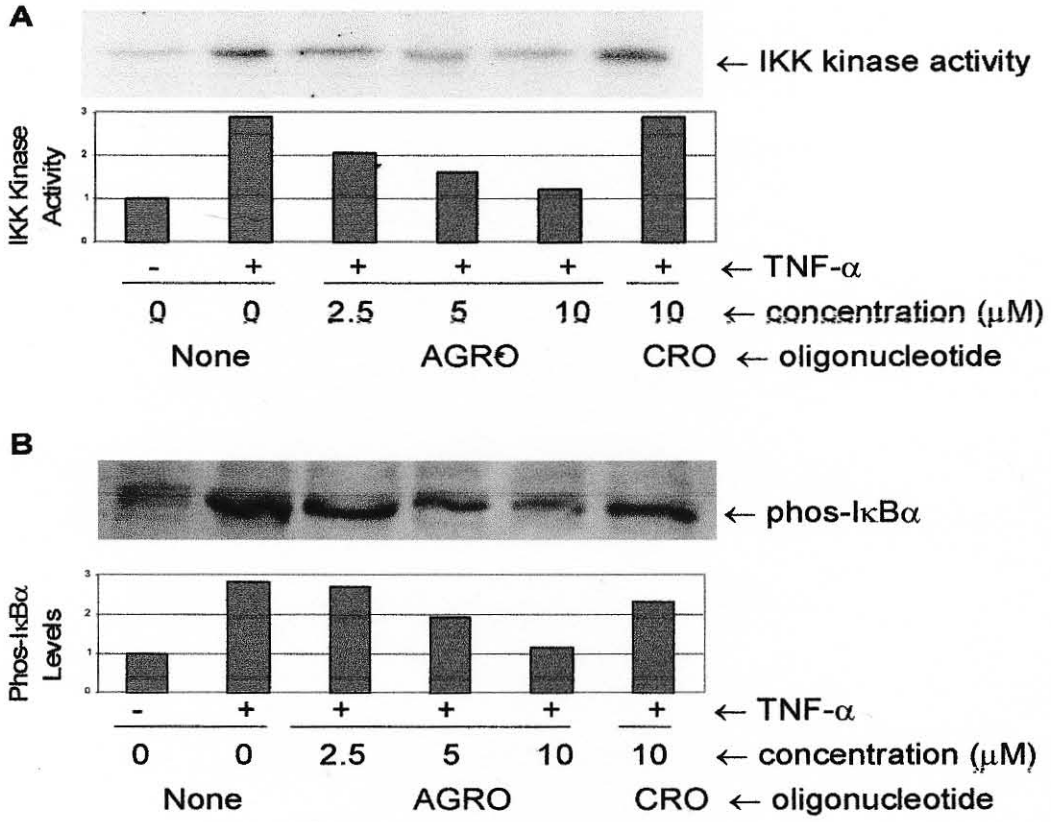

Lysate
Streptavidin precipitated
-
-
AGRO
CRO
← Biotin-oligonucleotide
← NEMO
← nucleolin

Figure 11: AGRO100 inhibits activity of the IKK complex.

Lysates were prepared as described from HeLa cells that were treated for 1 h with AGRO100 (AGRO) or CRO26 (CRO), at the concentrations shown, then stimulated with TNFα for 30 min. (A) Lysates were used in an IKK kinase assay in the presence of γ-^{32}P-ATP and GST-IκBα as a substrate. The upper panel is an autoradiograph showing IKK kinase activity, as represented by phosphorylation of precipitated GST-IκBα. The lower panel represents quantified IKK kinase activity determined by densitometry using ImageQuant 5.2 software (Amersham Biosciences) with values shown relative to background activity; (B) Lysates were subjected to western blot analysis using an antibody specific for the phosphorylated form of IkBα in order to detect IKK-mediated phosphorylation of the endogenous substrate. The lower panel represents densitometric analysis of the blot, as described for the kinase assay.


A
← IKK kinase activity
IKK Kinase Activity
- + + + + +
← TNF-α
0 0 2.5 5 10 10
← concentration (μM)
None AGRO CRO
← oligonucleotide
B
← phos-IκBα
Phos-IκBα Levels
- + + + + +
← TNF-α
0 0 2.5 5 10 10
← concentration (μM)
None AGRO CRO
← oligonucleotide

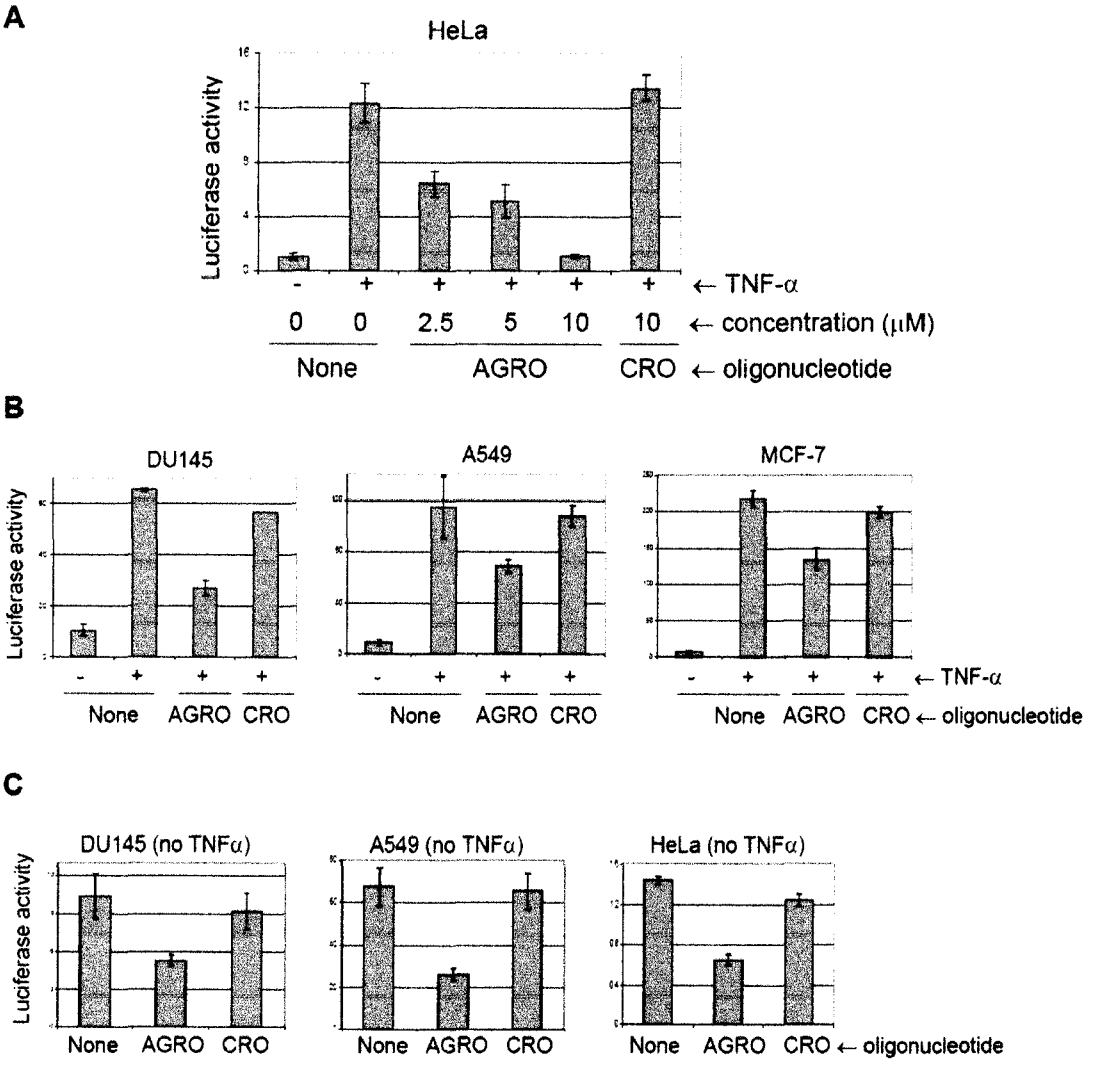
Incubation of cells with AGRO100 abrogated the activation of the IKK complex in a dose-dependent manner, whereas treatment with the control CRO had little effect. To validate these findings, we next used an additional method to examine the IKK-mediated phosphorylation of IκBα. Western blot analysis using an antibody specific for the phosphorylated form of IκBα was performed using lysates from cells that had been treated for 1 h with AGRO100 or CRO26 and then stimulated with TNFα. The membrane was subsequently stained (India Ink) to ensure equal amounts of protein were loaded and transferred. The results (Figure 11B) indicated that phosphorylation of IκBα occurred upon stimulation with TNFα, but was blocked by AGRO100, whereas CRO had no effect. These observations correlated well with the results from the IKK kinase assay.

Inhibition of NF-κB activation in AGRO100-treated cells

Based on the previous result that IKK activity is inhibited, NF-κB signaling would be expected to be blocked in cancer cells treated with AGRO100. To confirm this prediction, we examined the activity of NF-κB in cells that were transiently transfected with a NF-κB-driven luciferase reporter construct. Cells were first transfected for 24 h with the reporter construct (expressing firefly luciferase) plus an internal control vector (expressing *Renilla* luciferase) and then were treated with AGRO100 or CRO26 for 30 min. TNFα was then added to the medium and cells were incubated for a further 6 h followed by measurement of luciferase activity. As shown in Figure 12A, treatment of HeLa cells with AGRO100 reduced TNFα-induced NF-κB transcriptional activity in a dose-dependent manner, but treatment with the CRO26 control had no effect on NF-κB-driven luciferase activity. Next, we used the reporter gene assay to determine the effects

Figure 12: AGRO100 inhibits NF-κB transcriptional activity.

(A) HeLa cells were transiently transfected with a NF-κB reporter construct expressing firefly luciferase and a control plasmid expressing *Renilla* luciferase. After 24 h, cells were treated for 1 h with AGRO100 (AGRO) or control oligonucleotide, CRO26 (CRO) at the concentration indicated, then stimulated for an additional 6 h with TNFα and luciferase activity was then measured. The luciferase activity shown was calculated as firefly luciferase activity divided by *Renilla* luciferase activity and shown relative to the unstimulated activity. The bars represent the mean and standard error of triplicate experiments; (B) Similar experiments were carried out in the cell lines indicated at the top of each graph using 10 μM AGRO100 or CRO26; (C) The same assays were carried out in the absence of TNFα simulation to determine the effect of AGRO100 on constitutive NF-κB activity.


A
HeLa
Luciferase activity
- + + + + + ← TNF-α
0 0 2.5 5 10 10 ← concentration (μM)
None AGRO CRO ← oligonucleotide
B
DU145
A549
MCF-7
Luciferase activity
- + + + ← TNF-α
None AGRO CRO ← oligonucleotide
C
DU145 (no TNFα)
A549 (no TNFα)
HeLa (no TNFα)
Luciferase activity
None AGRO CRO ← oligonucleotide

of AGRO100 treatment on other types of cancer cells. Figure 12B shows that AGRO100 was able to inhibit TNFα-induced NF-κB activation in cell lines derived from human prostate cancer (DU145), breast cancer (MCF-7) and non-small cell lung cancer (A549). The effect on Hs27 cells was not determined because transfection efficiency in these cells was poor. Furthermore, as demonstrated by similar experiments carried out in the absence of TNFα, AGRO100 could also inhibit constitutive NF-κB signaling in those cell lines that had significant basal NF-κB activity (Figure 12C). In either the absence or presence of TNFα, there was no significant effect of AGRO100 on the activity due to the control *Renilla* luciferase plasmid or on cell number, therefore excluding the possibility of non-specific effects of AGRO100 on transcription, translation or cell viability (data not shown).

Co-precipitation of NEMO and nucleolin from AGRO100-treated cells

Because both nucleolin and NEMO were precipitated with AGRO100, we proceeded to investigate the possibility that these proteins were simultaneously present in the same AGRO100-containing complex within the cell. Immunoprecipitation with nucleolin antibody was performed using extracts from HeLa cells that had been treated with AGRO100 or CRO26 and then stimulated with TNFα. The immunoprecipitates were then subjected to western blot analysis to determine if NEMO was co-precipitated with nucleolin. Figure 13A demonstrates that in untreated cells, or cells treated with the control oligonucleotide, a very small amount of NEMO was detected in the nucleolin immunoprecipitate. However, when the cells were treated with AGRO100 and then stimulated with TNFα, significantly more NEMO co-precipitated with nucleolin,

indicating that NEMO, nucleolin and AGRO100 are present in the same complex. Although we cannot definitively exclude that AGRO100 is bound independently to both proteins, a more likely explanation for this result is that AGRO100 binds directly to nucleolin and stabilizes a protein-protein interaction between nucleolin and NEMO. This idea is supported by the weak interaction between nucleolin and NEMO in untreated cells, which was confirmed in additional experiments (Figure 13B).

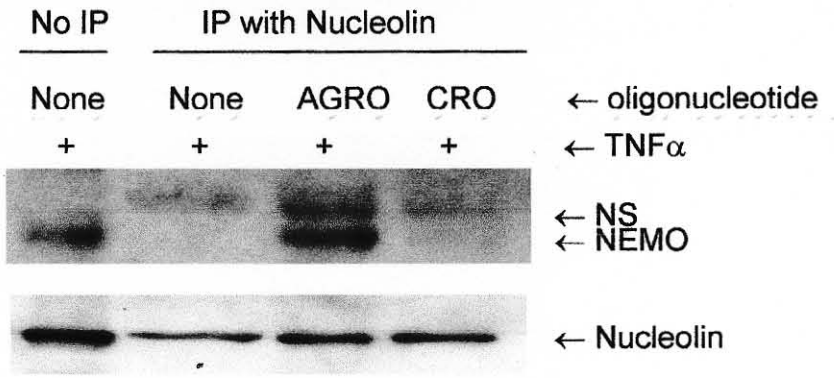
DISCUSSION

It has become clear in recent years that NF-κB signaling is frequently deregulated in many types of cancer [110, 135, 136]. NF-κB target genes encode a variety of pro-proliferation and anti-apoptotic proteins, and it seems likely that malignant cells have acquired constitutive activation of this pathway in order to bypass the normal physiological signals that prevent uncontrolled proliferation or to resist apoptosis induced by radiation, chemotherapy or hormonal agents [110, 135, 136]. Constitutive NF-κB activation may have particular significance with respect to advanced cancers and their resistance to therapeutic manipulation. For example, constitutive activity of NF-κB was not detected in hormone-responsive prostate cancer cell lines, but was detected in hormone-insensitive prostate cancer lines and in tumor tissue from patients with advanced prostate cancer, a disease that is very often resistant to therapeutic intervention [118, 141, 142]. Inhibition of NF-κB activation has also been linked to the chemopreventive properties of several compounds with activity in cancer, such as selenium, green tea, and silymarin [140, 143, 144]. Down-regulation of NF-κB activity is therefore considered a very attractive strategy for developing new cancer treatments

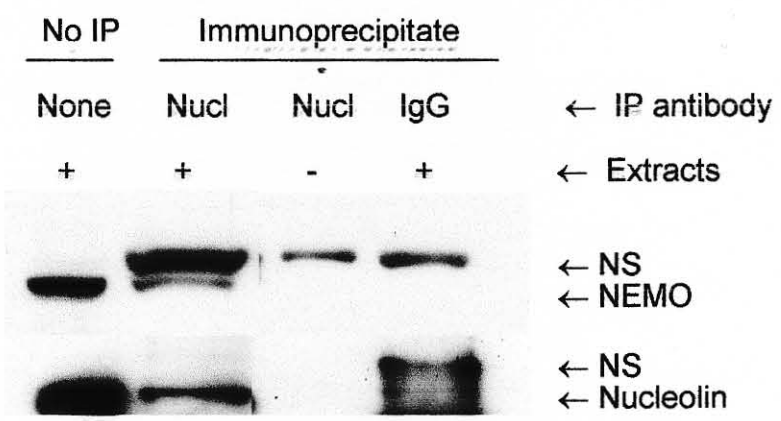
Figure 13: Co-precipitation of NEMO by nucleolin in AGRO100-treated cells.

(A) Cytoplasmic (S-100) extracts were prepared from HeLa cells that had been treated for 1 h with 10 μM AGRO100 (AGRO) or CRO26 (CRO), then stimulated with TNFα for 30 min. Immunoprecipitation with nucleolin antibody was performed and western blot analysis was used to assess the presence of NEMO and nucleolin. Non-precipitated extract (5 μg) was used for comparison, in the left lane; (B) To confirm the interaction of NEMO and nucleolin in the absence of AGRO100, a similar experiment was performed. Whole cell lysates from HeLa cells that were stimulated 30 min with TNFα were subjected to immunoprecipitation with anti-nucleolin antibody, and western blot analysis revealed the presence of NEMO and nucleolin, indicated by arrows. Mock immunoprecipitation (no cellular extracts, lane 3) and immunoprecipitation with non-specific IgG were done in parallel to ensure the specificity of the interaction (lane 4). Non-precipitated extract (5 μg) was used for comparison (left lane). Non-specific bands that were artifacts of the immunoprecipitation are indicated by "NS".

A


No IP
IP with Nucleolin
None
None
AGRO
CRO
← oligonucleotide
+
+
+
+
← TNFα
← NS
← NEMO
← Nucleolin

B

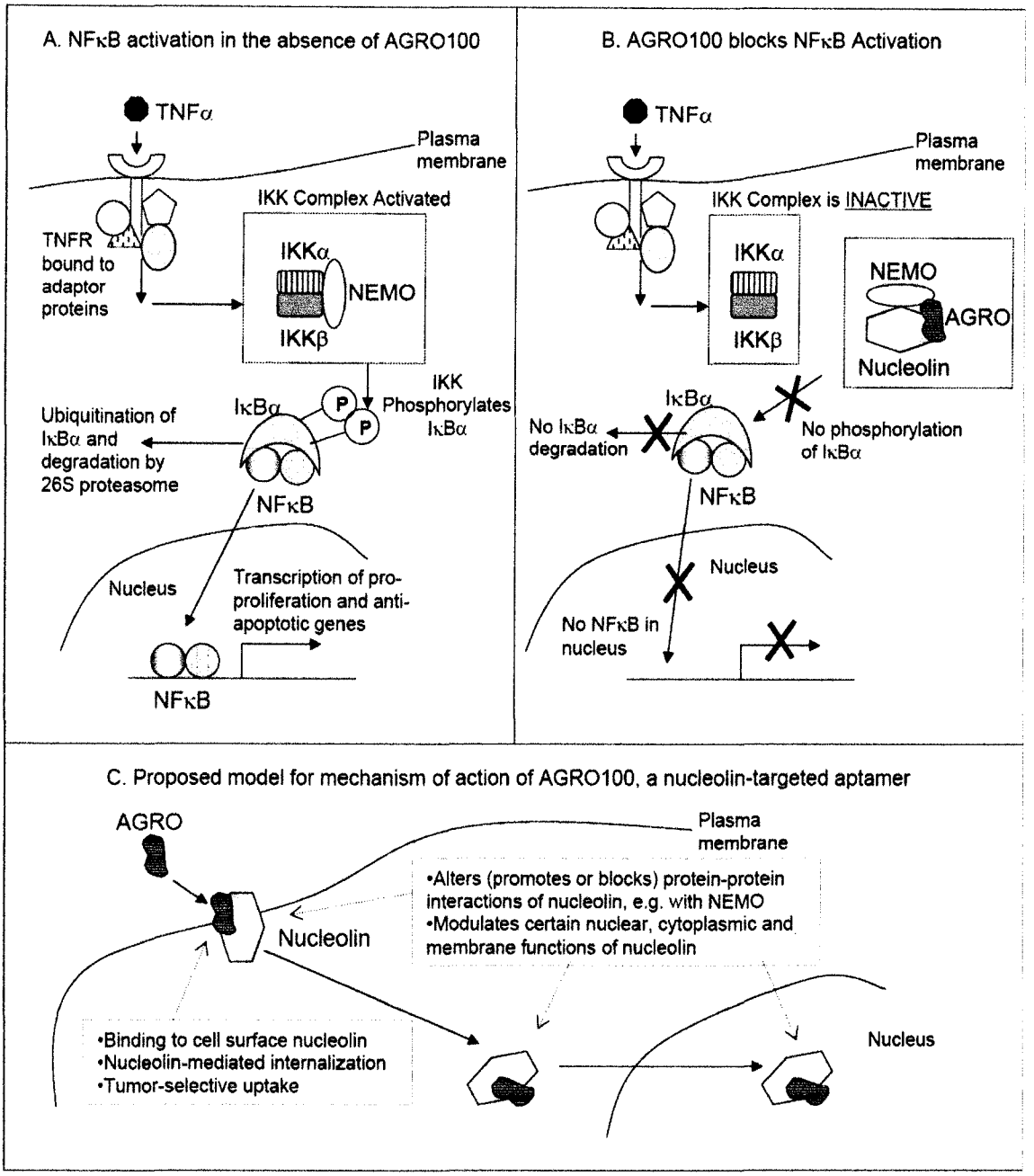

No IP
Immunoprecipitate
None
Nucl
Nucl
IgG
← IP antibody
+
+
-
+
← Extracts
← NS
← NEMO
← NS
← Nucleolin

[136]. The components of the IKK complex are of particular interest for drug discovery [139] and peptide inhibitors of NEMO have recently been shown to have anticancer activity [145], in addition to anti-inflammatory properties [52].

We have now demonstrated that AGRO100 is an inhibitor of both constitutive and TNFα-induced NF-κB signaling in a variety of cancer cell lines derived from prevalent solid tumor types. Moreover, we have defined a probable mechanism of this activity by showing that AGRO100 sequesters NEMO in a complex with nucleolin, thereby preventing activation of IKK and precluding phosphorylation of IκBα and subsequent release of NF-κB (Figure 14B). Although it has not been ascertained that the inhibition of NF-κB is responsible for the anticancer activity of AGRO100, it seems likely that blocking this pro-survival, anti-apoptotic pathway in cancer cells that have constitutive activation would lead to antiproliferative effects. It is apparent from Figures 9 and 12 that the ability of AGRO100 to inhibit TNFα-induced NF-κB signaling does not correlate directly with its antiproliferative activity. However, this is not surprising since TNFα is absent in the experiments shown in Figure 9 and the effects of inhibiting *constitutive* NF-κB activity may depend complexly on numerous factors, including the basal level of NF-κB signaling and the extent of its inhibition by AGRO100. It may be significant that AGRO100 has the least growth inhibitory effect on Hs27 cells, which have no detectable constitutive activation of NF-κB (confirmed by an EMSA to detect NF-κB in unstimulated nuclear extracts). In any case, as discussed below, it appears that inhibition of NF-κB signaling is one of several potential anticancer effects induced by AGRO100.

Figure 14: Schematic representations of NF-κB activation and proposed model for its inhibition in the presence of AGRO100.

(A) Upon ligation of an appropriate surface receptor, such as the TNFα receptor (TNFR), a signal is transduced to activate the IKK complex, which consists of IKKα, IKKβ and NEMO. The complex then phosphorylates IκBα, which normally holds NF-κB in an inactive state in the cytoplasm, and causes it to become ultimately degraded by the 26S proteasome. Thus, the nuclear localization signal of NFκB is unmasked and the transcription factor translocates to the nucleus to activate gene expression; (B) In the presence of AGRO100, NEMO is sequestered in a complex containing nucleolin and AGRO100. The IKK complex is therefore inactive and phosphorylation of IκBα cannot occur, which prevents release and activation of NF-κB.


A. NFκB activation in the absence of AGRO100
TNFα
Plasma membrane
IKK Complex Activated
TNFR bound to adaptor proteins
IKKα
NEMO
IKKβ
IKK Phosphorylates IκBα
Ubiquitination of IκBα and degradation by 26S proteasome
IκBα
P
P
NFκB
Nucleus
Transcription of pro-proliferation and anti-apoptotic genes
NFκB
B. AGRO100 blocks NFκB Activation
TNFα
Plasma membrane
IKK Complex is INACTIVE
IKKα
IKKβ
NEMO
AGRO
Nucleolin
IκBα
No IκBα degradation
No phosphorylation of IκBα
NFκB
Nucleus
No NFκB in nucleus
C. Proposed model for mechanism of action of AGRO100, a nucleolin-targeted aptamer
AGRO
Plasma membrane
Nucleolin
•Alters (promotes or blocks) protein-protein interactions of nucleolin, e.g. with NEMO
•Modulates certain nuclear, cytoplasmic and membrane functions of nucleolin
•Binding to cell surface nucleolin
•Nucleolin-mediated internalization
•Tumor-selective uptake
Nucleus

Clearly, more research is needed to fully characterize the mechanism of GROs such as AGRO100, but the results described herein are consistent with our theory that they work primarily as nucleolin-targeted aptamers. While investigating the role of nucleolin in GRO activity, we have observed by farwestern blotting that the presence of AGRO100 can induce changes in the protein-protein interactions of nucleolin, which include some enhanced and some reduced interactions (unpublished observations). Therefore, our hypothesis regarding the mechanism of GROs is that their antiproliferative effects result from their binding to nucleolin and modulation of its molecular interactions. We predict that this consequently alters certain activities of this highly multifunctional protein, leading to pleiotropic biological effects. Such effects may include inactivation of NF-κB signaling, inhibition of DNA replication and induction of cell death, which have been reported here or previously [35]. We have now shown that AGRO100 inhibits NF-κB activation by stabilizing a complex containing nucleolin and NEMO. Thus, we have identified NEMO as one of the partners of nucleolin whose binding is affected (enhanced, in this case) by the presence of AGRO100.

The complex interactions that regulate NF-κB signaling are an area of intense interest and much has been learned regarding these in recent years. However, the full details of the pathway, especially the mechanisms leading to IKK activation, are not yet fully understood. Although nucleolin has not previously been implicated in NF-κB signaling, our present findings that a nucleolin-binding aptamer inhibits NF-κB activity and that nucleolin is associated with NEMO are suggestive of a role for nucleolin in regulation of the IKK complex. In conclusion, further studies appear to be warranted in

order to define the precise nature of the nucleolin-NEMO interaction and to determine the role of nucleolin in NF-κB signaling, which is further examined in Chapter 4.

CHAPTER IV

NUCLEOLIN AND NEMO: POSSIBLE ROLE FOR NUCLEOLIN IN NF-KB SIGNALING

INTRODUCTION

The NF-κB/Rel family of transcription factors has intensively studied as an important model system for how extracellular stimuli, such as tumor necrosis factor α (TNFα), cause the activation of transcription factors through signal transduction cascades. NF-κB regulates the expression of many genes critical for cellular processes, including inflammation, immune responses, and apoptosis [150]. NF-κB is normally kept inactive in the cytoplasm of unstimulated cells by its inhibitor proteins, IκBα. When an extracellular signal binds to the appropriate cell surface receptor, the signal results in the release of the NF-κB dimer from IκBα, and subsequent translocation to the nucleus to regulate gene transcription [110].

Central to the activation of NF-κB by extracellular stimuli is the IκB kinase (IKK) complex. The IKK complex is composed of two catalytic subunits IKKα and IKKβ, and a regulatory subunit NEMO (NF-κB essential modulator). Signaling pathways lead to the activation of IKK, which then results in the phosphorylation of IκB and its degradation by the ubiquitin-proteosome pathways allowing the release of NF-κB

[110]. NEMO is essential in regulating IKK and the activation of NFκ-B; its importance has been demonstrated in NEMO knockout mice experiments, where there is a complete loss of NF-κB activity in response to stimuli [150]. NEMO has multiple distinct domains which are thought to be used as scaffolding for the binding of IKKα and IKKβ, and possible binding of other unknown proteins as well [110].

Nucleolin is predominantly known as a nucleolar protein that is highly multifunctional. This 110-kD protein has been implicated in several cellular functions like cell growth and proliferation [40-42], ribosome biogenesis [40, 43], DNA replication [44], cytokinesis and nuclear division [45], and apoptosis [46, 47]. Although nucleolin is generally thought of as a nucleolar protein, there has been evidence supporting the role of nucleolin in transporting proteins between the cytoplasm and nucleus [60-62], and several reports demonstrating the mobility of nucleolin in the nucleoplasm, cytoplasm, and cell surface [34, 48-53]. The translocation of nucleolin to the cell surface has been shown in response to stimuli such as heat shock [44, 54], T-cell activation [55], mitosis [56], treatment with cyclin-dependent kinase inhibitor [57], and viral infection [58-60].

We report here that decreased expression of nucleolin by siRNA results in increased NF-κB transcriptional activity. Additionally, upon stimulation with TNFα, nucleolin and NEMO precipitate in the same complex in a time-dependent manner. These results suggest a possible role of nucleolin in the signaling cascade that leads to the transcription of genes regulated by NF-κB.

MATERIALS AND METHODS

Cell culture and treatment

HeLa cells (cervical cancer cells) in were obtained from American Type Culture Collection (ATCC). Cells were grown to 50% confluence in 6-well plates in a standard incubator in DMEM (Gibco) supplemented with 10% heat-inactivated (65°C for 20 min) fetal bovine serum (Gibco), 100 units/ml penicillin and 100 units/ml streptomycin. TNFα (R & D Systems, Inc.) was added to the culture medium for a final concentration of 10 ng/ml.

siRNA

The siRNA duplex sequence is 5'-d(GUUGCAGCAGCCUUCUUGCUU)-3' (antisense) and 5'-d(GCAAGAAGGCUGCUGCAACUU)-3' (sense) (Oligos Etc.). The antisense and sense strands of siRNA were suspended in Diethyl-pyrocarbonate (DEPC)-treated water. All siRNAs were annealed at 90°C for 2 min, then 37°C for 1 hr in Buffer R (100 mM potassium acetate, 30 mM HEPES-KOH pH 7.4, 2 mM magnesium acetate). HeLa cells were transfected with siRNA (100 nM) using Oligofectamine transfection reagent (Invitrogen) in Opti-MEM 1 reduced serum medium (Invitrogen) according to manufacturer's instructions. Cells were lysed with the addition of M-PER Mammalian protein extraction buffer (Pierce).

Western blot analysis

Whole cell lysates were electrophoresed on SDS-polyacrylamide (8%) gels and transferred to PVDF membranes. Membranes were blocked with 5% nonfat dried milk in PBS plus 0.05% Tween20 (PBST) then incubated with primary antibody in PBST (1

μg/ml anti-nucleolin, sc-8031, Santa Cruz or 1 μg/ml anti-NEMO, sc-8032, Santa Cruz). Following incubation with secondary antibody (0.5 μg/ml anti-mouse HRP-conjugated, Santa Cruz) bands were visualized by chemiluminescence (ECL, Amersham Biosciences).

Transient transfection and measurement of luciferase activity

Cells were plated in 24-well plates at a density of 2 x 10^4 cells per well and were transiently transfected with an NF-κB reporter plasmid and an internal control plasmid (described below) using Superfect reagent (Qiagen), according to the manufacturer's protocol. At 24 h post-transfection, the cells were treated with siRNA as described above. After an incubation of 48 h, TNFα (10 ng/ml) was added to the medium for an additional 6 h and cell lysates were prepared using reporter lysis buffer (Promega). Lysates were added to the substrate reagents supplied in the Dual-Luciferase reporter system (Promega) and luciferase activity was measured using a luminometer (Zylux corporation). The NF-κB reporter plasmid contained tandem repeats of the κB sequence upstream of the minimal simian virus 40 (SV40) promoter and firefly luciferase gene in pLuc-MCS vector (Stratagene) and was a kind gift from Dr. Fajan Yang. As an internal control to account for variations in transfection efficiency, pRLnull (Promega), which expresses *Renilla* luciferase by basal transcription, was used (a gift from Dr. Robert Mitchell). Luciferase activity is expressed as the activity of firefly luciferase divided by *Renilla* luciferase.

Immunoprecipitation of NEMO

HeLa cells were plated as described above and treated with TNFα (10 ng/ml) for various from 5 min to 180 min. Cells were lysed with the addition M-PER Mammalian protein extraction buffer (Pierce). The extracts were incubated with 10 μg of anti-NEMO antibody (sc-8330, Santa Cruz) for 2 h at 4°C in RIPA buffer (150 mM NaCl, 10 mM Tris.Hcl pH 7.5, 0.1% SDS, 1% Triton X-100, 1% Deoxycholate, 5 mM EDTA, 1mM PMSF, 2 μg/ml leupeptin). Protein G coated magnetic beads (Magnabind, Pierce) were then added to the samples and incubated for 1 h at 4°C. The beads were captured and unbound sample was removed by washing three times with RIPA buffer. The precipitated proteins were eluted from the beads by the addition of SDS sample loading buffer (4% SDS, 2% glycerol, 5% β-mercaptoethanol, 0.75 M Tris.HCl, pH 8.8) and heating at 65°C for 15 min. Western blot analysis, as described above, was then utilized to detect the presence of NEMO and nucleolin.

RESULTS

Decrease of nucleolin expression increases NF-κB transcriptional activity

HeLa cells were transfected with siRNA (100 nM) corresponding to nucleolin or a nonspecific sequence as a control. Following an incubation of 48 h, the cells were stimulated with TNFα (10 ng/ml) for 2 h, then lysed and subjected to western blot analysis to detect levels of nucleolin and β-actin as a loading control. Sufficient knockdown of nucleolin was achieved as seen in Figure 15. To examine NF-κB transcriptional activity, HeLa cells were first transfected with a NF-κB luciferase reporter plasmid and a null renilla plasmid as an internal control. 24 h after transfection, the cells

were treated with siRNA as described above. Following stimulation with TNFα for 6 h, luciferase activity was measured revealing an increase in NF-κB transcriptional activity in response to the knockdown of nucleolin. (Figure 15).

Asscociation of nucleolin and NEMO increases in the presence of TNFα

We previously observed co-precipitation of nucleolin and NEMO in the presence of GRO in the S-100 fraction of cell lysates (see Chapter 3). This association suggested nucleolin may be involved in the signaling events leading to NF-κB transcription. To examine this possibility, cells were treated with TNFα from 5 to 180 min. Cells were lysed and subjected to immunopreciptation with NEMO antibody. Western blotting revealed the association of nucleolin and NEMO increases in the presence of TNFα (Figure 16).

Re-localization of nucleolin in response to TNFα stimulation

We then investigated the effects of stimulation with TNFα on the levels and localization of nucleolin. Western blot analysis of lysates from HeLa cells (Figure 17) revealed an increase in S-100 nucleolin and a concomitant reduction in nuclear nucleolin, suggesting that there is rapid translocation of nucleolin in response to TNFα.

Figure 15: Decreased expression of nucleolin results in increased NF-κB transcriptional activity.

(A) Western blot analysis probing for nucleolin and β-actin from cell lysates transfected with siRNA (100 nM) corresponding to nucleolin or a non-specific sequence then stimulated with TNFα (10 ng/ml); (B) NF-κB transcriptional activity in the presence of siRNA for nucleolin or a non-specific sequence measured by luciferase assay.

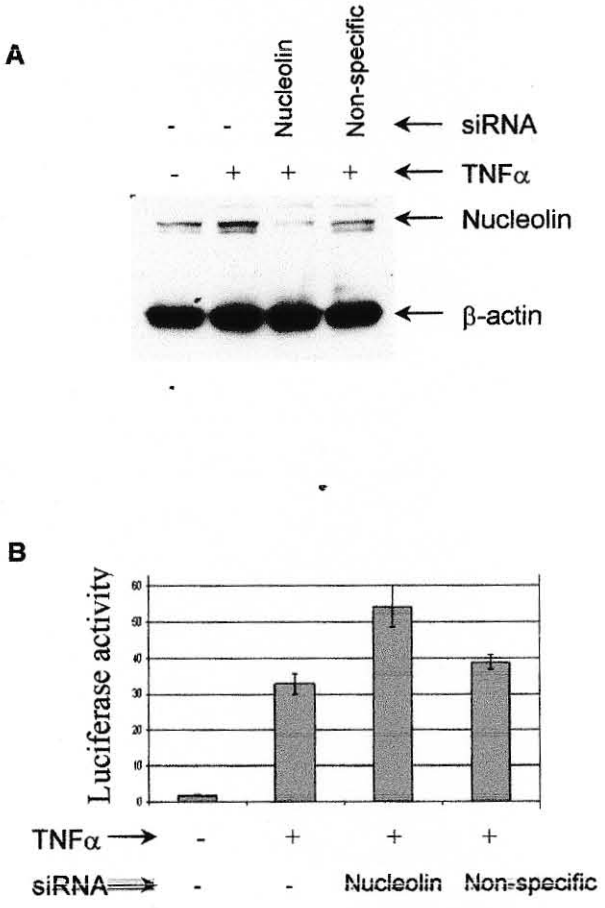

A
Nucleolin
Non-specific
siRNA
TNFα
Nucleolin
β-actin
B
Luciferase activity
60
50
40
30
20
10
0
TNFα
siRNA
Nucleolin
Non-specific

Figure 16: Association of nucleolin and NEMO increases in the presence of TNFα.

HeLa cells were treated with TNFα (10 ng/ml), lysed, and subjected to immunopreciptiation with NEMO antibody. Western blot anlaysis revealed the presence of nucleolin and NEMO.

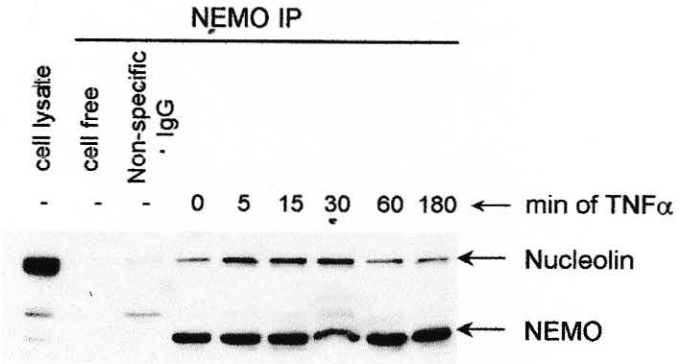
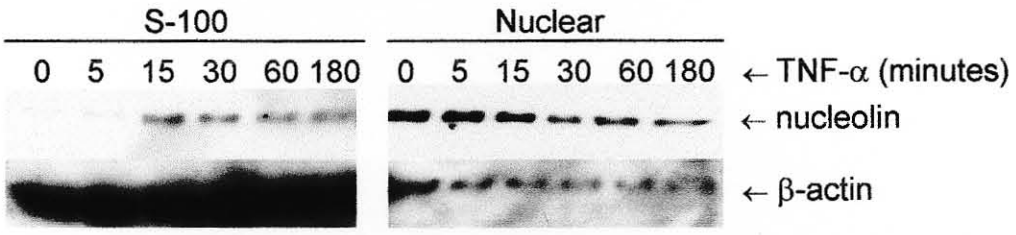

NEMO IP
cell lysate
cell free
Non-specific IgG
- - - 0 5 15 30 60 180 ← min of TNFα
← Nucleolin
← NEMO

Figure 17: Translocation of nucleolin in response to TNFα stimulation.

Nuclear and S-100 fractions were prepared from HeLa cells that had been stimulated with TNFα (10 ng/ml) for the time indicated. These extracts were subjected to western blot analysis to determine the levels of nucleolin and β-actin, which was used as a control for equal loading.


S-100
Nuclear
0 5 15 30 60 180
0 5 15 30 60 180
← TNF-α (minutes)
← nucleolin
← β-actin

DISCUSSION

There has been a lot of interest in NF-κB signaling recently due to its involvement in several cellular processes. Although research has revealed much information about the events leading to NF-κB transcription, there are still unanswered questions in the signaling cascade, especially in the activation of the IKK complex.

We report here that nucleolin may play a role in the signaling cascade of NF-κB transcription; although there needs to be additional research to validate this hypothesis, we show evidence suggesting that nucleolin may be involved in the signaling cascade. Down-regulation of nucleolin by siRNA results in an increase in NF-κB transcriptional activity (Figure 15). Additionally, the association of nucleolin and NEMO increases in the presence of TNFα in a time-dependent manner (Figure 16), and nucleolin translocates in response to TNFα (Figure 17). This evidence suggests nucleolin may play a role in the activation of IKK.

There has been no previous reports describing the interaction of nucleolin and NEMO in NF-κB signaling; there is other circumstantial evidence supporting a role in this pathway. For example, it has been reported that nucleolin is phosphorylated in response to TNFα [151], and several other stimuli known to activate NF-κB can also induce mobilization of nucleolin [47, 55]. Nucleolin is a protein that shuttles between the nucleus and cytoplasm [50], and NEMO also undergoes nucleocytoplasmic shuttling [150]. Because of the NEMO-nucleolin colocalization in response to TNFα, it is possible

that nucleolin may be involved in its trafficking. In conclusion, further studies are needed to identify the role of nucleolin in NF-κB signaling and the interaction between nucleolin and NEMO.

CHAPTER V

GLOBAL ASSESSMENT OF GENE AND PROTEIN EXPRESSION IN CELLS TREATED WITH GUANINE-RICH OLIGONUCLEOTIDES

INTRODUCTION

Cancer is a major public health problem in the United States being the second leading cause of death after heart disease. Currently, one in four deaths in the United States is due to cancer [66]. Although there have been improved screening and treatment regimens, there is still a need for novel therapies in patients that do not respond to the treatment and for those patents who develop resistance to traditional chemotherapy.

We have previously described a novel therapy known as guanine-rich oligonucleotides (GROs). GROs contain sequences that are capable of forming G-quartets, which are four coplanar guanines stabilized with Hoogsteen hydrogen bonds. They exhibit potent antiproliferative properties in several malignant cell lines, inducing S-phase cell cycle arrest and cell death exclusively in cancer cells, leaving non-malignant cells primarily unaffected [35]. GROs have also demonstrated considerable potential in phase I clinical trials in patients with advanced cancer, showing that the drug is well tolerated and exhibits promising clinical activity [38]. The primary target of GROs has previously been identified as nucleolin [34], which is a multifunctional protein involved in a variety of cellular processes like ribosome biogenesis [40, 43], DNA replication [44],

cell growth and proliferation [40-42], apoptosis [46, 47], and cytokinesis and nuclear division [45]. Nucleolin can also function as a cell surface receptor, where it acts as a shuttling protein between cytoplasm and nucleus [60-62].

Recent advances in high-throughput differential gene expression technologies have revolutionized research. The ability to obtain expression data for tens of thousands of genes from the same sample in a single assay has made it possible to study complicated interactions between networks of genes and gene products [146, 147]. Although much insight can be gained from analyzing global gene expression, this approach is limited in that it fails to monitor posttranscriptional mechanisms; so it imperative to investigate global protein expression as well [148].

In this study, we applied cDNA microarray and two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) technologies to analyze malignant cells that have treated with GROs. We propose that the use of combined global gene and protein expression technologies will allow for a better understanding of the mechanism of GROs. Although these techniques generate excessive data, our results reveal important aspects into the antiproliferative properties of GROs.

MATERIALS AND METHODS

Cell culture and treatment

DU145 cells (prostrate cancer cells) in were obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells were grown to 50% confluence in a standard

incubator in DMEM (Gibco) supplemented with 10% heat-inactivated (65°C for 20 min) fetal bovine serum (Gibco), 100 units/ml penicillin and 100 units/ml streptomycin. The cells were then treated by addition of oligonucleotide directly to the culture medium to give the final concentration of 10 μM. The oligonucleotides used in these experiments were as follows: GRO26B, 5'-d(GGTGGTGGTGGTTGTGGTGGTGGTGG)-3', and an inactive control CRO, 5'-d(CCTCCTCCTCCTTCTCCTCCTCCTCC)-3'. The oligonucleotides were purchased in the desalted form from Integrated DNA Technologies Inc. and used without further purification. They were then resuspended in water, sterilized by filtration through a 0.2 μm filter, then diluted with sterile water to give stock solutions of 400-500 μM that were stored in aliquots at -20 °C.

Preparation of labeled cRNA, array hybridization, and global gene analysis

DU145 cells were grown to 50% confluence in T150 culutre flasks and samples were prepared from cells that were untreated or treated with GRO26B (10 μM) or CRO (10 μM) for either 2 h or 18 h. Total RNA was isolated from the cells with Trizol reagent (Invitrogen) according to manufacturer's protocol. Subsequent mRNA isolation was done using an mRNA purification kit (Qiagen) using the instructions provided by the manufacturer. The protocols for sample preparation are described in the Affymetrix technical manual. But briefly, mRNA was converted to double-stranded cDNA using Superscript Reverse transcriptase (Invitrogen) and an oligo(dT) primer linked to the T7 RNA polymerase-binding sequence. The cDNA was then converted to labeled cRNA using T7 RNA polymerase in the presence of biotinylated UTP and CTP (ENZO Diagnostics). The labeled cRNA was purified with an RNeasy column (Qiagen), and

fragmented by heat and ion-mediated hydrolysis. The fragmented cRNA was hybridized for 16 h at 45°C to Affymetrix GeneChip Human HG_U133, followed by washing, staining, signal amplification with biotinylated antistreptavidin antibody, and final staining. The array was then scanned using a laser confocal genearray scanner, and the scanned images were analyzed using Affymetrix Analysis Suite 4.0. The differences in the levels of fluorescence intensity, representing the levels of hybridization, were analyzed by multiple stringencies to determine the alterations in levels of gene expression. Background and noise corrections were made to account for nonspecific binding and minor variations in hybridization conditions. The online data mining tool, NetAffx Analysis Center (Affymetrix), was then used to analyze to the results.

Sample preparation and protein separation by 2D electrophoresis

DU145 cells were grown to 50% confluence and treated with 10 μM GRO26B, CRO, or left untreated as a control. After 4 or 24 h of treatment, nuclear and S-100 extracts were isolated with ReadyPrep Protein Extraction Kit (Bio-Rad) according to the manufacturer's protocol. To ensure the lysates were suitable for 2D electrophoresis, the extracts were subjected to ReadyPrep 2D Cleanup Kit (Bio-Rad) following the protocol provided by the manufacturer. Samples (100 μg) were run on non-linear pH 3-10 IPG strips (Invitrogen) for the first dimension. The proteins were then run on 4-12% gradient acrylamide gels (Invitrogen) for the second dimension. Following electrophoresis, the gels were visualized with colloidal Coomassie blue stain (Genomic Solutions).

RESULTS

Microarray analysis reveals many genes affected by treatment with GRO

Microarray chip analysis was used to investigate alterations in global gene expression in cells treated with GRO. DU145 cells were treated with active GRO, inactive CRO, or left untreated for mRNA extraction after 2 and 18 h of treatment. The samples were prepared and subjected to microarray chip analysis to reveal alterations in the global gene expression of the cells treated with GRO compared to CRO treated and untreated cells. The analysis revealed 63 genes altered after only 2 hours of treatment with GRO, and 238 genes altered after 18 hours of treatment with GRO. Tables 3 and 4 list the genes altered by treatment with GRO, and their fold changes relatives to the controls.

Although the expression of these genes needs to be validated by another technique, such as RT-PCR, there are some interesting observations that can been seen in the global expression data from cells treated with GROs. One observation is the expression of nucleolin is not affected by treatment with GROs. Because nucleolin is the primary target of GROs [34], it is important to note that GROs apparently do not influence the transcription of nucleolin which is consistent with previous work indicating that total levels of nucleolin protein are not changed in GRO-treated cells. This observation leads to two likely possibilities: either GROs are affecting the function of nucleolin directly, or nucleolin binds to GROs on the surface of the cell, and aids in shuttling the GRO inside the cell where it affects function of other proteins.

There are several other observations that can be seen from the global gene expression data. There are several changes in metabolic enzymes, particularly those involved in fatty acid and glycerolipid metabolism. These include the expression of acetyl coA carboxylase (-8.9), g-butyrobetaine hydroxlyase (-4.6), NADH ubiquinone oxidoreductase (-4.9), and NAD synthetase (-2.1). There is also a marked decrease in expression in many cancer-associated proteins including telomerase (-5.7), estrogen receptor α (-8.0), cathespin S (-4.4), and EpCAM/TROP2 tumor marker (-20.5). A strong induction can be seen in HUS1 checkpoint analog (+15.6), which is a cell cycle checkpoint protein. There is also a dramatic decrease in specific transcription factors such as T-box 1 (-78.8), achaete-schute-like 2 (-27.3), and single minded 2/SIM2 (-12.4). A strong induction of several helicases can be observed including DDX3X (+13.3), SMARCA2 (+10.1), DDX17 (+4.4), and RecQ-like helicase (+2.2).

Microarray analysis reveals many biological systems affected by treatment with GRO

Upon further analysis, the affected genes are involved in several different cellular functions. Tables 5 and 6 lists these biological processes, the number of genes involved in the processes, and the number of GRO-altered genes in each process. After two hours of treatment with GRO, there are several biological processes affected including transcription, transport, and metabolism, particularly nucleic acid metabolism. GRO treatment for 18 hours leads to the alteration of many more cellular functions. The same processes seen after two hours of treatment are also seen after 18 hours, with the addition

of many more affected cellular functions. These functions include signal transduction, cell communication, apoptosis, cell motility, cell adhesion, programmed cell death, and regulation of cell growth.

Alterations in global protein expression in GRO treated cells

DU145 cells were treated with 10 μM GRO26B, CRO, or left untreated as a control. After 4 or 24 h of treatment, nuclear and S-100 extracts were isolated and subjected to separation by 2D electrophoresis. The individual proteins were then visualized by colloidal Coomasie stain. As seen in Figures 18 and 19, global protein expression does not appear to be greatly affected by treatment with GRO. We do expect to see the expression of several proteins affected, given that the global gene expression shows several genes affected by the GRO. This is most likely due to the sensitivity of this technique which limits the detection of these proteins. Using this technique, 63 nuclear and 41 cytoplamsic proteins were visualized, which is clearly not representative of the entire cellular proteome. Therefore, further investigation is needed to explore the global protein expression affected by GROs.

DISCUSSION

The success of the genome project and technological advances in functional genomics and proteomics has facilitated the transition to large-scale systemic approaches in studying global mechanisms. mRNA expression profiling by microarray has been used in a wide range of cancer-related studies and provided important insights into disease. More recently, proteomic analysis by 2D electrophoresis and mass spectrometry

have provided additional clues about the post-transcriptional mechanisms underlying disease. Utilizing these techniques can give significant insight in investigating cellular mechanisms on the global scale [148].

We have previously reported guanine-rich oligonucleotides (GROs) exhibiting potent antiproliferative properties in several malignant cell lines [34-37], and demonstrating considerable potential as a novel therapeutic agent for cancer [38]. Although some important aspects of GRO activity are known (i.e. S phase cell cycle arrest and cell death) [35], the precise molecular mechanism remains unclear. However, it is known that the activity of GROs correlate with its ability to bind to nucleolin, and there is evidence to support that nucleolin is the primary target of GROs [34].

The goal of this study is to gain a better understanding of the mechanism of GRO antiproliferative activity by utilizing global gene and protein expression technologies. Unfortunately, the 2D electrophoresis used to detect global protein expression in cells treated with GROs was not able to detect any alterations in the proteins isolated, probably due to the sensitivity of the technique. However, microarray analysis revealed the expression of several genes changed upon treatment with GROs. After only two hours of treatment with GROs, the cell responds by increasing or decreasing the expression of 63 different genes, and 238 genes after 18 hours of treatment (Tables 3 and 4). These genes are involved in a variety of cellular functions (Tables 5 and 6), the most prominent being metabolism, transcription, and transport.

Microarray analysis also revealed that the expression of nucleolin did not change in the cells treated with GRO. Because nucleolin is presumably the primary target of GROs [34], it is most likely that GROs either modify the function of nucleolin directly, or nuceolin shuttles the GRO inside the cell where it affects the function of other proteins. Nucleolin is an abundant protein that is implicated in several different cellular functions. Interestingly, some of the same functions that are affected by GROs have been implicated in the function of nucleolin; these include transcription [42], apoptosis [46, 47], helicase activity [42], and regulation of cell growth [40-42]. This evidence is consistent with our hypothesis that GROs directly affect the function of nucleolin; however it appears that GROs are not exclusively affecting nucleolin. Because of the abundance of genes and functions altered by treatment with GROs, it appears that the functions of proteins other than nucleolin are altered as well.

Clearly, more work is needed to decipher the molecular mechanism of the antiproliferative activity of GROs. In this present study, we have gained insight into how the cell responds to GRO treatment by utilizing microarray technology. The results presented here are only a foundation in which to continue the investigation into the mechanism of GROs. Other techniques should be utilized to validate the expression changes seen in the microarray data. Additionally, it is important to investigate the expression levels of proteins as well since posttranscriptional modifications can alter a presumed function of a gene. However, our results are a notable starting point in which to continue research.

Table 3: Genes altered after 2 hours of GRO treatment.

Fold change	Gene title
17.4	Homo sapiens clone 24540 mRNA sequence
11.7	RAB9, member RAS oncogene family, pseudogene 1
8.3	nuclear antigen Sp100
7.0	EGF-like repeats and discoidin I-like domains 3
6.1	KIAA1068 protein
4.9	Homo sapiens mRNA; cDNA DKFZp434J193 (from clone DKFZp434J193); partial cds
4.9	thymus high mobility group box protein TOX
4.0	HIV-1 inducer of short transcripts binding protein
4.0	ADP-ribosylation factor interacting protein 1 (arfaptin 1)
3.2	likely ortholog of mouse and zebrafish forebrain embryonic zinc finger-like
2.9	I factor (complement)
2.8	TAF6-like RNA polymerase II, p300/CBP-associated factor (PCAF)-associated factor, 65kDa
2.8	21383_at
2.8	hypothetical protein MGC11266
2.6	hypothetical protein FLJ11142
2.6	macrophage stimulating, pseudogene 9
2.6	hypothetical protein FLJ32389
2.5	leukocyte Ig-like receptor 9
2.5	216688_at
2.4	zinc finger protein, Y-linked
2.3	hypothetical protein FLJ13646
2.3	eukaryotic translation initiation factor 4E
2.3	APG12 autophagy 12-like (S. cerevisiae)
2.3	zinc finger protein 45 (a Kruppel-associated box (KRAB) domain polypeptide)
2.2	polymerase delta interacting protein 46
2.2	F-box and WD-40 domain protein 1B
2.2	amiloride binding protein 1 (amine oxidase (copper-containing))
2.2	RNA binding motif protein 3
2.2	CD34 antigen
2.1	nescient helix loop helix 2
2.1	211074_at
2.1	211506_s_at
2.1	transient receptor potential cation channel, subfamily A, member 1
2.1	protein tyrosine phosphatase type IVA, member 2
2.1	hypothetical protein MGC3067
2.0	solute carrier family 35 (UDP-N-acetylglucosamine (UDP-GlcNAc) transporter), member A3
2.0	superoxide dismutase 2, mitochondrial
-12.0	calponin homology (CH) domain containing 1
-8.9	acetyl-Coenzyme A carboxylase alpha
-6.8	B-cell CLL/lymphoma 7C
-5.1	chromosome 6 open reading frame 11
-4.7	protein kinase C and casein kinase substrate in neurons 3
-4.5	chromosome 14 open reading frame 34
-3.6	peptidylprolyl isomerase (cyclophilin)-like 2

-2.8	autoantigen
-2.7	cholinergic receptor, nicotinic, epsilon polypeptide
-2.7	keratin 15
-2.4	hypothetical protein MGC5178
-2.3	hypothetical protein 24432
-2.3	transmembrane 4 superfamily member 7
-2.2	hypothetical protein FLJ22341
-2.2	host cell factor C1 regulator 1 (XPO1 dependant)
-2.2	7-dehydrocholesterol reductase
-2.2	transmembrane 7 superfamily member 2
-2.1	pleiomorphic adenoma gene-like 1
-2.1	proline dehydrogenase (oxidase) 1
-2.1	PISC domain containing hypothetical protein
-2.1	inhibitor of DNA binding 2, dominant negative helix-loop-helix protein
-2.1	jagged 2
-2.1	hepatitis delta antigen-interacting protein A
-2.1	stearoyl-CoA desaturase (delta-9-desaturase)
-2.0	filamin B, beta (actin binding protein 278)
-2.0	hypothetical protein FLJ21347

Table 4: Genes altered after 18 hours of GRO treatment.

Fold change	Gene title
15.6	HUS1 checkpoint homolog (S. pombe)
14.5	hypothetical protein FLJ10849
13.5	hypothetical protein FLJ10970
13.2	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked
10.9	EGF-like repeats and discoidin I-like domains 3
10.1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 2
8.0	hypothetical protein PRO1853
6.5	PTB domain adaptor protein CED-6
6.2	SEC10-like 1 (S. cerevisiae)
5.9	v-rel reticuloendotheliosis viral oncogene homolog (avian)
5.5	glucosamine (N-acetyl)-6-sulfatase (Sanfilippo disease IIID)
5.5	RAB3B, member RAS oncogene family
5.4	golgi SNAP receptor complex member 2
5.2	zinc finger protein 37a (KOX 21)
5.2	hypothetical protein FLJ12994
5.1	prenylcysteine oxidase 1
5.0	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2
4.9	actin filament associated protein
4.9	wingless-type MMTV integration site family, member 7B
4.4	DEAD (Asp-Glu-Ala-Asp) box polypeptide 17
4.3	zinc finger RNA binding protein
4.1	paraneoplastic antigen
4.0	PTK9 protein tyrosine kinase 9
3.8	211506_s_at
3.7	216383_at
3.6	similar to Caenorhabditis elegans protein C42C1.9
3.5	guanine nucleotide binding protein (G protein), alpha activating activity polypeptide, olfactory type
3.4	suppression of tumorigenicity
3.3	Homo sapiens cDNA FLJ31439 fis, clone NT2NE2000707.
3.3	tumor necrosis factor receptor superfamily, member 10d, decoy with truncated death domain
3.2	ring finger protein 125
3.1	fumarate hydratase
3.1	stress-induced-phosphoprotein 1 (Hsp70/Hsp90-organizing protein)
3.1	zinc finger RNA binding protein
3.1	NGFI-A binding protein 1 (EGR1 binding protein 1)
3.0	paternally expressed 10
3.0	poly(A) polymerase alpha
3.0	steroid sulfatase (microsomal), arylsulfatase C, isozyme S
3.0	Homo sapiens, clone IMAGE:5294815, mRNA
2.9	secretory carrier membrane protein 1
2.9	endothelial and smooth muscle cell-derived neuropilin-like protein
2.8	aryl hydrocarbon receptor nuclear translocator-like 2
2.8	208844_at
2.8	met proto-oncogene (hepatocyte growth factor receptor)
2.8	SOCS box-containing WD protein SWiP-1

2.8	PCTAIRE protein kinase 2
2.7	vesicle-associated membrane protein 3 (cellubrevin)
2.7	Bcl-2-associated transcription factor
2.7	cyclin E2
2.7	hypothetical protein H41
2.6	cell division cycle 27
2.6	solute carrier family 7, (cationic amino acid transporter, y+ system) member 11
2.6	NDRG family member 3
2.5	progesterone receptor membrane component 1
2.5	mitogen-activated protein kinase kinase kinase kinase 5
2.5	zinc finger protein 426
2.5	secretory carrier membrane protein 1
2.5	heat shock 70kDa protein 4
2.5	APG12 autophagy 12-like (S. cerevisiae)
2.5	CD164 antigen, sialomucin
2.5	AFFX-r2-Hs18SrRNA-M_x_at
2.4	REV3-like, catalytic subunit of DNA polymerase zeta (yeast)
2.4	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 2
2.4	zinc finger protein 45 (a Kruppel-associated box (KRAB) domain polypeptide)
2.4	septin 10
2.4	Homo sapiens hypothetical LOC133993 (LOC133993), mRNA
2.4	Sec23 homolog A (S. cerevisiae)
2.4	polymerase (RNA) III (DNA directed) (32kD)
2.4	hypothetical protein KIAA1164
2.3	histone 1, H3h
2.3	Ras-GTPase activating protein SH3 domain-binding protein 2
2.3	RIO kinase 3 (yeast)
2.3	interleukin 6 signal transducer (gp130, oncostatin M receptor)
2.3	HIV-1 Rev binding protein
2.3	hypothetical protein MGC3067
2.3	calumenin
2.3	SEC24 related gene family, member D (S. cerevisiae)
2.3	core-binding factor, beta subunit
2.3	insulin-like 5
2.3	AFFX-HUMRGE/M10098_5_at
2.2	erythrocyte membrane protein band 4.1-like 1
2.2	calumenin
2.2	butyrate-induced transcript 1
2.2	hypothetical protein MGC11061
2.2	lectin, mannose-binding, 1
2.2	NCK-associated protein 1
2.2	RecQ protein-like (DNA helicase Q1-like)
2.2	chromosome 20 open reading frame 30
2.2	secretory carrier membrane protein 1
2.2	chromosome 6 open reading frame 62
2.2	AFFX-HUMISGF3A/M97935_MA_at
2.1	calnexin
2.1	muscleblind-like (Drosophila)
2.1	SBBI26 protein
2.1	sphingosine-1-phosphate phosphatase 1

2.1	GM2 ganglioside activator protein
2.1	oculocerebrorenal syndrome of Lowe
2.1	catalase
2.1	nucleolar and spindle associated protein 1
2.1	Homo sapiens cDNA FLJ35853 fis, clone TESTI2007078, highly similar to MEMBRANE COMPONENT, CHROMOSOME 17, SURFACE MARKER 2.
2.1	DKFZP586N0721 protein
2.1	cleavage and polyadenylation specific factor 5, 25 kDa
2.1	leukocyte-derived arginine aminopeptidase
2.1	transducin (beta)-like 1X-linked
2.1	hypothetical protein MGC14799
2.1	ROD1 regulator of differentiation 1 (S. pombe)
2.1	promethin
2.1	phosphoglycerate kinase 1
2.1	M-phase phosphoprotein, mpp8
2.1	RIO kinase 3 (yeast)
2.1	thioredoxin domain containing
2.1	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 3
2.1	tumor rejection antigen (gp96) 1
2.1	PTD016 protein
2.0	Homo sapiens transcribed sequence with weak similarity to protein ref:NP_060312.1 (H.sapiens) hypothetical protein FLJ20489 [Homo sapiens]
2.0	216899_s_at
2.0	AFFX-HUMRGE/M10098_M_at
2.0	solute carrier family 35 (UDP-N-acetylglucosamine (UDP-GlcNAc) transporter), member A3
2.0	lamina-associated polypeptide 1B
2.0	hypothetical protein FLJ12806
2.0	Homo sapiens transcribed sequence with strong similarity to protein ref:NP_055485.1 (H.sapiens) basic leucine-zipper protein BZAP45; KIAA0005 gene product [Homo sapiens]
2.0	adenovirus 5 E1A binding protein
2.0	solute carrier family 16 (monocarboxylic acid transporters), member 1
2.0	serum/glucocorticoid regulated kinase-like
-78.8	T-box 1
-62.2	semenogelin II
-27.3	achaete-scute complex-like 2 (Drosophila)
-27.1	hypothetical protein LOC157697
-20.5	tumor-associated calcium signal transducer 2
-15.5	Homo sapiens similar to dJ309K20.1.1 (novel protein similar to dysferlin, isoform 1) (LOC375095), mRNA
-15.2	208278_s_at
-13.5	216737_at
-12.4	single-minded homolog 2 (Drosophila)
-12.3	217451_at
-12.0	EphA5
-11.6	Homo sapiens transcribed sequence with weak similarity to protein ref:NP_060219.1 (H.sapiens) hypothetical protein FLJ20294 [Homo sapiens]
-11.6	217093_at
-11.1	superoxide dismutase 2, mitochondrial
-11.0	insulin-like growth factor 1 (somatomedin C)
-10.8	Homo sapiens transcribed sequence with moderate similarity to protein

	ref:NP_060219.1 (H.sapiens) hypothetical protein FLJ20294 [Homo sapiens]
-10.1	Homo sapiens transcribed sequences
-9.8	histamine receptor H3
-9.5	alkaline phosphatase, placental-like 2
-9.4	G protein-coupled receptor 17
-9.4	cardiac ankyrin repeat kinase
-8.6	dachshund homolog (Drosophila)
-8.4	A kinase (PRKA) anchor protein 5
-8.3	ankyrin repeat domain 1 (cardiac muscle)
-8.1	estrogen receptor 1
-8.0	tight junction protein 3 (zona occludens 3)
-7.6	transmembrane protease, serine 4
-7.6	cold autoinflammatory syndrome 1
-7.5	glutathione S-transferase theta 2
-7.2	glutamate receptor, ionotropic, N-methyl D-aspartate 1
-7.1	hypothetical protein FLJ10786
-6.8	CD1E antigen, e polypeptide
-6.6	zinc finger protein 157 (HZF22)
-6.6	Homo sapiens cDNA: FLJ21911 fis, clone HEP03855
-6.5	hypothetical protein FLJ22688
-6.5	tissue inhibitor of metalloproteinase 3 (Sorsby fundus dystrophy, pseudoinflammatory)
-6.4	major histocompatibility complex, class II, DO beta
-6.4	gasdermin-like
-6.3	inversin
-6.0	KIAA0685
-5.9	small muscle protein, X-linked
-5.8	zinc finger protein 254
-5.7	cadherin, EGF LAG seven-pass G-type receptor 1 (flamingo homolog, Drosophila)
-5.7	telomerase reverse transcriptase
-5.5	Nef associated protein 1
-5.4	glycoprotein Ib (platelet), beta polypeptide
-5.1	a disintegrin and metalloproteinase domain 28
-4.9	high density lipoprotein binding protein (vigilin)
-4.9	NADH:ubiquinone oxidoreductase MLRQ subunit homolog
-4.8	5-hydroxytryptamine (serotonin) receptor 2C
-4.7	family with sequence similarity 12, member B (epididymal)
-4.6	butyrobetaine (gamma), 2-oxoglutarate dioxygenase (gamma-butyrobetaine hydroxylase) 1
-4.5	tripartite motif-containing 3
-4.4	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3F
-4.4	211218_at
-4.4	cathepsin S
-4.1	homeo box D3
-4.1	FK506 binding protein 12-rapamycin associated protein 1
-3.9	217311_at
-3.8	ubiquitin protein ligase E3A (human papilloma virus E6-associated protein, Angelman syndrome)
-3.7	dystrophin (muscular dystrophy, Duchenne and Becker types)
-3.7	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4

-3.7	tyrosine kinase with immunoglobulin and epidermal growth factor homology domains
-3.7	aquaporin 4
-3.6	forkhead box D3
-3.5	homeo box A6
-3.4	adipose specific 2
-3.4	T-cell leukemia, homeobox 2
-3.4	caspase recruitment domain family, member 10
-3.3	ribosomal protein S11
-3.3	agouti signaling protein, nonagouti homolog (mouse)
-3.3	arginine vasopressin receptor 2 (nephrogenic diabetes insipidus)
-3.2	diacylglycerol kinase, epsilon 64kDa
-3.0	eukaryotic translation initiation factor 3, subunit 5 epsilon, 47kDa
-3.0	Homo sapiens transcribed sequences
-2.9	granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3)
-2.8	erythrocyte membrane protein band 4.1 (elliptocytosis 1, RH-linked)
-2.8	G protein-coupled receptor 8
-2.8	potassium inwardly-rectifying channel, subfamily J, member 12
-2.8	histone 1, H4f
-2.8	leukocyte immunoglobulin-like receptor, subfamily A (without TM domain), member 5
-2.7	Homo sapiens transcribed sequences
-2.7	chromodomain helicase DNA binding protein 3
-2.7	solute carrier family 22 (organic anion/cation transporter), member 11
-2.7	221018_s_at
-2.6	ATPase, H+ transporting, lysosomal 9kDa, V0 subunit e
-2.6	fibroblast growth factor 18
-2.6	LOC92346
-2.6	Homo sapiens transcribed sequences
-2.6	prostaglandin D2 synthase 21kDa (brain)
-2.5	KIAA1922 protein
-2.5	hypothetical protein LOC339047
-2.5	IMP (inosine monophosphate) dehydrogenase 2
-2.5	Homo sapiens mRNA; cDNA DKFZp564P142 (from clone DKFZp564P142)
-2.4	transient receptor potential cation channel, subfamily C, member 3
-2.4	zinc finger protein 165
-2.3	carnitine palmitoyltransferase 1B (muscle)
-2.3	tripartite motif-containing 31
-2.3	221720_s_at
-2.3	leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 1
-2.2	mitogen-activated protein kinase 8 interacting protein 3
-2.2	cholinergic receptor, nicotinic, epsilon polypeptide
-2.2	chorionic somatomammotropin hormone-like 1
-2.2	UDP glycosyltransferase 2 family, polypeptide B17
-2.2	viperin
-2.2	hypothetical protein FLJ12443
-2.2	calponin homology (CH) domain containing 1
-2.2	growth differentiation factor 11
-2.1	calcium channel, voltage-dependent, L type, alpha 1B subunit
-2.1	CD84 antigen (leukocyte antigen)
-2.1	cysteine knot superfamily 1, BMP antagonist 1
-2.1	NAD synthetase 1

-2.1	growth arrest and DNA-damage-inducible, beta
-2.1	ribosomal protein L17
-2.1	hypothetical protein HSPC109
-2.0	chromosome 12 open reading frame 6
-2.0	CDC28 protein kinase regulatory subunit 1B
-2.0	interleukin 24
-2.0	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 11 (CHL1-like helicase homolog, S. cerevisiae)
-2.0	E4F transcription factor 1
-2.0	protocadherin beta 8

Table 5: Biological processes that are associated with genes altered by treatment with GRO after 2 hours.

biological_process	Altered genes	Total genes on array	%
pyrimidine nucleotide-sugar transport	1	5	20.0%
nucleotide-sugar transport	1	5	20.0%
cell proliferation	2	1341	0.1%
lipid metabolism	2	578	0.3%
induction of programmed cell death	1	173	0.6%
programmed cell death	2	512	0.4%
organic acid biosynthesis	1	56	1.8%
cell activation	1	67	1.5%
regulation of cell proliferation	1	366	0.3%
regulation of endocytosis	1	2	50.0%
cell cycle arrest	1	71	1.4%
response to oxidative stress	1	44	2.3%
sexual reproduction	1	182	0.5%
cation transport	1	454	0.2%
ion transport	2	625	0.3%
transport	5	1853	0.3%
cell organization and biogenesis	2	632	0.3%
carboxylic acid metabolism	2	394	0.5%
regulation of protein biosynthesis	1	25	4.0%
cell cycle	2	872	0.2%
translational initiation	1	79	1.3%
protein biosynthesis	1	565	0.2%
T-cell activation	1	31	3.2%
regulation of biosynthesis	1	49	2.0%
histogenesis	1	129	0.8%
organogenesis	3	1090	0.3%
oxygen and reactive oxygen species metabolism	1	61	1.6%
lymphocyte activation	1	59	1.7%
neurogenesis	1	492	0.2%
ectoderm development	1	95	1.1%
vesicle-mediated transport	1	325	0.3%
proline metabolism	1	3	33.3%
transcription from Pol II promoter	1	671	0.1%
epidermal cell differentiation	1	2	50.0%
hair cell fate commitment	1	2	50.0%
non-covalent chromatin modification	1	48	2.1%
immune response	1	903	0.1%
defense response	1	963	0.1%
response to stress	1	972	0.1%
physiological process	25	10507	0.2%
protein metabolism	5	2584	0.2%
negative regulation of endocytosis	1	1	100.0%

cytoplasm organization and biogenesis	1	355	0.3%
carbohydrate transport	1	26	3.8%
regulation of transcription from Pol II promoter	1	360	0.3%
regulation of transcription, DNA-dependent	7	2120	0.3%
transcription initiation	1	59	1.7%
transcription, DNA-dependent	7	2262	0.3%
transcription	7	2313	0.3%
carboxylic acid biosynthesis	1	56	1.8%
central nervous system development	1	120	0.8%
N signaling pathway	1	3	33.3%
amine metabolism	2	295	0.7%
cell fate commitment	1	11	9.1%
microtubule-based movement	1	39	2.6%
microtubule-based process	1	94	1.1%
chromatin modification	1	90	1.1%
protein ubiquitination	1	15	6.7%
cytoskeleton organization and biogenesis	1	237	0.4%
hearing	1	100	1.0%
glutamine family amino acid metabolism	1	44	2.3%
regulation of cell cycle	1	505	0.2%
muscle contraction	1	208	0.5%
siderochrome transport	1	197	0.5%
sensory perception	1	327	0.3%
positive regulation of programmed cell death	1	173	0.6%
endocytosis	1	144	0.7%
regulation of programmed cell death	1	173	0.6%
morphogenesis	3	1210	0.2%
positive regulation of apoptosis	1	173	0.6%
T-cell differentiation	1	13	7.7%
cholesterol biosynthesis	1	28	3.6%
cell surface receptor linked signal transduction	1	1095	0.1%
steroid biosynthesis	1	62	1.6%
signal transduction	2	2915	0.1%
chromatin remodeling	1	48	2.1%
chromosome organization and biogenesis (sensu Eukarya)	1	256	0.4%
macromolecule biosynthesis	3	885	0.3%
nucleobase, nucleoside, nucleotide and nucleic acid metabolism	9	3310	0.3%
biosynthesis	3	1042	0.3%
cell motility	2	413	0.5%
regulation of apoptosis	1	275	0.4%
cell adhesion	1	651	0.2%
amino acid metabolism	1	206	0.5%
cell communication	3	3643	0.1%
lipid biosynthesis	2	171	1.2%
establishment and/or maintenance of chromatin architecture	1	209	0.5%
DNA packaging	1	222	0.5%
cell death	2	550	0.4%
induction of apoptosis by p53	1	3	33.3%

induction of apoptosis	1	173	0.6%
apoptosis	2	511	0.4%
autophagy	1	4	25.0%
cell fate determination	1	10	10.0%
obsolete biological process	1	395	0.3%
embryonic morphogenesis	1	11	9.1%
organic acid metabolism	2	396	0.5%
amino acid and derivative metabolism	1	254	0.4%
embryonic development	1	30	3.3%
ubiquitin cycle	1	95	1.1%
perception of sound	1	102	1.0%
cell differentiation	2	163	1.2%
cellular process	11	7049	0.2%
cholesterol metabolism	1	76	1.3%
steroid metabolism	1	153	0.7%
response to abiotic stimulus	1	587	0.2%
translation	1	183	0.5%
immune cell activation	1	67	1.5%
perception of abiotic stimulus	1	326	0.3%
regulation of cellular process	1	439	0.2%
perception of external stimulus	1	354	0.3%
nucleotide-sugar metabolism	1	12	8.3%
protein modification	2	1140	0.2%
protein complex assembly	1	160	0.6%
sterol biosynthesis	1	34	2.9%
alcohol metabolism	1	226	0.4%
sterol metabolism	1	82	1.2%
male gamete generation	1	124	0.8%
metabolism	19	7171	0.3%
cell growth and/or maintenance	9	3939	0.2%
regulation of biological process	1	516	0.2%
biological_process	28	12339	0.2%
regulation of cell migration	1	3	33.3%
protein folding	1	181	0.6%
spermatogenesis	1	124	0.8%
DNA metabolism	1	636	0.2%
epidermal differentiation	1	79	1.3%
cell migration	1	54	1.9%
lymphocyte differentiation	1	26	3.8%
hemopoiesis	1	52	1.9%
response to biotic stimulus	2	1039	0.2%
response to external stimulus	2	1526	0.1%
reproduction	1	182	0.5%
limb morphogenesis	1	3	33.3%
gametogenesis	1	155	0.6%
RNA metabolism	1	428	0.2%
development	6	1956	0.3%
carbohydrate metabolism	1	372	0.3%

regulation of translation	1	98	1.0%
synaptic transmission, cholinergic	1	13	7.7%
nerve-nerve synaptic transmission	1	15	6.7%
thymic T-cell selection	1	3	33.3%
transmission of nerve impulse	1	287	0.3%
regulation of metabolism	1	58	1.7%
UDP-N-acetylglucosamine metabolism	1	4	25.0%
death	2	557	0.4%
N-acetylglucosamine metabolism	1	6	16.7%
nuclear organization and biogenesis	1	263	0.4%
glucosamine metabolism	1	9	11.1%
organelle organization and biogenesis	1	298	0.3%
amino sugar metabolism	1	15	6.7%
synaptic transmission	1	281	0.4%
T-cell selection	1	6	16.7%
cell-cell signaling	2	665	0.3%
fatty acid biosynthesis	1	48	2.1%
fatty acid metabolism	1	141	0.7%
hair cell differentiation	1	2	50.0%
mechanoreceptor differentiation	1	2	50.0%
RNA processing	1	406	0.2%
UDP-N-acetylglucosamine transport	1	2	50.0%
intracellular transport	1	597	0.2%
regulation of transcription	7	2131	0.3%
cytoskeleton-dependent intracellular transport	1	39	2.6%

Table 6: Biological processes that are associated with genes altered by treatment with GRO after 18 hours.

biological_process	Altered genes	Total genes on array	%
regulation of JNK cascade	1	7	14.3%
membrane fusion	1	22	4.5%
circadian rhythm	1	20	5.0%
rhythmic behavior	1	29	3.4%
striated muscle contraction	1	42	2.4%
excretion	2	55	3.6%
frizzled-2 signaling pathway	1	17	5.9%
physiological process	122	10507	1.2%
G-protein signaling, adenylate cyclase activating pathway	1	41	2.4%
G-protein signaling, coupled to cAMP nucleotide second messenger	1	95	1.1%
G-protein signaling, coupled to cyclic nucleotide second messenger	2	140	1.4%
G-protein coupled receptor protein signaling pathway	7	636	1.1%
nuclear division	2	215	0.9%
mevalonate transport	1	4	25.0%
phospholipid biosynthesis	1	39	2.6%
muscle contraction	4	208	1.9%
behavior	2	109	1.8%
neuropeptide signaling pathway	1	90	1.1%
endocytosis	1	144	0.7%
glutamate signaling pathway	1	19	5.3%
sexual reproduction	3	182	1.6%
M phase	2	218	0.9%
mitotic cell cycle	5	448	1.1%
serotonin receptor signaling pathway	1	4	25.0%
nucleobase, nucleoside, nucleotide and nucleic acid metabolism	37	3310	1.1%
tricarboxylic acid cycle	1	30	3.3%
cell motility	5	413	1.2%
monocarboxylic acid transport	1	21	4.8%
main pathways of carbohydrate metabolism	1	97	1.0%
energy pathways	2	265	0.8%
hearing	2	100	2.0%
carbohydrate transport	1	26	3.8%
amino acid derivative biosynthesis	1	32	3.1%
phototransduction	1	22	4.5%
vision	2	185	1.1%
sensory perception	3	327	0.9%
organic anion transport	1	43	2.3%
ER to Golgi transport	1	28	3.6%
exocytosis	1	55	1.8%
intracellular protein transport	4	459	0.9%
pregnancy	1	69	1.4%

di-, tri-valent inorganic cation transport	3	142	2.1%
modification-dependent protein catabolism	1	141	0.7%
monovalent inorganic cation transport	4	284	1.4%
transmembrane receptor protein tyrosine kinase signaling pathway	1	137	0.7%
RNA transport	1	38	2.6%
nucleic acid transport	1	38	2.6%
enzyme linked receptor protein signaling pathway	1	214	0.5%
response to endogenous stimulus	3	274	1.1%
cell surface receptor linked signal transduction	12	1095	1.1%
protein amino acid glycosylation	1	115	0.9%
signal transduction	33	2915	1.1%
regulation of circadian sleep/wake cycle, sleep	1	3	33.3%
response to radiation	3	217	1.4%
organic acid metabolism	5	396	1.3%
induction of apoptosis	1	173	0.6%
positive regulation of programmed cell death	1	173	0.6%
apoptosis	9	511	1.8%
regulation of programmed cell death	1	173	0.6%
autophagy	1	4	25.0%
nucleocytoplasmic transport	2	136	1.5%
positive regulation of apoptosis	1	173	0.6%
phagocytosis, engulfment	1	12	8.3%
amino acid and derivative metabolism	1	254	0.4%
cyclic-nucleotide-mediated signaling	2	140	1.4%
cAMP-mediated signaling	1	95	1.1%
second-messenger-mediated signaling	2	146	1.4%
ubiquitin-dependent protein catabolism	1	141	0.7%
heterophilic cell adhesion	1	100	1.0%
protein metabolism	25	2584	1.0%
electron transport	2	365	0.5%
homophilic cell adhesion	3	93	3.2%
cell adhesion	8	651	1.2%
cell communication	39	3643	1.1%
amine biosynthesis	1	63	1.6%
amine metabolism	3	295	1.0%
induction of programmed cell death	1	173	0.6%
protein secretion	1	21	4.8%
phagocytosis	1	28	3.6%
programmed cell death	9	512	1.8%
DNA damage response, signal transduction	1	28	3.6%
vesicle docking during the process of exocytosis	1	14	7.1%
lipid transport	1	70	1.4%
cell-cell adhesion	4	218	1.8%
proteolysis and peptidolysis	7	606	1.2%
amino acid transport	1	53	1.9%
regulation of cell growth	1	76	1.3%
ribonucleotide biosynthesis	1	68	1.5%
protein amino acid phosphorylation	7	612	1.1%

fumarate metabolism	1	3	33.3%
protein modification	9	1140	0.8%
protein complex assembly	3	160	1.9%
TCA intermediate metabolism	1	25	4.0%
alcohol metabolism	1	226	0.4%
negative regulation of cell growth	1	10	10.0%
morphogenesis	16	1210	1.3%
biogenic amine biosynthesis	1	26	3.8%
ribonucleotide metabolism	1	73	1.4%
cell death	9	550	1.6%
lipid biosynthesis	2	171	1.2%
water-soluble vitamin biosynthesis	1	6	16.7%
mesoderm development	2	31	6.5%
protein folding	2	181	1.1%
mitotic metaphase/anaphase transition	1	6	16.7%
regulation of circadian rhythm	1	3	33.3%
translation	1	183	0.5%
cholesterol metabolism	1	76	1.3%
steroid metabolism	3	153	2.0%
proton transport	3	86	3.5%
phosphorylation	8	648	1.2%
regulation of translational initiation	1	49	2.0%
regulation of translation	1	98	1.0%
regulation of circadian sleep/wake cycle	1	3	33.3%
activation of MAPKKK	1	6	16.7%
UDP-N-acetylglucosamine metabolism	1	4	25.0%
circadian sleep/wake cycle	1	3	33.3%
N-acetylglucosamine metabolism	1	6	16.7%
glucosamine metabolism	1	9	11.1%
amino sugar metabolism	1	15	6.7%
NF-kappaB-nucleus import	1	8	12.5%
negative regulation of NF-kappaB-nucleus import	1	7	14.3%
regulation of NF-kappaB-nucleus import	1	8	12.5%
muscle development	2	168	1.2%
perception of sound	2	102	2.0%
small molecule transport	2	66	3.0%
energy derivation by oxidation of organic compounds	1	158	0.6%
negative regulation of transcription, DNA-dependent	3	98	3.1%
phosphate metabolism	8	808	1.0%
death	9	557	1.6%
metabolism	81	7171	1.1%
phosphorus metabolism	8	808	1.0%
cell growth and/or maintenance	46	3939	1.2%
biological_process	139	12339	1.1%
mRNA processing	1	200	0.5%
response to abiotic stimulus	4	587	0.7%
cellular process	80	7049	1.1%
RNA processing	2	406	0.5%

nucleotide-sugar metabolism	1	12	8.3%
heart development	1	30	3.3%
perception of light	3	191	1.6%
epidermal differentiation	2	79	2.5%
perception of abiotic stimulus	4	326	1.2%
perception of external stimulus	4	354	1.1%
prostaglandin biosynthesis	1	11	9.1%
anion transport	1	116	0.9%
mitosis	2	170	1.2%
MAPKKK cascade	1	58	1.7%
glycosaminoglycan catabolism	1	5	20.0%
aminoglycan catabolism	1	5	20.0%
transcription from Pol III promoter	1	36	2.8%
response to pest/pathogen/parasite	1	543	0.2%
innate immune response	1	214	0.5%
aminoglycan metabolism	1	26	3.8%
response to wounding	1	323	0.3%
hydrogen transport	3	86	3.5%
vesicle docking	1	14	7.1%
calcium ion transport	3	97	3.1%
potassium ion transport	1	145	0.7%
regulation of neurotransmitter levels	1	45	2.2%
cation transport	6	454	1.3%
ion transport	8	625	1.3%
transport	22	1853	1.2%
skeletal development	2	171	1.2%
cytolysis	1	17	5.9%
translational initiation	1	79	1.3%
protein biosynthesis	3	565	0.5%
chromosome segregation	3	34	8.8%
response to biotic stimulus	12	1039	1.2%
response to external stimulus	15	1526	1.0%
fatty acid oxidation	1	24	4.2%
carboxylic acid transport	2	78	2.6%
nitrogen metabolism	1	42	2.4%
purine ribonucleoside monophosphate biosynthesis	1	13	7.7%
purine ribonucleoside monophosphate metabolism	1	13	7.7%
nicotinamide metabolism	1	15	6.7%
nucleotide biosynthesis	1	119	0.8%
oxygen and reactive oxygen species metabolism	2	61	3.3%
water-soluble vitamin metabolism	2	26	7.7%
vitamin metabolism	2	35	5.7%
ribonucleoside monophosphate metabolism	1	22	4.5%
mRNA-nucleus export	1	31	3.2%
RNA-nucleus export	1	38	2.6%
RNA localization	1	38	2.6%
cell cycle	12	872	1.4%
oncogenesis	1	164	0.6%

transcription from Pol II promoter	11	671	1.6%
vesicle-mediated transport	5	325	1.5%
negative regulation of protein-nucleus import	1	7	14.3%
regulation of protein-nucleus import	1	9	11.1%
glycosaminoglycan metabolism	1	25	4.0%
ribonucleoside monophosphate biosynthesis	1	22	4.5%
purine ribonucleotide biosynthesis	1	58	1.7%
protein catabolism	7	610	1.1%
coenzyme and prosthetic group biosynthesis	1	82	1.2%
purine ribonucleotide metabolism	1	63	1.6%
neurogenesis	8	492	1.6%
ectoderm development	2	95	2.1%
regulation of transcription from Pol III promoter	1	9	11.1%
regulation of transcription from Pol II promoter	9	360	2.5%
regulation of transcription, DNA-dependent	25	2120	1.2%
transcription, DNA-dependent	26	2262	1.1%
transcription	26	2313	1.1%
glycolipid catabolism	1	11	9.1%
nucleobase, nucleoside, nucleotide and nucleic acid transport	1	47	2.1%
cell differentiation	3	163	1.8%
regulation of transcription	25	2131	1.2%
peripheral nervous system development	1	12	8.3%
siderochrome transport	2	197	1.0%
brain development	1	34	2.9%
negative regulation of transcription from Pol II promoter	3	81	3.7%
secretory pathway	2	145	1.4%
pyridine nucleotide biosynthesis	1	2	50.0%
pyridine nucleotide metabolism	1	15	6.7%
cytokinesis	1	113	0.9%
male gamete generation	3	124	2.4%
sphingolipid catabolism	1	15	6.7%
chromatin modification	1	90	1.1%
central nervous system development	3	120	2.5%
oxidoreduction coenzyme metabolism	1	20	5.0%
Golgi vesicle transport	1	72	1.4%
coenzyme metabolism	1	100	1.0%
coenzyme and prosthetic group metabolism	1	120	0.8%
nucleosome assembly	1	79	1.3%
prostaglandin metabolism	1	23	4.3%
prostanoid metabolism	1	23	4.3%
carboxylic acid metabolism	5	394	1.3%
chromatin assembly/disassembly	2	125	1.6%
icosanoid metabolism	1	43	2.3%
histogenesis	4	129	3.1%
intracellular transport	6	597	1.0%
organogenesis	14	1090	1.3%
cytoplasmic sequestering of transcription factor	1	8	12.5%
secretion	2	52	3.8%

negative regulation of transcription factor-nucleus import	1	8	12.5%
transcription factor-nucleus import	1	9	11.1%
regulation of transcription factor-nucleus import	1	9	11.1%
purine nucleoside monophosphate biosynthesis	1	13	7.7%
purine nucleoside monophosphate metabolism	1	13	7.7%
nucleoside monophosphate biosynthesis	1	25	4.0%
nucleoside monophosphate metabolism	1	25	4.0%
membrane lipid biosynthesis	1	50	2.0%
membrane lipid catabolism	1	19	5.3%
negative regulation of cell cycle	2	82	2.4%
glycosphingolipid metabolism	1	25	4.0%
establishment and/or maintenance of chromatin architecture	2	209	1.0%
telomere binding	1	1	100.0%
DNA packaging	2	222	0.9%
chromosome organization and biogenesis (sensu Eukarya)	2	256	0.8%
regulation of apoptosis	1	275	0.4%
DNA repair	3	236	1.3%
nucleotide metabolism	1	159	0.6%
vitamin biosynthesis	1	6	16.7%
prostanoid biosynthesis	1	11	9.1%
icosanoid biosynthesis	1	26	3.8%
mitotic anaphase	1	23	4.3%
DNA recombination	1	86	1.2%
coenzyme biosynthesis	1	65	1.5%
M phase of mitotic cell cycle	2	171	1.2%
G2/M transition of mitotic cell cycle	2	69	2.9%
steroid catabolism	1	12	8.3%
glycoprotein biosynthesis	1	120	0.8%
S phase of mitotic cell cycle	3	180	1.7%
glycoprotein metabolism	1	134	0.7%
G1/S transition of mitotic cell cycle	1	83	1.2%
sphingolipid metabolism	1	44	2.3%
glycolipid metabolism	1	30	3.3%
regulation of behavior	1	3	33.3%
regulation of cellular process	5	439	1.1%
regulation of enzyme activity	1	80	1.3%
DNA dependent DNA replication	1	87	1.1%
DNA replication	2	177	1.1%
regulation of CDK activity	1	53	1.9%
organic acid transport	2	78	2.6%
DNA damage response, signal transduction resulting in cell cycle arrest	1	20	5.0%
cell cycle checkpoint	1	55	1.8%
macromolecule biosynthesis	6	885	0.7%
regulation of cell cycle	6	505	1.2%
biosynthesis	8	1042	0.8%
macromolecule catabolism	7	643	1.1%
catabolism	10	873	1.1%
mitotic chromosome segregation	1	15	6.7%

regulation of heart rate	1	34	2.9%
circulation	2	134	1.5%
steroi metabolism	1	82	1.2%
carbohydrate metabolism	3	372	0.8%
obsolete biological process	4	395	1.0%
GMP metabolism	1	3	33.3%
negative regulation of transcription	3	117	2.6%
regulation of biological process	7	516	1.4%
carboxylic acid biosynthesis	1	56	1.8%
DNA metabolism	7	636	1.1%
nicotinamide adenine dinucleotide metabolism	1	2	50.0%
negative regulation of nucleocytoplasmic transport	1	7	14.3%
DNA replication and chromosome cycle	5	226	2.2%
regulation of nucleocytoplasmic transport	1	9	11.1%
amine/polyamine transport	1	58	1.7%
glycolate metabolism	1	4	25.0%
coagulation	2	111	1.8%
phospholipid metabolism	1	75	1.3%
membrane lipid metabolism	2	123	1.6%
spermatid development	1	6	16.7%
spermatogenesis	3	124	2.4%
mRNA metabolism	1	212	0.5%
RNA metabolism	2	428	0.5%
protein transport	4	486	0.8%
carnitine metabolism	1	1	100.0%
nicotinamide adenine dinucleotide biosynthesis	1	2	50.0%
UDP-N-acetylglucosamine transport	1	2	50.0%
nuclear organization and biogenesis	2	263	0.8%
fatty acid beta-oxidation	1	22	4.5%
fatty acid biosynthesis	1	48	2.1%
circadian sleep/wake cycle, sleep	1	3	33.3%
pyrimidine nucleotide-sugar transport	1	5	20.0%
fatty acid metabolism	2	141	1.4%
nucleotide-sugar transport	1	5	20.0%
gametogenesis	3	155	1.9%
development	28	1956	1.4%
synaptic transmission, cholinergic	1	13	7.7%
nerve-nerve synaptic transmission	1	15	6.7%
sleep	1	4	25.0%
lipid metabolism	8	578	1.4%
carnitine biosynthesis	1	1	100.0%
neurotransmitter secretion	1	23	4.3%
synaptic transmission	6	281	2.1%
cell-cell signaling	9	665	1.4%
RAS protein signal transduction	2	50	4.0%
small GTPase mediated signal transduction	4	275	1.5%
Wnt receptor signaling pathway	1	39	2.6%
organic acid biosynthesis	1	56	1.8%

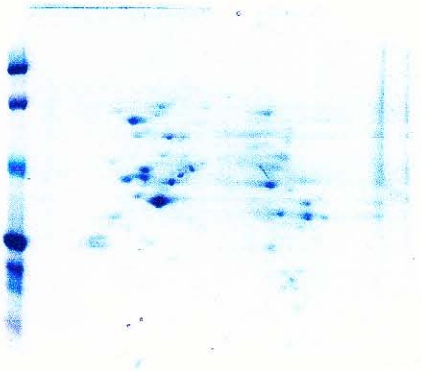
carbohydrate catabolism	1	67	1.5%
response to light	3	198	1.5%
regulation of cell proliferation	4	366	1.1%
response to oxidative stress	2	44	4.5%
response to DNA damage stimulus	3	274	1.1%
cell growth	1	96	1.0%
activation of JUNK	2	12	16.7%
growth	1	33	3.0%
biogenic amine metabolism	1	50	2.0%
amino acid derivative metabolism	1	59	1.7%
JNK cascade	3	45	6.7%
cytoplasmic sequestering of NF-kappaB	1	7	14.3%
cell organization and biogenesis	2	632	0.3%
lipid catabolism	2	81	2.5%
activation of NF-kappaB-inducing kinase	1	12	8.3%
GMP biosynthesis	1	3	33.3%
protein-nucleus import	1	90	1.1%
protein targeting	2	173	1.2%
NIK-I-kappaB/NF-kappaB cascade	3	28	10.7%
negative regulation of cell proliferation	1	201	0.5%
positive regulation of cell proliferation	3	165	1.8%
cell proliferation	18	1341	1.3%
reproduction	3	182	1.6%
protein kinase cascade	6	197	3.0%
intracellular signaling cascade	13	1102	1.2%
transmission of nerve impulse	6	287	2.1%
purine nucleotide biosynthesis	1	62	1.6%
purine nucleotide metabolism	1	70	1.4%
immune response	9	903	1.0%
inflammatory response	1	211	0.5%
defense response	10	963	1.0%
hemostasis	3	120	2.5%
feeding behavior	1	24	4.2%
metal ion transport	4	351	1.1%
response to stress	10	972	1.0%
blood coagulation	2	111	1.8%
adenylate cyclase activation	1	38	2.6%

Figure 18: 2D separation of nuclear proteins from cells treated with GRO, CRO or left untreated.

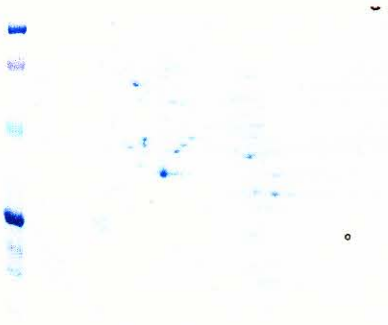
Untreated

GRO treatment after 4 hours

GRO treatment after 24 hours



CRO treatment after 4



CRO treatment after 24 hours

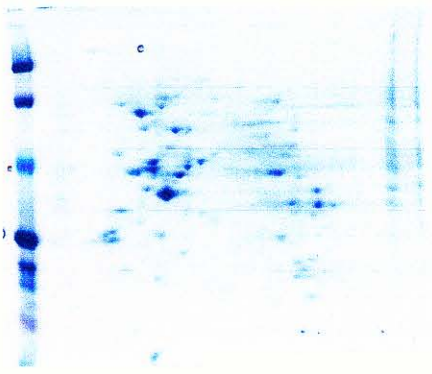
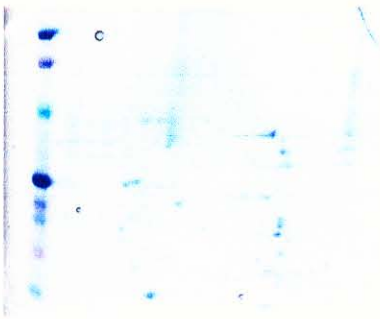


Figure 19: 2D separation of cytoplasmic proteins from cells treated with GRO, CRO or left untreated.

Untreated

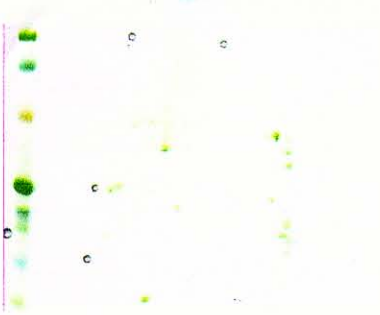
GRO treatment after 4 hours

GRO treatment after 24 hours



CRO treatment after 4 hours

CRO treatment after 24 hours



CHAPTER VI

CLOSING REMARKS

Oligonucleotide therapeutics are an emerging class of drugs that are gaining interest due to their apparent high target specificity and few toxic side effects. Therapy utilizing oligonucleotides is now being developed as treatment in a variety of diseases including cancer, genetic disorders, coronary disease, and HIV [1-4]. The strategies used by oligonucleotides include triplex formation (which targets DNA), antisense (which targets RNA), siRNA (which targets RNA), and aptamer (which involve target proteins and shape recognition) [5-13].

An oligonucleotide therapy thought to function using the aptamer mechanism is guanine-rich oligonucleotides (GROs). GROs are now being investigated as a new therapeutic against cancer, viral diseases, and other illnesses. GROs are capable of forming the secondary structure known as a G-quartet. G-quartets contain four coplanar guanines stabilized by Hoogsteen hydrogen bonds. These G-quartets are capable of stacking upon one another which increases the stability of the structure. Sequences capable of forming G-quartets have been identified throughout nature including the *c-myc* promoter, the fragile X repeat, and HIV1 RNA [14-19, 22-28, 30-33, 149].

GROs exhibit potent antiproliferative properties against several cancer cell lines [34-37], and demonstrated the same anticancer effects in an *in vivo* tumor mouse model (Figure 6D). Phase I clinical trials have also demonstrated that GROs could be a promising clinical agent against cancer [38]. The antiproliferative activity of GROs apparently has a novel mechanism of action, although some aspects of its function have previously been shown. Upon treatment with GRO, cancer cells undergo S phase cell cycle arrest, DNA replication inhibition, and cell death [35]. It has also been shown that the activity of GROs requires the formation of G-quartets, but the secondary structure alone cannot predict the antiproliferative activity of GRO. The only predictive factor was the GROs' ability to bind to complex of proteins, one of which has been identified as nucleolin which has been identified as the primary target of GROs [34, 37]. GROs could exhibit their antiproliferative effects by either modifying the function of nucleolin directly or GROs could bind to nucleolin on the surface of the cell which shuttles the GRO inside the cell where the functions of proteins other than nucleolin are affected.

To gain insight into the mechanism of GROs, we first identified the proteins that bind to an active GRO by utilizing biotynlated oligonucleotides for precipitation and mass spectrometry for the identification of the bound proteins. In Chapter II, I identified 14 nuclear proteins and 3 cytoplasmic proteins that are able to bind to an active GRO but not able to bind to an inactive GRO (see Table 2 for complete list). Nucleolin was identified as a GRO-binding protein, validating the technique. The functions of the additional proteins are diverse; however, several of their functions correlate with functions of nucleolin. These include apoptosis, ribosome assembly, DNA repair, rRNA maturation,

and nucleocytoplasmic transport. Furthermore, several identified proteins have previously been shown to have the ability to bind to nucleolin. This data supports that nucleolin is the primary target of GROs, and an important aspect of the mechanism of GROs is modifying the function of nucleolin.

One important GRO-binding protein identified in Chapter II is NEMO (NF-κB essential modulator). I chose to further investigate the possible role of NEMO in the mechanism of GRO antiproliferative activity because of its important role in NF-κB signaling. NF-κB is often deregulated in cancer cells [135, 136], and it was previously reported that NF-κB transcription is inhibited by polyguanine oligonucelotides [137]. As seen in Chapter III, I report here that GROs do inhibit NF-κB transcription. I show that GROs bind to NEMO, inhibit the activation of the IKK complex, and inhibit the phoshphorylation of IκBα. I also show that when cells are treated with the GRO, NEMO and nucleolin co-precipitate. Because nucleolin is the primary target of GROs, the co-precipiation of the nucleolin-NEMO complex suggests nucleolin may play a role in NF-κB transcription (which is further investigated in Chapter IV).

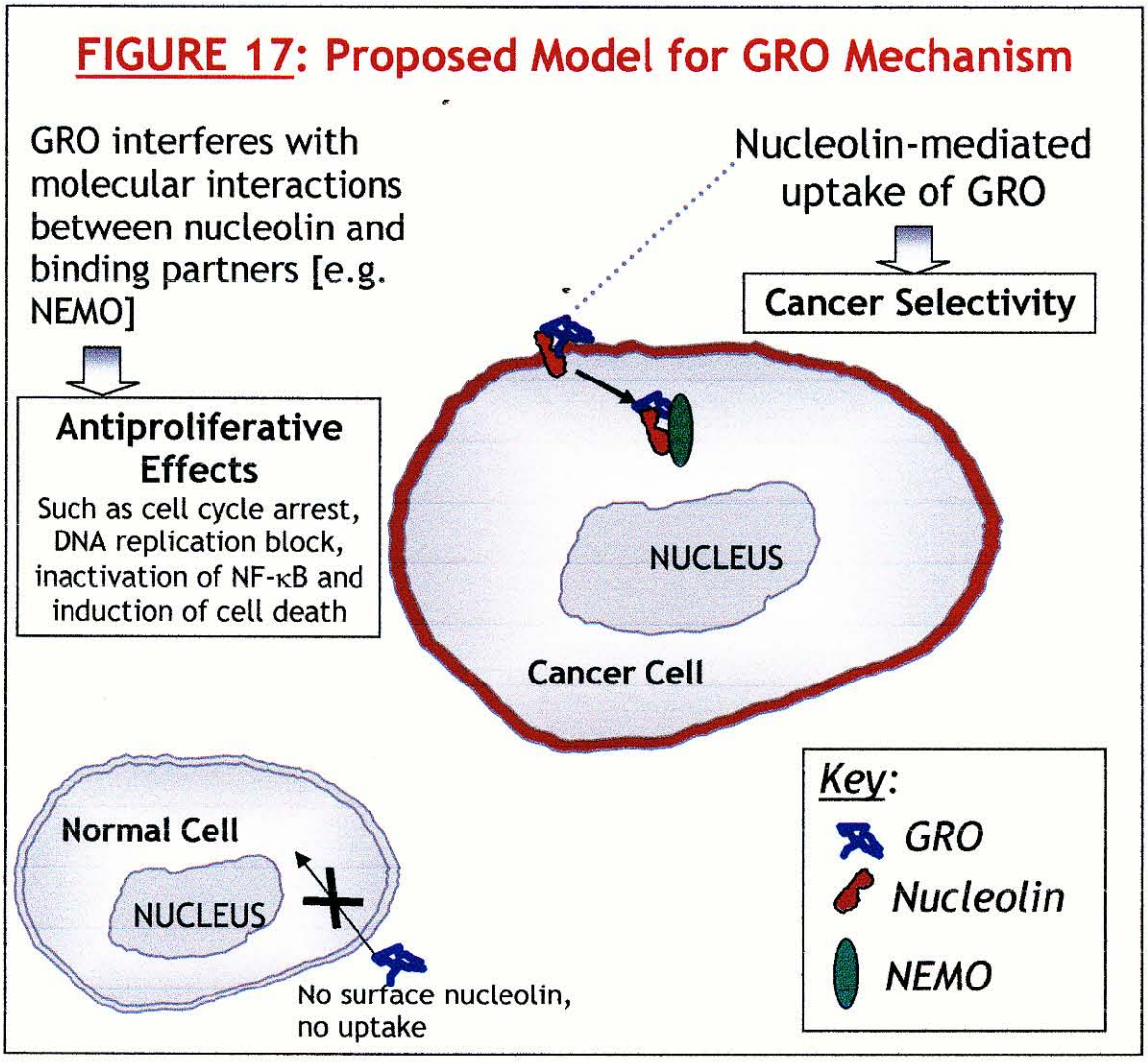
For further insight into the mechanism of GRO antiproliferative activity, we assessed the global gene and protein expression in cells treated with an active GRO compared to untreated cells and cells treated with an inactive GRO. This was achieved by utilizing recent advances in global expression technology with microarray and 2D electrophoresis; the results are reported in Chapter V. Although the global protein expression analysis did not reveal any changes (see Figures 18 and 19), the global gene

expression data revealed the alteration of many genes when a cell is treated with the GRO, as seen in Tables 3 and 4. The analysis revealed 63 genes changed after 2 h of treatment with the GRO, and 238 genes changed after 18 h of treatment. The expression of nucleolin was not changed, suggesting that GROs do not apparently affect the transcription of nucleolin. The GRO-altered genes are involved in several different cellular processes (see Tables 5 and 6) including transcription, transport, apoptosis, cell motility, and regulation of cell growth. Interestingly, some of the same cellular functions that are affected by GROs have been implicated in the function of nucleolin, which is consistent with our hypothesis that GROs exert their activity by modifying the function of nucleolin.

The data presented in this dissertation is consistent with our functional model which is represented by the schematic in Figure 20. Nucleolin that is present on the surface of cancer cells binds to the GRO which mediates its uptake into the cell. Upon internalization, the GRO interferes with molecular interactions of nucleolin and other proteins in the nucleus, cytoplasm, and plasma membrane. The alteration of these nucleolin-protein interactions leads to antiproliferative effects including cell cycle arrest, inactivation of NF-κB, and induction of cell death. The selectivity of GRO comes from the presence of nucleolin on the surface of cancer cells. Nucleolin is absent on the surface of normal cells, thus preventing the uptake of GRO and leaving the cell unaffected.

Figure 20: Proposed model for GRO mechanism.

FIGURE 17: Proposed Model for GRO Mechanism



In conclusion, more work is needed to decipher the precise molecular mechanism of the antiproliferative activity of GROs. However, in the present study, important new insights into the mechanism have been revealed. All of the data presented here supports the hypothesis that GROs bind primarily to nucleolin, and modulate the normal function of nucleolin. Although the detailed mechanism of GROs was not elucidated in this study, there are several aspects of its mechanism reported here that should be further investigated.

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APPENDIX I

ABBREVATIONS

GRO(s)	guanosine-rich oligonucletide(s)
CRO(s)	cytosine-rich oliognucleotide(s)
NF-κB	nuclear factor kappa B
NEMO	NF-κB essential modulator
IKK	IκB kinase
TNFα	tumor necrosis factor alpha
AgNORs	silver-staining nucleolar organizer regions
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
EMSA	electrophoretic mobility shift assay
DMEM	Dulbecco's modified Eagle's Medium
PBS	phosphate buffered saline
PVDF	polyvinylidene difluoride
SDS	sodium dodecyl sulfate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
EDTA	ethylenediaminetetraacetic acid
PMSF	phenylmethylsulfonyl fluoride

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