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14. ABSTRACT

The focus of this research is to discern the structural and biophysical features of small deoxyoligonucleotides that have significant biological properties including the inducement of Toll-like receptor 9 (TLR9) in the mediation of cellular invasion. Cell invasion (metastasis) is a significant problem in the control and treatment of breast cancer. Recent research from our laboratory has demonstrated enhanced cellular invasion in the MDA-MB-231 breast cancer cells by ODN-M362, a 25-base single-stranded CpG-containing deoxyoligonucleotide. Although the mechanism(s) for this induction is unknown, our studies reveal key insights into the structural and sequence requirements for DNA activation of this cellular invasion process. The deoxyoligonucleotides that are most effective in eliciting an invasion response have been shown to adopt stable structural motifs including stem-loops or hairpins or G-quadruplex structures. Sequence modifications have been designed to probe base sequence, structure, and stabilities that are required for initiating TLR-9 mediated cellular invasion. Our results demonstrate that these small deoxyoligonucleotides and the stability of their secondary structures play a pivotal role in eliciting the TLR9-induced invasion process.

15. SUBJECT TERMS
Metastasis, TLR9, deoxyoligonucleotide, hairpin, quadruplex

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Introduction:

The focus of this research is to correlate the influence of secondary structure and stability of nucleic acids (particularly short deoxyoligonucleotides) on their ability to induce toll-like receptor 9 (TLR9) mediated cancer cell invasion. Cell invasion (metastasis) is a significant problem in the control and treatment of breast cancer. Our laboratory has demonstrated enhanced cellular invasion in the MDA-MB-231 breast cancer cells by CpG rich deoxyoligonucleotides such as ODN-M362, a 25-base single-stranded CpG-and known agonist for TLR9. Although the mechanism(s) for this induction is unknown, our studies reveal key insights into the structural and sequence requirements for DNA activation of this cellular invasion process. More importantly, recent studies from our laboratory have demonstrated that DNA fragments isolated from breast apoptotic cancer cells (treated with doxorubicin) were effective in eliciting TLR9-mediated cancer cell invasion. Our most recent studies indicate that the deoxyoligonucleotides that are most effective in eliciting an invasion response have been shown to adopt stable structural motifs including stem-loops or hairpins or G-quadruplex structures. Sequence modifications have been designed to probe base sequence, structure, and stabilities that are required for initiating TLR-9 mediated cellular invasion. Our results demonstrate that these small deoxyoligonucleotides and the stability of their secondary structures play a pivotal role in eliciting the TLR9-induced invasion process.

BODY

Statement of Work (Aim 3 – Graves) Characterization of the base sequence, secondary structure, and stabilities required for deoxyoligonucleotides for the induction of TLR9-mediated cellular invasion

Rationale: CpG deoxyoligonucleotides such as ODN-M362 has been known to exert significant cellular responses since reported by Krieg in 1995 (Nature 374, 546-549). In 2001, Bauer and coworkers reported the linkage between ODN-M362 and hTLR9 through activation of a response to bacterial DNA in the cells innate immune response. In 2005, apoptotic DNA was demonstrated to exert similar hTLR9 activation of cellular invasion. Of fundamental interest to our research is the nature of the deoxyoligonucleotide-hTLR9 interaction. Aim 3 will utilize a biophysical approach to directly measure these interactions with respect to synthetic DNAs designed to examine base sequence, secondary structure, and structural stabilities in their interactions with TLR9. High resolution NMR will be used to characterize the structural properties of many of these DNAs (hairpins) as well as their structural stabilities (along with DSC). Surface Plasmon Resonance (BiaCore) will be used in conjunction with ITC studies to determine the binding properties associated with hTLR9 constructs with both synthetic and native DNAs. These biophysical studies will be directly correlated with cell-invasion assays to determine the linkage between DNA properties and their abilities to modulate TLR9 induced cellular invasion.

Aim # 3: Probing the Role of DNA Sequence, Structure and Stability in TLR9 Meditated Induction of Cellular Invasion.

The mechanism(s) through which deoxyoligonucleotides exert influence in TLR9 mediated biological responses remains unknown. The CpG deoxyoligonucleotide (ODN-M362) has long been demonstrated to influence TLR9-mediated biological activites; however, recent studies from this laboratory have shown that ODN-M362 to be highly influential in stimulating TLR9-mediated cellular invasion. Our laboratory recently demonstrated comparable cellular invasion using vertebrate DNA obtained from apoptotic cancer cells. Hence, the initial characterizatio of ODN-M362 as a TLR9 ligand may need to be expanded to more general terms of sequence, DNA secondary structure, and/or stabilities. A major focus of our research is to correlate these structural and biophysical properties of short nucleic acids with their effectiveness in modulating TLR9 mediated cellular activities. A better understanding of the nature of the interaction(s) between TLR9 and short deoxyoligonucleotides may provide key insights into strategies for circumventing activation of TLR9-mediated invasion processes.

Approach: The studies proposed in AIM 3 will focus on examination and characterization of base sequence, secondary structural features, and stabilities of synthetic deoxyoligonucleotides. These deoxyoligonucleotides will be designed to probe effects of changes in base sequence (CpG versus non-CpG), secondary structural properties (single-stranded, hairpin, duplex) and stabilities of concomitant secondary structures on interactions with TLR9, activation of TLR9-induced cellular invasion, and nuclease susceptibility. Our multifaceted approach will encompass a myriad of biophysical approaches including surface plasmon resonance (SPR),

isothermal titration calorimetry (ITC), and differential scanning calorimetry (DSC) to gain insights into whether deoxyoligonucleotides of different sequences and/or structural motifs exhibit altered binding to TLR9. These studies will parallel cellular invasion assays to determine correlations between TLR9 activation by deoxyoligonucleotides with their induction of cellular invasion. From these studies using synthetic deoxyoligonucleotide systems we will expand our efforts to include apoptotic DNAs derived from breast cancer cells.

Task 3.1. Purification of TLR9. We have developed an inducible expression system for the portion of the extracellular domain of hTLR9 in HEK cells. This domain has a FLAG-his tag on the C-terminus portion. The hTLR9 will be purified by AKTA-FPLC System (GE Healthcare) with the final purification using a nikel column.

Task 3.2. Design and Characterization of Selected deoxyoligonucleotides to probe sequence, secondary structures, and stabilities as TLR9-mediating binding ligands. Deoxyoligonucleotides will be designed to determine the role of base sequence, structural, and stabilities as binding ligands for TLR9. Preliminary studies have demonstrated that the parent ODN-M362 (25-mer) can be reduced in size to a blunt-end hairpin (16-mer) with no loss in TLR9-mediated induction of cell-invasion. Further studies are underway to discern the structural and thermodynamic nature of the deoxynucleotide(s) and to determine the lower-limit in size that is needed for TLR9-activation. DSC and NMR (Bruker Avance II-700 with Cryoprobe) are used to characterize the secondary structures and stabilities associated with the deoxyoligonucleotides. Recent studies in our laboratory reveal secondary structure to play a critical role in TLR9 activiation; however, this may be a secondary response due to the nuclease resistance imposed by secondary structural features, allowing the deoxyoligonucleotide to reach its intracellular target.

Task 3.3. SPR and ITC studies to determine whether DNA sequence, structure, or stability modulates TLR9 interactions. Current studies in our laboratory examining the role of deoxyoligonucleotides in inducing TLR9-mediated cellular invasion demonstrate a clear correlation; however, direct evidence of this interaction is required. With the ability to overexpress hTLR9 in inducible HEK cells, we know propose to directly examine the interactions of various DNAs (both demonstrated TLR9-ligands such as ODN-M362 as well as deoxyoligonucleotides that show no activity in the cellular invasion assay). From these studies, we hope to discern the structural and biophysical nature of the deoxyoligonucleotide-TLR9 interaction and gain insights as to how this interaction influences TLR9 mediated cellular invasion.

Progress to Date (July 15, 2010 – July 14, 2011)

Task 3.1. Purification of TLR9. Rather than express and purify TLR9 in-house, recombinant hTLR9 is now available for purchase from BioClone, Inc., San Diego, CA (http://www.bioclone.us/CD-Marker-cDNA-recombinant-protein-3.html) (PVP-0222, 100 micrograms @ $1,995). This company provides human TLR9 (a.a. 26-818) that is expressed as a recombinant protein produced from Sf9 insect cell line. The protein is pure and has been demonstrated quite stable for our biophysical studies which probe the preferential binding properties of selected DNA structural motifs and sequences.

Task 3.2. Design and characterization of selected deoxyoligonucleotides to probe sequence, secondary structures, and stabilities as TLR9-mediating binding ligands. CpG rich deoxyoligonucleotides such as ODN-M362 have been known to exert significant cellular responses since reported by Krieg in 1995 (Nature 374, 546-549). (1) In 2001, Bauer and coworkers reported the linkage between ODN-M362 and hTLR9 through activation of a response to bacterial DNA in the cells innate immune response. In 2005, apoptotic DNA was demonstrated to exert similar hTLR9 activation of cellular invasion.(2) Of fundamental interest to our research is the nature of the deoxyoligonucleotide-hTLR9 interaction. Aim 3 will utilize a biophysical approach to directly measure these interactions with respect to synthetic DNAs designed to examine base sequence, secondary structure, and structural stabilities in their interactions with TLR9. High resolution NMR will be used to characterize the structural properties of many of these DNAs (hairpins) as well as their structural stabilities (along with DSC). Surface Plasmon Resonance (BiaCore) will be used in conjunction with ITC studies to determine the binding properties associated with hTLR9 constructs with both synthetic and native DNAs. These biophysical studies will be directly correlated with cell-invasion assays to determine the linkage between DNA properties and their abilities to modulate TLR9 induced cellular invasion.

In our work over the past year, we have focused our investigations on the role of stable secondary structures of deoxyoligonucleotides on their ability to induce toll-like receptor 9 (TLR9) mediated cancer cell invasion.

Earlier studies from our laboratory *(Ilvesaro, et al (2008) "TLR9 mediates Cp G-Oligonucleotide-Induced Cellular Invasion" Molecular Cancer Research 6, 1534-1543)* have demonstrated small deoxyoligonucleotides to have significant role in breast cancer metastasis (cell invasion) and may play an important role in the control and treatment of breast cancer (3). TLR9 is a mediator of the innate immune system that recognizes both microbial and vertebrate DNAs. Members of the Toll-like receptor (TLR) family contain leucine rich repeat domains in the extracellular portion and an intracellular TIR (Toll-1L-1R) domain. There are at least eleven members in the TLR family that can be divided into five subfamilies, one of which is TLR9 (including TLRs 7, 8 and 9). These proteins recognize pathogen derived RNAs and DNAs. Non-specific endocytosis of ODNs is required to activate antigen-presenting cells (APCs). Members of the TLR9 subfamily are expressed intracellularly in the endosomal-lysosomal compartment as opposed to other subfamilies that are bound to the cell surface. After ligand binding, TLRs and their adapter proteins, such as MyD88 and TRAM, recruit intracellular signaling mediators, which in turn activate transcription factors, such as nuclear factor-κB (NF-κB. (4) TLR activation results in an immune reaction, which is characterized by increased production of inflammatory mediators, such as cytokines and interleukins.(5) Furthermore, the association of ODNs with TLR9 has been shown to promote tumor progression in human breast cancer and lung cancer cells (Ilvesaro, 2008; Ren, 2009).(3, 6).

Over the past decade, there have been a number of studies conducted to examine the effects of base sequence on the binding of ODNs to TLR9. It is commonly accepted in the literature that the ODN sequence must contain CpG nucleotides in order to bind to and activate TLR9. Rutz, et al, have demonstrated binding of CpG ODNs to TLR9 via surface plasmon resonance (SPR) biosensor technology. (7) A sequence-specific recognition of and response to a specific CpG containing ODNs by TLR9 has been reported in many studies. (2) The ODN known as M362 is a 25-base long sequence that has been well characterized as a TLR9 agonist.(8,9).

Our hypothesis is that the activation of TLR9 is not only dependent on a specific CpG containing sequence but is manifested on the stability of the secondary structure of the deoxyoligonucleotide. As shown in Figure 1, the classic TLR9 agonist, ODN M362, is a 25-mer rich in CpG sequences and contains a central 16-base sequence (shown in red) that is self-complementary. This region of complementarity enables the deoxyoligonucleotide to adopt several stable secondary structures including a hairpin with dangling ends (four base overhang on the 5' end and five base overhang on the 3' end) as well as a bimolecular duplex (with dangling ends). We have performed a series of sequence modifications to correlate whether particular secondary structures and/or stabilities of the deoxyoligonucleotides are required to activate TLR9 induced cancer cell invasion.

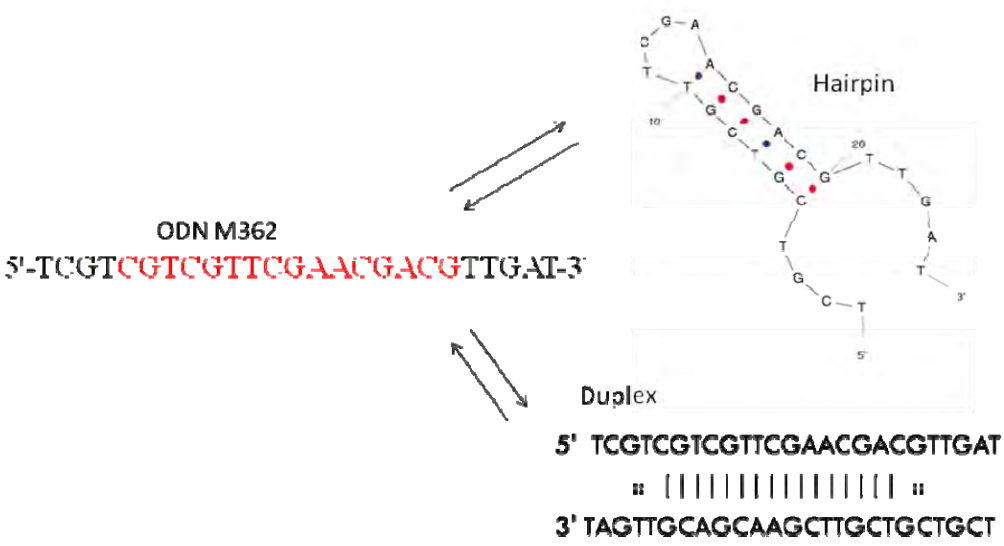


Figure 1. The 25-mer deoxyoligonucleotide ODN M362 exists in equilibrium between a duplex, single strand, and hairpin structure due to the internal sixteen self-complementary bases (shown in red).

With ODN M362 as our primary positive control TLR9 agonist, numerous additional deoxyoligonucleotides have been designed and used to probe TLR9-mediated cancer cell invasion. Specifically, nucleotide

sequences that result in stable secondary structures such as hairpins, duplexes, and G-quadruplexes have been examined. A key linkage that we have discovered is that stable secondary structures resulting in nuclease resistance are more effective at inducing TLR9 mediated cellular invasion. An additional focus of our research is to determine how the deoxyoligonucleotides are brought into the cancer cell and specifically to TLR9 in the endosomal compartment. LL-37, an antimicrobial peptide has been shown to associate with self-DNA and interact with TLR9 in plasmacytoid dendritic cells (10) and to enhance the immunostimulatory effects of CpG-ODNs against ovarian cancer (11). We are investigating whether LL-37 differentially forms complexes with deoxyoligonucleotides having well-defined secondary structures such as single-strand, duplex, hairpin, or G-quadruplexes.

Analysis of secondary structures and stabilities of deoxyoligonucleotides: The molecular modeling programs MFOLD and Discovery Studio (Accelrys) were used to predict structural features and stabilities of deoxyoligonucleotides that were shown to exert biological activity, including the induction of TLR9 mediated cellular invasion. This software is used to predict minimal energy structures of DNA and RNA oligonucleotides based on the base sequence, propensity for forming base pairs, base stacking, mismatches, and dangling ends. Using MFOLD, the most stable secondary structures for these oligodeoxynucleotides were determined and probed for biophysical stabilities of base pairing patterns within stems, loop structural and sequence features, and base pair mismatches within the hairpin stems. Results obtained from the secondary structure prediction allowed us to use rational design to incorporate subsequent changes into the base sequence to probe the effects of stem stability, loop sequence and size, and base pair mismatches within the stem on influencing the biological activity of these ODNs in the cell invasion. Accelrys Discovery Studio 3.1 was used to model DNA secondary structures and to evaluate their energetic stabilities.

Table 1. Sequence variations of ODN M362 and other deoxyoligonucleotides.

Name	Length	Sequence
ODN M362	25	5'-TCGTCGTCGTTCGAACGACGTTGAT-3'
Truncated	16	5'-CGTCGTTCGAACGACG-3'
5' end	20	5'-TCGTCGTCGTTCGAACGACG-3'
3' end	21	5'-CGTCGTTCGAACGACGTTGAT-3'
Trunc + T	17	5'-CGTCGTTCTGAACGACG-3'
Trunc + TT	18	5'-CGTCGTTCTTGAACGACG-3'
9mer Hairpin	9	5'-CGCGAAGCG-3'
16mer purines	16	5'-CGTCGTGAAAACGACG-3'
h-Tel22	22	5’-TGGGTTAGGGTTAGGGTTAGGG-3’

Cellular Invasion Assays: MDA-MB-231 breast cancer cells were plated onto Matrigel matrices at a cell density of 1 x 10^4 cells per well in 500 µL of culture medium. Oligonucleotide treatments containing a phosphorothioate backbone (PS) were added at a concentration of 5 µM. When noted, the oligonucleotides were left unmodified with a phosphodiester backbone (PD). A vehicle treatment of TE buffer was used as the negative control. The cells were allowed to invade for 22 hours after which the inserts were removed and stained with Hema 3 Stain set according to manufacturer recommendations. The number of invaded cells was counted microscopically at five preselected fields using a 40X objective. The results are given as mean ± sd, unless otherwise stated. Student’s t test was used to calculate statistically significant differences between the various study groups.

DSC Studies: DSC experiments were performed with a Microcal VP-DSC from 10 °C to 90 °C at a heating rate of 0.5 °C/min against the appropriate buffer. All samples were prepared to 100 µM (strand) and degassed prior to use. At least five scans of buffer scans were run to acquire an adequate baseline, followed by a minimum of five deoxyoligonucleotide melts.

Circular Dichroism Studies: CD experiments were performed on an Aviv 400 CD spectropolarimeter using a 1 cm pathlength cell at 25 °C. Samples were prepared to 6 μM in 0.01 M sodium phosphate, 0.001 M disodium EDTA, and 100 mM NaCl (BPES) buffer at the designated pH. Data were collected from 215 to 320 nm at every 1 nm with a bandwidth of 1 nm. Time course experiments were monitored at 250 nm over 30 minutes. Spectra were corrected for buffer contributions, and the data were normalized to molar ellipticity ($deg \cdot cm^{2} \cdot decimol^{-2}$).

Isothermal Titration Calorimetry Studies: ITC experiments were performed with a Microcal VP-ITC at 25 °C in Na-BPES buffer (0.01 M NaH_2PO_4, 0.01 M Na_2HPO_4, 0.001 M EDTA, 0.1 M NaCl) at pH 7. All samples were degassed prior to use. The sample cell was filled to capacity (~1.6 mL) with LL-37 (10 μM) and ODN (100 μM) was injected in 25-40 aliquots of 2 μL each with 300 seconds resting time between injections. The resulting data were integrated and are shown with the measured heats of injections.

Results: In order to discern the structural characteristics of the various deoxyoligonucleotides that are necessary to induce invasion in breast cancer cells, several sequence variations on the parent ODN M362 and other deoxyoligonucleotides were performed as shown in Table I. As shown in Figure 1, ODN M362 may exist in equilibrium as a single-stranded DNA, as a duplex with sixteen base pairs centrally located, and as a hairpin structure with six base pairs in the stem and four bases within the loop (all structures are shown in Figure 1). The sequence modifications are summarized in Table 1.

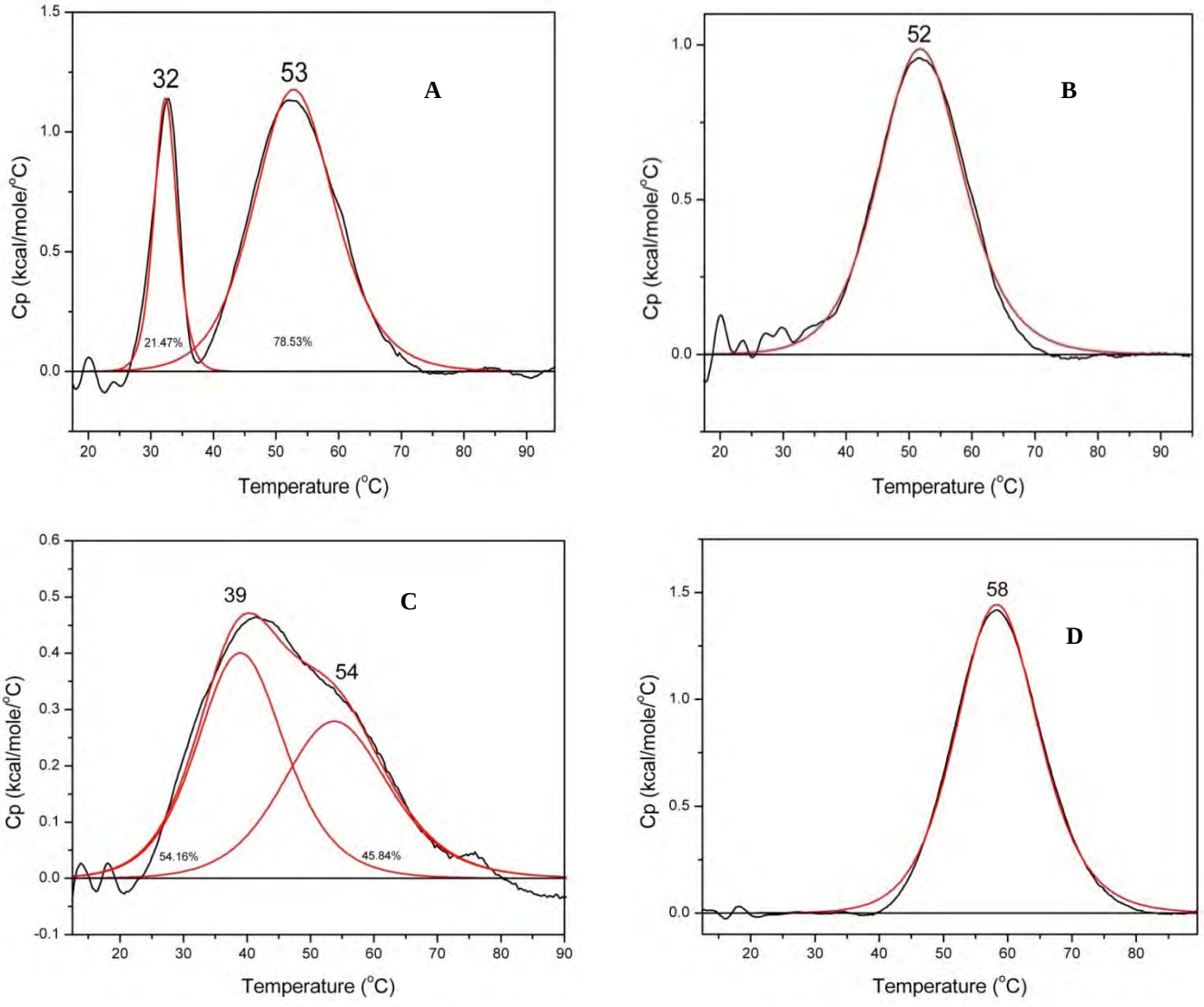


Figure 2. DSC melting profiles of 100 μM truncated 16mer (PS) (A: TE buffer, 1st melt; B: TE buffer, successive melts; C: 100 mM NaCl BPES buffer) and D: the 17mer (PS) (Trunc+T, 100 mM NaCl BPES buffer).

The base sequence of the parent ODN M362 (25-mer) was truncated to an entirely self-complementary 16-mer ODN. This 16-mer can adopt both a hairpin and a duplex structure, as predicted by MFOLD. The sequence was further modified by introducing additional bases – one thymine (+T) and two thymines (+TT) – into the loop of the hairpin structure. The addition of the thymine bases into the loop pushes the equilibrium of the DNA structure to a hairpin, which is more likely to be the dominant species than a duplex with unpaired bases in the center of the sequence.

This shift in equilibrium is demonstrated by DSC melting data. The first melt of the truncated 16-mer in the absence of salt revealed two species in solution, with melting temperatures (T_m) of 32 °C and 53 °C (Figure 2A). This is indicative of two structures of the self-complementary ODN in solution, which supports the theory of equilibrium between a duplex and hairpin. The less stable structure in the absence of salt is the duplex, and the more stable is the hairpin. After the sample was cooled and melted again, the DSC profile revealed one melting transition at a T_m of approximately 52 °C (Figure 2B). This corresponds to the annealing of the DNA into its most stable form, the hairpin structure. However, in the presence of salt, the duplex structure is stabilized more than the hairpin. The DSC melting profile of the truncated 16-mer in 100 mM NaCl indicated the presence of two structures, with T_m's of 39 °C and 54°C (Figure 2C).

The presence of salt stabilized the duplex structure by approximately seven degrees, and thereby altered the position of equilibrium between duplex and hairpin. Addition of T nucleotides bases into the loop of the hairpin structure (T = 17-mer and TT = 18-mer), in order to push the equilibrium to the hairpin. The DSC melting profile of the 17-mer, containing an additional T in the loop of the hairpin, revealed that this modification does favor the hairpin structure. The 17-mer in 100 mM NaCl melted in a single transition, with a T_m of approximately 58°C (Figure 2D). Even in the presence of salt, the 17-mer does not adopt a stable duplex structure. The hairpin structure is further stabilized for the 18-mer, with two additional T's in the loop. This is evidenced by the increase in melting temperatures determined by DSC, as summarized in Table 2.

Table 2. The melting temperature (T_m) of DNA hairpin structures in 100 mM NaCl BPES as determined by DSC.

Sequence	T_m (°C)
16mer (PS)	54.8 ± 0.53
17mer (PS)	58.15 ± 0.028
18mer (PS)	60.58 ± 0.038

Characterization of DNA structure by CD spectroscopy. The equilibrium between duplex and hairpin of the truncated 16-mer was further evaluated by CD spectrophotometry. The change in the CD spectrum of the 16-mer at pH 7 in the presence of 100 mM NaCl was monitored over an increase in temperature. The shift in the spectrum with increasing temperature resulted in two isoelliptical points (Figure 3). This is indicative of two species in solution, presumably the hairpin and the duplex. However, because TLR9 is expressed in the endosomal and lysosomal compartments, the interactions between TLR9 and the ODNs may take place in an acidic environment.

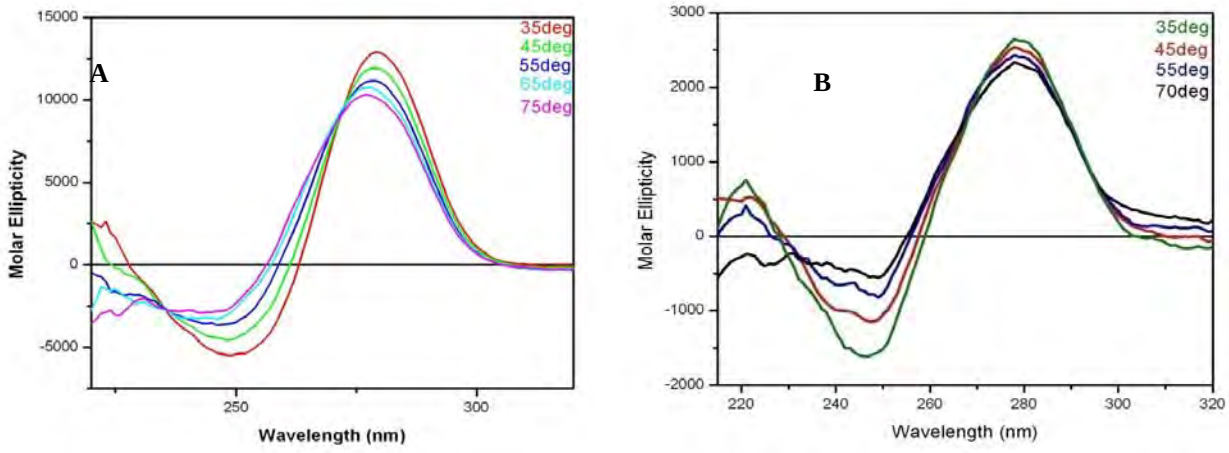
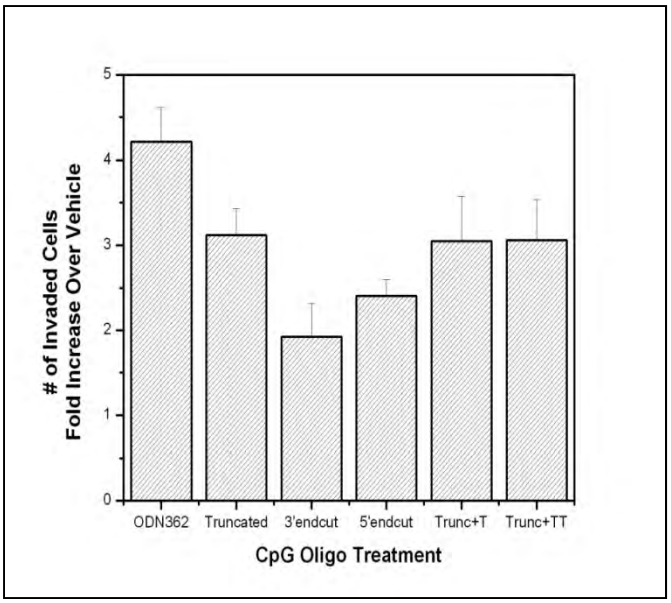


Figure 3. CD spectra of the truncated 16mer (PS) in 100mM NaCl BPES buffer at (**A**) pH 7 and (**B**) pH 5.

Therefore, the CD spectrum of the 16-mer at pH 5 was monitored over an increase in temperature. At low pH, there was no shift in the spectrum and no isoelliptical points were observed. This demonstrates that at pH 5, the equilibrium between the two structures is shifted predominantly to one. Because hairpin structures are favored at lower pH values, it is likely that the hairpin structure dominates at lower pH, even in the presence of salt.



Correlation of DNA structure and stabilit y with TLR9-mediated cancer cell in vasion. Invasion assays were performed to evaluate the ability of each ODN to induce invasion in MDA-MB-231 breast cancer cells. The resulting fold increase in invasion over a buffer vehicle for each sequence is shown in Figure 4. The 16-mer induced invasion at a level comparable to that of the parent ODN M362. It has been demonstrated that the 16-mer exists in equilibrium between a duplex and hairpin form. However, the 17-mer (Truncated + T) and 18-mer (Truncated + TT) are more likely to adopt hairpin structures.

Figure 4. The effects of various deoxyoligonucleotides (5μM) on invasion were studied in invasion assays *in vitro* using MDA-MB-231 cells. The results are expressed as the normalized fold increase in invasion over vehicle. Columns: mean (n = 8) ± SD.

These sequences also induced invasion comparable to the parent 25-mer. Therefore, the 16-mer and the 25-mer may also adopt hairpin structures in order to produce the invasive response. In order to perform the invasion assays, the ODNs were modified to a phosphorothioate (P=S) backbone, which contains phosphate-sulfur double bonds in place of the native phosphate-oxygen double bonds in phosphodiester (P=D) backbone. This modification was implemented to make the deoxyoligonucleotides resistant to nuclease digestion.(12,13)

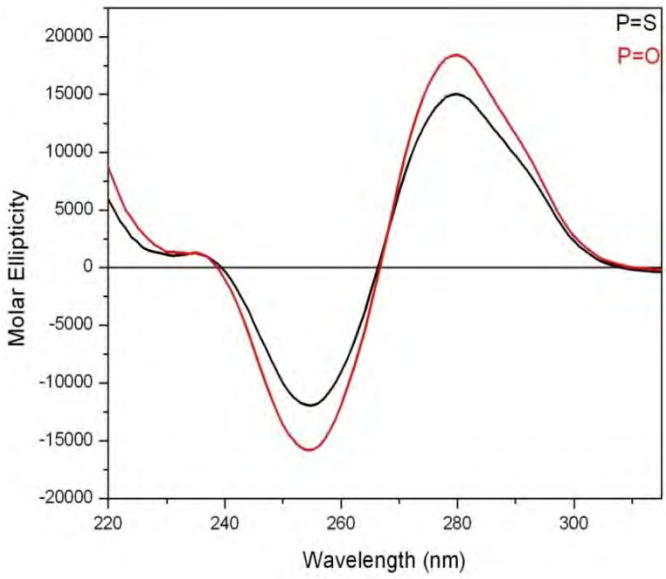


Figure 5. CD spectra of PS (P=S) and PD (P=O) ODN (16-mer) at 25 °C, demonstrating that the phosphothioate linkage does not impact the structural features of the 16-mer hairpin.

Because the overall structures of the deoxy-oligonucleotides are not significantly affected by the modification to the PS backbone as indicated by the CD spectra this variation was not believed to significantly affect the structural properties of these deoxyoligonucleotides.

Apoptotic DNA (derived from cancer cells treated with doxorubicin) and shown to induce invasion by the same TLR9 mechanism as these deoxyoligonucleotides; however, they do not contain the modified P=S backbone, and are therefore not resistant to nucleases by the P=S mechanism.(13) We sought to determine if a stable hairpin structure would protect the deoxyoligonucleotides from nuclease digestion. (14,15)

It has been reported that hairpin structures may offer resistance to nuclease digestion (12, 13). To The effects of a very stable 9-mer hairpin with the sequence 5'-d(CGCGAAGCG)-3' on invasion with P=O and P=S backbone were compared.

In addition, the truncated 16-mer sequence was modified to contain only purine bases in the loop, in order to provide more favorable stacking interactions to make the hairpin more stable.

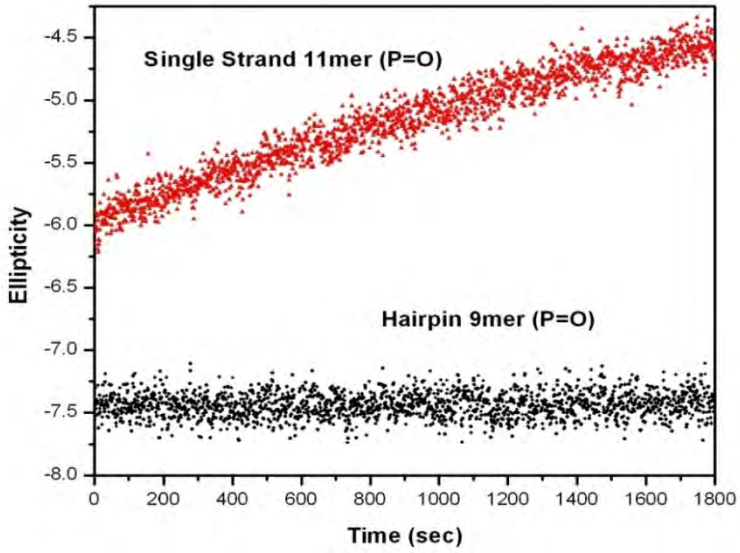


Figure 6. CD signal at 250 nm over 30 minutes performed at 25 °C after addition of S1 nuclease to a single strand 11-mer and the hairpin 9-mer (P=O indicates PD backbone).

The effects of this sequence (5'-d(CGTCGTGAAAACGACG)-3', termed "16-mer purines") with P=O and P=S backbones were also studied in invasion assays. The results are summarized in Figure 7. The induction of TLR9 mediated invasion by both the P=O and P=S 9-mer hairpins were surprisingly similar. The P=O and P=S 16-mer with purines in the loop also induced invasion at similar levels. This demonstrates that a stable hairpin structure offers resistance to digestion, which allows the ODNs to interact with TLR9 and induce the invasive response, with or without the modified PS backbone. The hairpin resistance to nuclease digestion was confirmed via CD time course experiments. The CD signal at 250 nm was monitored for a single strand 11mer (PD) that cannot adopt a hairpin structure and for the 9mer (PD) after the addition of S1 nuclease (Figure 6). There was a significant change in CD signal for the single strand ODN, while the signal remained constant over thirty minutes for the hairpin ODN.

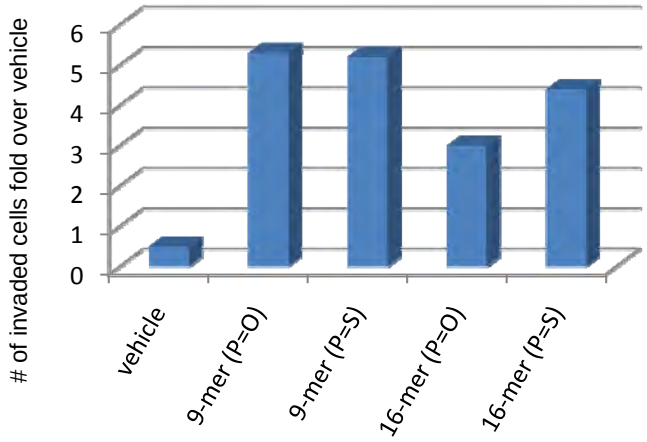


Figure 7. Comparison of native (P=O) and phosphothioate (P=S) deoxyoligonucleotide hairpins in inducing TLR9-mediated cell invasion.

Summary findings of this study as shown in Figure 4 through 7 are as follows:

- ODN-M362 (25-mer) which exerts maximal activity for induction of TLR9-mediated cancer cell invasion could be reduced to the 16-mer (truncated hairpin, i.e. removal of 5' and 3' overhangs) with minimal loss in TLR9 mediated response.
- Stabilization of the hairpin with addition of T or TT in the hairpin loop results in a 4 and 6 (°C) stabilization, respectively of the hairpin structure and retains TLR9 mediated response.
- The more stable the hairpin, the greater resistance to nuclease digestion.
- Hairpin stabilization resulted in elimination for the need of phosphothioate modification of the DNA backbone (Figure 7) for nuclease resistance.

Characterization of structural and energetic properties of deoxyoligonucleotides that activate TLR9-mediated cancer cell invasion. One of the key issues in examining the variety of deoxyoligonucleotides (and concomitant structural motifs) as shown in Table I was to discern the minimal deoxyoligonucleotide size and/or structural motif that is required for TLR9-mediated cancer cell invasion. Starting with the parent 25-mer, ODN M362, the size was trimmed to the core 16-mer and further refined to the 9-mer hairpin, 5'-CGC GAA GCG-3'. One of the most intriguing features of this 9-mer hairpin was its unusually high thermal stability as well as its proficiency for inducing TLR9-mediated cancer cell invasion.

NMR Studies to discern the solution structure of the 9-mer hairpin. The 9-mer deoxyoligonucleotide consists of a three base-pair stem and a three-base loop. In addition to determining the solution structure of this hairpin by NMR, we also analyzed the thermodynamic properties associated with the melting of the hairpin

by differential scanning calorimetry (DSC). To probe the role of the loop sequence in stabilization of the hairpin, we designed novel 9-mers incorporating specific mutations within the loop to allow us to differentiate the thermodynamic stabilities attributed from both base pairing and base stacking within the loop. (16-18).

Purified synthetic oligonucleotides referenced in Table 3 were purchased from Midland Certified Reagent Company; Midland, TX. Samples were dissolved directly into 100mM NaCl BPES buffer (10mM sodium phosphate buffer with 1mM EDTA pH 7.0), allowed to hydrate and then allowed to anneal by heating the sample above the melting point and then slowly cooling back to room temperature.

Table 3. 9mer deoxyoligonucleotide sequences analyzed by DSC. C= cytosine, G=guanine, A=adenine, T=thymine, I=ionosine, N=nebularine, U= uracil. For the structure of I and N reference Figure 8.

Loop Name	Sequence
GAA	d(CGCGAAGCG)
GTA	d(CGCGTAGCG)
GUA	d(CGCGUAGCG)
GAT	d(CGCGATGCG)
GAU	d(CGCGAUGCG)
GAN	d(CGCGANGCG)
IAA	d(CGCIAAGCG)
IAN	d(CGCIANGCG)
GTT	d(CGCGTTGCG)
GUU	d(CGCGUUGCG)

Sample concentrations were determined by spectroscopically at 260 nm on a Cary 100 UV-vis spectrophotometer at 90 °C. Mutations of the bases within the loop sequence seen in Figure 8 were chosen so as to perturb either loop base stacking and/or hydrogen bonding between G4 and A6. By choosing to mutate G4 to an ionosine or A6 to nebularine, we were able to remove their hydrogen bond donating capabilities either separately or together while maintaining optimal base stacking conditions. Mutations of adenine to thymine or uracil allowed us to analyze disruptions in base stacking within the loop sequence.

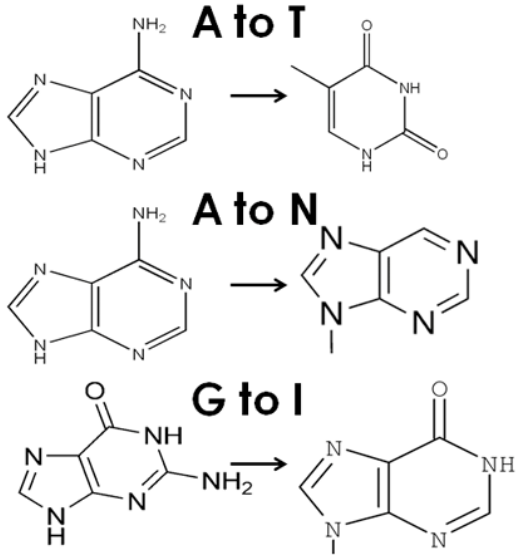


Figure 8. Mutations used to probe base stacking (and/or hoogstein base pairing within the hairpin loop.

Hairpin stability is dependent on the loop sequence. The melting temperatures and the associated heats of unfolding for each hairpin were determined using a Microcal VP-DSC (GE Healthcare, Northampton, MA). Oligonucleotide samples were prepared at strand concentrations from 200 – 400 μM in BPES buffer and verified by UV-Vis spectroscopy. Each sample was melted at a rate of 90 °C /hour from 5-120 °C and repeated a minimum of 5 times. Reference scans were also produced by analyzing buffer versus buffer in the same manner as the samples. Data was analyzed using Origin 7.0 VP-DSC software by first subtracting a reference scan (buffer versus buffer) from the raw data and the normalizing to strand concentration, producing heat capacity versus temperature plots. The data was then baseline corrected by connecting the pre and post-transition baselines with the cubic function provided within the software and subtracting the resulting baseline from the data. From the thermodynamic relationship, $\Delta H_{cal} = \int \Delta C_P(T) dT$, integrating the total area under the resultant melting curve provides the enthalpy of unfolding (ΔH_{unfold}), while the midpoint of the transition provides the melting temperature (T_m). The change in Gibb's free energy (ΔG) can then be derived by using $\Delta G^0(T) = \Delta H[1 - \frac{T}{T_m}]$ at any reference temperature, for our calculations the Gibb's free energy was calculated at 37°C.

Molecular Dynamics Simulations. Theoretical structural models were obtained from molecular dynamics simulations using explicit solvation by AMBER starting from the in-vacuum minimized Watson-Crick based paired 9-mer hairpin structures. These models were then used with NMR derived distance restraints for generalized Born (GB) implicit solvation minimization with AMBER to give the solution structure of the wild time 9mer hairpin.

Structure of the 9-mer hairpin. Samples for NMR analysis were prepared at single strand concentrations of 2mM in BPES buffer. For analysis of exchangeable protons, samples were prepared in H_2O buffer and 10% D_2O was then added. For NMR analysis in 100% D_2O, the water samples were lyophilized and reconstituted into the same initial volume with D_2O, lyophilization and reconstitution in D_2O was repeated three times to remove trace amounts of H_2O. All of the NMR experiments were performed on a Bruker Avance III 700-mHz NMR spectrophotometer with Bruker TCI-cyroprobe. Proton assignments are provided in Table 4. NOESY spectra for 90% water and 100% D_2O samples were obtained at various mixing times ranging from 50 to 400 ms at 298K. COSY, DQFCOSY and TOCSY spectra were also obtained at 298K to determine scalar coupled protons. Solvent suppression was achieved using the Bruker derived excitation sculpting gradient pulse. All spectra were then processed using Bruker Topspin 2.1.

Table 4. ^{1}H chemical shifts of non-exchangeable protons of d(CGCGAAGCG). Sample prepared in 10mM BPES with 100mM NaCl, pH 7.0.

	H1'	H2'	H2"	H3'	H4'	H5'	H5"	H2	H5	H6	H8
C1	5.663	1.919	2.309	4.589	4.088	3.638	3.957	-	5.794	7.548	-
G2	5.847	1.915	2.263	4.550	3.900	3.380	-	-	-	-	7.876
C3	5.839	1.916	2.388	4.795	4.238	3.387	3.987	-	4.945	6.883	-
G4	6.073	2.276	2.537	4.577	3.874	-	-	-	-	-	7.854
A5	5.879	2.141	2.197	4.464	2.050	2.958	3.280	7.960	-	-	8.018
A6	6.197	2.783	2.783	4.730	-	-	-	7.910	-	-	7.924
G7	5.806	1.832	2.281	4.730	4.145	4.012	-	-	-	-	7.854
C8	5.347	2.386	2.514	4.799	4.296	4.022	-	-	5.293	7.300	-
G9	5.257	2.428	2.554	4.812	-	-	-	-	-	-	7.979

NOE peaks were assigned and gaussian fit integrated using SPARKY (Goddard). The peaks from each NOESY spectra were used for distant constraints while all other spectra were used to confirm peak assignments. Distances derived from non-overlapping peaks were assigned an error of +/- 10% while overlapping peaks were given an error of up to +/- 50%. The SPARKY derived integrated peaks from each spectrum were then averaged, normalized and converted to distance constrains using RANDMARDIGRAS from MARDIGRAS. The resulting distance restraints were then converted for use with AMBER by MARDIGRAS. The solution structure was then derived by coupling the resulting NMR distance restraints with a molecular dynamic simulated annealing procedure using generalized Born implicit solvation with AMBER utilizing the previously derived theoretical structure.

The resulting solution structure derived for the native 9-mer hairpin was determined by normalizing and averaging the assigned NOE peaks (Table 4) from three NOESY experiments ranging from a mixing time of 50 ms to 300 ms. These peak areas where then converted to distance constraints and used by AMBER to derive the solution structure seen in Figure 9. The resulting structure agrees well with the 6-mer hairpin derived by Hirao (Hirao 1994) where the loop G4 and A6 forms an unusual "Hoogstein-like" sheared base pair with the middle A5 base stacking above the G4. (19, 20) Direct evidence of the hydrogen bonding was difficult to

observe in NMR due to the co-resolution of the exchangeable amino protons that should be directly involved in such bonding; however, we were able to observe key NOEs between the H4' of the G4 sugar to the H2 of adenine. Based on the structure derived from AMBER, these protons should be 4.2 Å apart indicating the groups are within hydrogen bonding distance. The DSC data discussed below also supports the hypothesis that G4 and A6 are indeed forming an unusual Hoogstein-like base pair.

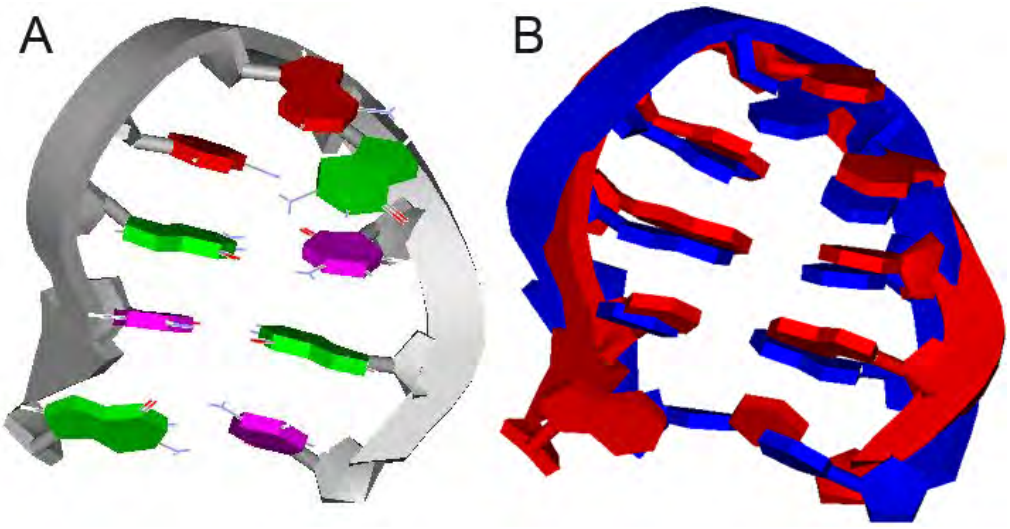


Figure 9. A. NMR resolved solution structure for d(CGCGAAGCG). B. Overlay of NMR restrained structure (red) with theoretical molecular dynamic simulated annealing structure (blue).

Energetic stability of the 9-mer h airpin and selected loop mutations. Differential scanning calorimetry allows us to monitor the change in heat capacity at constant pressure while increasing temperature. This data provides the melting temperature, the change in enthalpy upon melting and Gibbs free energy at a given temperature. As seen in table #, mutations within the loop sequence have significant effects on the thermodynamic stability of the 9mer hairpin.

Table 5. DSC analysis of 9-mer d($C_1G_2C_3G_4A_5A_6G_7C_8G_9$) and loop mutants. Each sample was analyzed at known concentrations between 200 – 400 uM. The samples were melted from 5 – 120 ºC at a rate of 90 ºC/hour. The listed results are an average and deviation of 6 scans per sample.

Loop Sequence	Tm (ºC)	ΔTm (kcal/mol)	ΔH (kcal/mol)	ΔΔH (kcal/mol)	$\Delta G_{37ºC}$ (kcal/mol)	$\Delta\Delta G_{37ºC}$ (kcal/mol)
GAA	88.5 ± 0.1	NA	19.1 ± 0.4	NA	13.7 ± 0.3	NA
GTA	84.1 ± 0.5	- 4.4	16.6 ± 0.4	- 2.5	11.7 ± 0.3	- 2.0
GUA	84.6 ± 0.5	- 3.9	16.8 ± 0.4	- 2.3	11.9 ± 0.2	- 1.8
GAT	63.3 ± 0.2	- 25.2	7.9 ± 0.4	- 11.2	4.8 ± 0.2	- 8.9
GAU	63.8 ± 1.9	- 24.6	4.9 ± 0.2	- 14.1	3.0 ± 0.2	- 10.7
GAN	77.3 ± 0.6	- 11.1	17.6 ± 0.2	- 1.5	11.9 ± 0.1	- 1.8
IAA	69.1 ± 0.4	- 19.4	15.5 ± 0.6	- 3.6	9.9 ± 0.4	- 3.8
IAN	64.7 ± 0.5	- 23.7	11.1 ± 0.5	- 8.0	6.8 ± 0.3	- 6.9
GTT	65.4 ± 0.5	- 23.0	8.4 ± 0.4	- 10.7	5.2 ± 0.2	- 8.5
GUU	66.1 ± 0.2	- 22.4	8.2 ± 0.4	- 10.9	5.1 ± 0.3	- 8.6

Mutation of A6 to T6, results in a 24 °C decrease in the melting temperature. In contrast, mutating A5 to T5 only decreases the T_m by 4^0C. However, if we analyze the changes in enthalpy we see that mutating either adenine to a thymine has a negative impact. The changes in melting temperature can be attributed to a

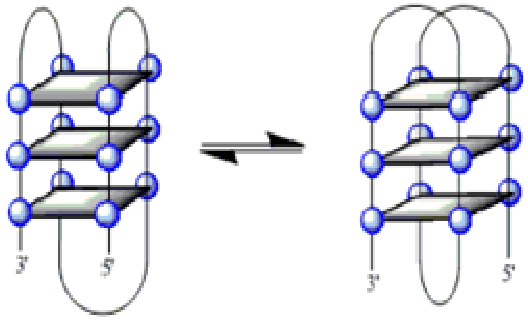
disruption of the “Hoogstein-like” hydrogen bonding between G4 and A6, as well as a disruption in stabilization provided by base stacking contributed by both purines.

In order to determine the magnitude that hydrogen bonding stabilize the native structure without disrupting the stabilizing base stacking effects, we selectively mutated G4 to an ionosine (I) residue and A6 to nebularine (N) residue. As seen in Figure 8, both ionosine and nebularine resemble their parent base structures of guanine and adenine perfectly except for the absence of their respective amino groups. As seen in Table 5, these mutations result in a decrease in melting temperature, enthalpy, and free energy. Mutating G4 to ionosine decreases the melting temperature by 19 °C, while mutating A6 to nebularine decreases the T_m by 11 °C. Mutation of both G4 and A6 to I4 and N6 results in a decrease in the melting temperature by 24 °C, similar to that of mutating A6 to T6. The enthalpy of melting is slightly higher for the loop containing IAN than for GAT, suggesting that the thermal stability of the loop is largely dependent on the base pairing of G4 and A6 as well as base stacking. It is also interesting to note that by removing the hydrogen bond donor capabilities of adenine by mutating to nebularine, we do not see the same effect on the melting temperature as we do when we mutate the guanine. We speculate that the orientation of G4 enhances both H-bonding and base stacking. This degree of tilt that is observed in the NMR structure is attributed to the stacking of A5 with its amino group juxtaposed over G4, constricting G4 between C3 and A5 as seen in the solution structure in Figure 9.

Mutation of A5 to either T5 or U5 results in only slightly decrease the hairpin stability; in contrast to the marked decrease in stability when A6 is mutated to T6. We initially presumed that mutations to thymine would have a larger effect on the loop stability when compared to uracil due to the presence of the methyl group on thymine stacking; however; however there seems to be no significant difference between the T5 or U5 mutations on thermal stability of the hairpin. This suggests that the methyl group on T5 is buried within the loop and is not molecule exposed to solvent as suggested by the theoretical models. Future studies by solving the solution structure for the GTA mutant will to verify this hypothesis.

Summary findings of this study as shown in Figure 7 through 9 and Table 5 are as follows:

- The 9-mer d($C_1G_2C_3G_4A_5A_6G_7C_8G_9$) is very proficient in inducing TLR9-mediated cancer cell invasion.
- This 9-mer does not require phosphothioate backbone modification to be effective in matrigel invasion assays; the phosphodiester (native DNA sugar-phosphate linkage) is sufficient.
- The 9-mer form an unusually stable hairpin structure, with a T_m of 88 °C.
- The structure of this 9-mer hairpin as determined by NMR shows increased stabilization due to the $G_4A_5A_6$ loop sequence wherein the G_4 and A_6 form a Hoogstein-like base pair and the A_5 stacks across this $G_4 : A_6$ base pair.
- The unusual stability of this GAA tri-nucleotide loop is now being implemented in other sequences to provide a “molecular staple”.



The G-quadruplex structure derived from human telomere sequence (h-Tel 22). In an effort to expand our studies of deoxyoligonucleotides with stable secondary structures as potential agonists in probing TLR9-mediated cancer cell invasion, the 22-mer repeating sequence known as human telomeric repeat sequence, d[AGGG($TTAGGG)_3$], was examined. This sequence is found at the ends of human chromosomes and due to the nature of the repetitive GGGs forms G-tetrads that consequently stabilize into a stable secondary structure known as the G-quadruplex (Figure 10). (21)

Figure 10. Chair (left) and basket (right) structures of h-Tel 22 in Na^+.

Human telomeric DNA is a non-coding region at the end of the chromosome that consists of a repetitive sequence that is approximately 150-200 kilobases in length. (21) The terminal end of this sequence has a 3’ single stranded overhang with the tandem repeat of 5’- TTAGGG-3’ that has been demonstrated to form stable G-quadruplex structures, *in vitro.* (22,23) The formation of G-quadruplex structure inhibits the attachment of telomerase and the enzymatic extension of the telomere that is often associated with most cancer cells. (24,

25) The human telomeric G-quadruplex structure has three stacked G-tetrads connected by -TTA- loops. The nature of the loop connectivity gives rise to the alternate conformations that have been observed by NMR and X-ray crystallographic methods under varying solution conditions. The most notable structural differences are between the sodium and potassium forms. Sodium based buffers result in an all antiparallel strand orientation with glycosidic bond angles that are all anti conformations. However, the loop connectivity may differ and either be all lateral loops, the "chair" conformation or have two lateral loops and one diagonal loop, the "basket" conformation, both Na^+ conformations and their loop connectivity can be seen in Figure 10. Potassium based buffers result in a more complex G-quadruplex structure that is characterized by two lateral loops and one loop that is a chain reversal oriented on the edge of the tetrad core (structure not shown). The result is a mixed strand polarity that has both antiparallel and parallel characteristics with one guanine in a syn conformation and the remaining three in an anti conformation. The hybrid structure was described by Phan, *et al*, (26) and an alternate structure exists for the crystal structure reported by Neidle, *et al*. (27)

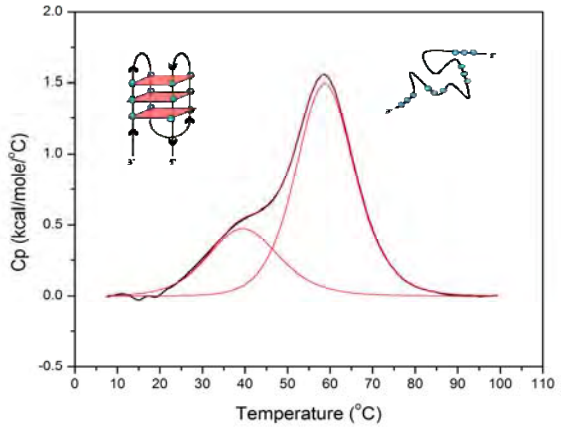


Figure 11. DSC experiment showing the thermal melting transition of G-quadruplex structure formed by hTel-22 in sodium phosphate buffer.

The energetics of G-quadruplex unfolding and the enthalpic (ΔH_{unfold}) and entropic (ΔS_{unfold}) contributions to the thermal stability have been reported by several research groups using a variety of methods. Shown in Figure 11 is a DSC analysis of h-Tel 22 G-quadruplex stability in Na^+ buffer. Our data shows this G-quadruplex structure to melt at approximately 60 °C and undergo two transitions. In other data (not shown, we have monitored the G-quadruplex structure by CD spectroscopy, showing characteristic positive peak at 295 nm. As described in Figure 6, we have monitored the hTel-22 G-quadruplex DNA in the presence of a variety of nucleases and found this structural motif to infer nuclease resistance to the DNA. We have also examined the ability of this DNA to induce TLR9-mediated cancer cell invasion.

Our data reveals the G-quadruplex DNA from the human telomeric repeat sequence to be more effective than the 9-mer and almost effective as the ODN M362 at eliciting TLR9 mediated cancer cell invasion.

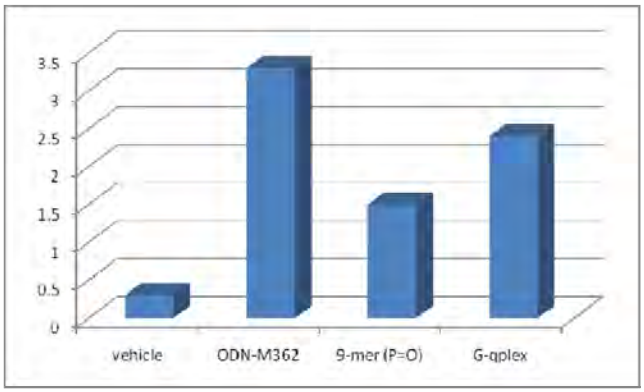


Figure 12. Parental MDA-MB-231 cells treated with 5 μM (strand) ODN-M362, 9-mer (wild type) or hTel-22 G-quadruplex in Matrigel-matrix.

Summary findings of G-quadruplex DNA study reveals:

- The native G-quadruplex is nuclease resistant in the folded form.
- The G-quadruplex DNA formed from the human telomeric repeat sequence is highly effective as a TLR9 agonist in inducing cancer cell invasion.

The roles of the human telomeric G-quadruplex as a TLR9 agonist in both the stimulation of cancer cell invasion as well as other potential activities in moderating innate cellular immunity are intriguing. Most cancer cells (including breast cancer cells) upregulate telomerase activity; hence, increasing the amount of telomeric DNA in these cells and potentially released upon cellular apoptosis resulting from chemotherapy. In contrast to most other DNAs released during apoptosis, the G-quadruplex DNA is nuclease resistant and may provide a suitable agonist for targeting TLR9 in surviving cancer cells. The direct interactions of these DNAs with TLR9 will be the focus of the upcoming years research.

Interactions of the antimicrobial peptide, LL-37 with deoxyoligonucleotides. As discussed earlier, LL-37, an antimicrobial peptide has been shown to associate with self-DNA and interact with TLR9 in plasmacytoid

dendritic cells (10, 11) and to enhance the immunostimulatory effects of CpG-ODNs against ovarian cancer (Chuang, 2009). Over the past year, we have investigated whether LL-37 differentially forms complexes with deoxyoligonucleotides having well-defined secondary structures such as single-strand, duplex, hairpin, or G-quadruplexes. The effects of deoxyribonucleic acid (DNA) length, sequence, structure, and ionic strength on the interactions with the antimicrobial peptide LL-37 were examined. The peptide under investigation, LL-37, has a positively charged amphipathic helix that is known to be involved in transporting DNA fragments into the cell. The oligonucleotides studied adopt many different secondary structures such as Watson-Crick duplex, hairpin, quadruplex, and single strand. When DNA and LL-37 are mixed, they immediately precipitate upon binding. This occurrence was monitored by observing the absorbance at 340 nm using UV-Visible Spectroscopy (UV-Vis), binding thermodynamics monitored using Isothermal Titration Calorimetry (ITC), and particle size and the rate of precipitation monitored using a Zetasizer.

To study the effects of DNA length on the binding of LL-37, a set of five DNA sequences were designed that would only exist as single stranded DNA (ssDNA). These five deoxyoligonucleotides are listed in Table 6 along with their compliments used to examine the effects of base composition, length, and single strand versus duplex structure on LL-37 binding. The primary base sequence (5'-AGTGTT-3') is a mutation from the base sequence for the G-quadruplex sequence of 5'-$(AGGGTT)_n$-3'. By changing the middle guanine to a thymine, the quadruplex structure is unable to form and the strand remains as ss-DNA. Upon binding, the LL-37/DNA complex precipates. Hence, to quantitate complex formation, light scattering was used. Using UV-vis spectroscopy, LL-37/DNA precipitation could be monitored to determine the effect of DNA length on binding.

Table 1: Oligonucleotide and Peptide Sequences

Name	**Sequence**	**Secondary Structure**
6mer	5'-AGTGTT-3'	Single Strand
6mer compliment	5'-AACACT-3'	Single Strand
12mer	5'- $(AGTGTT)_2$-3'	Single Strand
12mer compliment	5'- $(AACACT)_2$-3'	Single Strand
18mer	5'- $(AGTGTT)_3$-3'	Single Strand
18mer compliment	5'- $(AACACT)_3$-3'	Single Strand
24mer	5'- $(AGTGTT)_4$-3'	Single Strand
24mer compliment	5'- $(AACACT)_4$-3'	Single Strand
30mer	5'- $(AGTGTT)_5$-3'	Single Strand
30mer compliment	5'- $(AACACT)_5$-3'	Single Strand
9mer	5'-CGCGAAGCG-3'	Hairpin
16mer	5'-CGTCGTGAAAACGACG-3'	Hairpin
25mer	5'-TCGTCGTCGTTCGAACGACGTTGAT-3'	Hairpin
Quadruplex	5'-AGGGTTAGGGTTAGGGTTAGGG-3'	Quadruplex
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	Amphipathic Alpha Helical Peptide

Summary findings of LL-37 interactions with deoxyoligonucleotides.

- deoxyoligonucleotide length was critical determinant in LL-37 binding. The longer the DNA, the higher the binding affinity. This was true for both single-stranded deoxyoligonucleotides as well as duplexes.
- for single-stranded DNAs, LL-37 showed stronger binding to the GT rich strand in comparison to the AC rich complement strand.

Further studies are needed to assess the interactions of LL-37 with deoxyoligonucleotides and their role in facilitating transport of these deoxyoligonucleotides to the TLR9 target. Our cancer biology studies reveal that the deoxyoligonucleotides added to the MDA-MB-231 breast cancer cells accumulates in the cells in vitro and that this effect was enhanced by complexing the DNAs with LL-37 prior to addition to the cells. Hence, more studies are needed to determine how these biological effects overcome the apparent precipitative nature of the LL-37/DNA complex.

Task 3.3. SPR and ITC studies to determine whether DNA sequence, structure, or stability modulates TLR9 interactions. The focus of year 2 will be to examine the interactions of the selected deoxyoligonucleotides described in Task 3.2 as well as others that are currently under examination, with TLR9 using a variety of biophysical methods.

KEY RESEARCH ACCOMPLISHMENTS:

- ODN-M362 (25-mer) which exerts maximal activity for induction of TLR9-mediated cancer cell invasion could be reduced to the 16-mer (truncated hairpin, i.e. removal of 5' and 3' overhangs) with minimal loss in TLR9 mediated response.
- Stabilization of the hairpin with addition of T or TT in the hairpin loop results in a 4 and 6 (°C) stabilization, respectively of the hairpin structure and retains TLR9 mediated response.
- The more stable the hairpin, the greater resistance to nuclease digestion.
- Hairpin stabilization resulted in elimination for the need of phosphothioate modification of the DNA backbone (Figure 7) for nuclease resistance.
- The 9-mer d($C_1G_2C_3G_4A_5A_6G_7C_8G_9$) is very proficient in inducing TLR9-mediated cancer cell invasion.
- This 9-mer does not require phosphothioate backbone modification to be effective in matrigel invasion assays; the phosphodiester (native DNA sugar-phosphate linkage) is sufficient.
- The 9-mer form an unusually stable hairpin structure, with a T_m of 88 °C.
- The structure of this 9-mer hairpin as determined by NMR shows increased stabilization due to the $G_4A_5A_6$ loop sequence wherein the G_4 and A_6 form a Hoogstein-like base pair and the A_5 stacks across this $G_4 : A_6$ base pair.
- The unusual stability of this GAA tri-nucleotide loop is now being implemented in other sequences to provide a "molecular staple".
- The native G-quadruplex is nuclease resistant in the folded form.
- The G-quadruplex DNA formed from the human telomeric repeat sequence is highly effective as a TLR9 agonist in inducing cancer cell invasion.
- deoxyoligonucleotide length was critical determinant in LL-37 binding. The longer the DNA, the higher the binding affinity. This was true for both single-stranded deoxyoligonucleotides as well as duplexes.
- for single-stranded DNAs, LL-37 showed stronger binding to the GT rich strand in comparison to the AC rich complement strand.

REPORTABLE OUTCOMES:

- Manuscripts currently in preparation:

    - Hudson, J.S., Ding, L., Ma, J. Lewis, E. and Graves, D.E. (2011) "Recognition and Binding of Human Telomeric G-Quadruplex DNA by Unfolding Protein 1 (UP1)", manuscript in preparation for Nature Structural Biology.
    - Lanier, K.L., Ottenfeld, E., Hudson, J.S., and Graves, D.E. (2011) "Structural and Thermodynamic Characterization of a Highly Stable DNA Hairpin", manuscript in preparation for Nucleic Acids Research.
    - Mitchell, B., Hudson, J.S., and Graves, D.E. (2011) "Interactions of the Antimicrobial Peptide, LL-37, with Nucleic Acids. Effects of DNA Length, Sequence and Secondary Structure", manuscript in preparation for Biochemistry.
    - Hudson, J.S., Ding, L., Lewis, E., Graves, D.E. (2011) "Quadruplex Unfolding: Influence of Loop Mutations on Structural Stability", manuscript in preparation for Biochemistry.

- Abstracts from presentations at regional and national meetings:

    - Graves, D.E., Hudson, J.S., Ding, L., Ma, J., and Lewis, E. (2010) "Recognition and binding of the human telomeric G-quadruplex by UP1 (Unwinding Protein 1)". Joint 66th Southwest and 62nd Southeast Regional Meeting of the American Chemical Society, New Orleans, LA, Dec. 1-4, 2010.

    - Mitchell, B. A. and Graves, D.E. "Characterization of a DNA Binding Peptide". Joint 66th Southwest and 62nd Southeast Regional Meeting of the American Chemical Society, New Orleans, LA, Dec. 1-4, 2010.

- Lanier, K.L., Ottenfeld, E. and Graves, D.E. (2010) “Structural Analysis of a Highly Stable DNA Hairpin”. Joint 66th Southwest and 62nd Southeast Regional Meeting of the American Chemical Society, New Orleans, LA, Dec. 1-4, 2010.

- Brooks, S., Selander, K.S., Harris, K.W., and Graves, D.E. (2011) “Structure and Stability of Deoxyoligonucleotides that Induce TLR9-mediated Cancer Cell Invasion”. Era of Hope – Congressionally Directed Medical Research Programs, Orlando, FL, August 2-5, 2011.

- Degrees Awarded

    - Jason S. Hudson, Ph.D. (2010) – now employed as Associate Director of Toxicology by the Virginia Department of Forensic Science, Richmond, VA
    - Sonja Brooks, B.S. (2010) – currently in the Ph.D. program in Structural Biochemistry at Vanderbilt University.

CONCLUSION: The research that has been completed over the past year clearly demonstrates a direct correlation between structural stability of deoxyribonucleotides and their ability to induce TLR9-mediated cancer cell invasion. However, it is unclear whether this structural stability is (a) a determinant in TLR9 recognition and/or binding or (b) infers nuclease resistance so that the deoxyoligonucleotide can reach the TLR9 binding site. Experiments are currently underway to probe these questions. In the course of the past year’s research we have made significant strides in the characterization of structural and energetic properties of selected deoxyoligonucleotide of defined lengths, sequences, and secondary structures. We have determined that the phosphothioate modification of the sugar-phosphate linkage is not necessary for deoxyoligonucleotide induced cellular invasion; a stable secondary structure imparting nuclease resistance works just as well. Hence, DNA fragments from apoptotic cells that have sequences conducive for the formation of stable secondary structures may serve as TLR9 agonists. Of particular interest is our finding that the human telomeric sequence repeat is highly effective as a TLR9 agonist.

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23. Tsai, Y. C., Qi, H., and Liu, L. F. (2007) Protection of DNA ends by telomeric 3' G-tail sequences, J Biol Chem 282, 18786-18792.
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27. Parkinson, G. N., Lee, M. P., and Neidle, S. (2002) Crystal structure of parallel quadruplexes from human telomeric DNA, Nature 417, 876-880.

APPENDICES:

Curriculum Vitae

SUPPORTING DATA:

NA

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CURRICULUM VITAE (LAST UPDATE – APRIL, 2010)

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EDUCATION:

1970-1974	University of Alabama at Birmingham, B.S. (1974), Chemistry
1974-1979	University of Alabama at Birmingham, Ph.D. (1979), (Biochemistry & Molecular Biology in Laboratory of Dr. K. L. Yielding)
1980-1984	Postdoctoral - Department of Chemistry, University of Rochester, Rochester, New York. (Biophysical Chemistry in Laboratory of Dr. Thomas R. Krugh)

RESEARCH AND PROFESSIONAL APPOINTMENTS:

1972-1974	Teaching Assistant in Chemistry, University of Alabama at Birmingham
1974-1976	Graduate Research Assistant in Laboratory of Reproductive Biology, University of Alabama at Birmingham School of Medicine
1976-1979	Graduate Research Assistant in Laboratory of Molecular Biology, University of Alabama at Birmingham
1980-1984	Postdoctoral Fellow in Chemistry, University of Rochester
1984-1990	Assistant Professor of Chemistry, University of Mississippi
1984-1990	Assistant Professor of Pharmacognosy, University of Mississippi
1990-1996	Associate Professor of Chemistry, University of Mississippi
1990-1996	Associate Professor of Pharmacognosy, University of Mississippi
1996-2003	Professor of Chemistry, University of Mississippi
1996-2003	Director, Forensic Chemistry Program, University of Mississippi
1996-2003	Professor of Pharmacognosy, University of Mississippi
2002-2005	Distinguished Faculty Fellow, College of Liberal Arts, University of Mississippi
2003-present	Senior Scientist – Experimental Therapeutics Program, Comprehensive Cancer Center, University of Alabama at Birmingham
2003-present	Professor and Chair of Chemistry, University of Alabama at Birmingham
2003-present	Adjunct Professor, Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham
2010-July, 2011	Associate Dean for Research, College of Arts and Sciences, University of Alabama at Birmingham

FELLOWSHIPS, HONORS, AND AWARDS:

National Institutes of Health Research Service Award Traineeship (National Cancer Institute Postdoctoral Fellow) (1981-1984) in the laboratory of Professor Thomas R. Krugh, Department of Chemistry, University of Rochester
American Chemical Society Petroleum Research Award (1985-1988)
Cottrell Research Corporation Award (1985-1988)
Else U. Pardee Fellowship Award (1992-94)
Distinguished Faculty Fellow, College of Liberal Arts, University of Mississippi

PROFESSIONAL SOCIETIES:

American Chemical Society (1980-present)
American Association for Cancer Research (1988-present)
American Association for the Advancement of Science (1982-present)
Biophysical Society (1984-present)
Sigma Xi (1984-present)
Federation of American Society for Experimental Biology (1985-present)
American Society for Biochemistry and Molecular Biology (1986-present)

CONSULTANTSHIPS AND ADVISORY BOARDS:

Member, NIH Study Section – EBT (Enabling Bioanalytical and Biophysical Technologies) - (2008 – present)
Member, U.S. Army Breast Cancer Research Panel (USAMRMC) - (2001 - 2003)
Member, NSF, Molecular Biophysics Study Panel (1999 – 2003)
Member, Collaborative Research in Chemistry Study Panel, National Science Foundation (2002-2004)
Member, National Science Foundation Graduate Research Fellowship Review Panel (1999 – 2002)
Consultant, Shore Chan Bragalone, LLP
Chair, NIH Study Section - GGG-J (Genes, Genomes and Genetics) - (2007 - 2009)
Chair, NIH Study Section - IMST- (Genes, Genomes and Genetics) - (2009 - present)

RESEARCH INTERESTS:

DNA structural motifs as ligands for Toll-like receptors
DNA structure and stability as influenced by base sequence
Design and synthesis of novel topoisomerase I and II inhibitors as anticancer agents Mechanisms of action of anticancer agents
Structural and energetic properties of ligand-DNA interactions
Sequence and structurally selective interactions of DNA binding agents

UNIVERSITY COMMITTEE RESPONSIBILITIES:

(University of Mississippi)

Chairman, Institutional Biosafety Committee (Univ. Mississippi) (1989-2003)
Member, Graduate Council, University of Mississippi (1996-1998)
Alternate Member, Graduate Council, University of Mississippi (1998-2003)
Member, Environmental Safety Committee (Univ. Mississippi) (1989-2003)
Chairman, Biological Safety Sub-Committtee (Univ. of Mississippi) 1991-2003)
Member, University Faculty Senate (Univ. Mississippi) (1996-2000)
Chairman, Faculty Governance Committee (1999-2000)
Member, Executive Committee of the Faculty Senate (Univ. Mississippi) (1999-2000)
Chairman, Information Technologies Committee (Univ. Mississippi) (1998-2000)
Member, University of Mississippi SACS Self-Study Committee on Administrative Processes (1997-2003)
Member, Tenure and Promotion Review Committee, (Univ. Mississippi) (1998-2003)

Member, Research Advisory Committee (Univ. Mississippi) (1996-2003)

(University of Alabama at Birmingham)

Member, Graduate Program Directors (UAB) (2003-present)
Member, Center for Computational and Structural Biology (2003-present)
Member, Transinstitutional Advisory Committee for the Center for Computational and Structural Biology (UAB) 2003-present)
Member, Executive Committee for Center for Computational and Structural Biology (2003-present)
Chair, Transinstitutional Advisory Committee for the Center for Computational and Structural Biology (2009-present)
Member, Institutional Advisory Committee for the UAB NMR Core Facility (2003-present)
Chair, UAB Comprehensive Cancer Center NMR Facility Advisory Committee (2003-present)
Member, UAB Research Advisory Group (2003-present)
Member, Executive Committee for UAB Research Advisory Group (2006-present)
Member, Drug Discovery Development Group, UAB Comprehensive Cancer Center, 2008-present
Member, Alabama Drug Discovery Alliance, 2008-present
Member, Search Committee for recruitment of UAB Dean of the Graduate School (2005-2006)
Chair, Search Committee for recruitment of UAB Chair of Department of Biology (2006-07)
Member, Executive Committee, Center for Lung Health (2009-present)
Member, UAB Center for Nanoscale Materials and Biointegration (CNMB)
Member, Internal Advisory Committee, UAB Center for Nanoscale Materials and Biointegration
Member, Chemical Safety Committee, UAB Occupational Health and Safety (2009-present)
Chair, Chemical Safety Committee, UAB Occupational Health and Safety (2009-present)

PROFESSIONAL RESPONSIBILITIES:

Associate Editor

Anticancer Agents – Medicinal Chemistry, Bentham Publications, 2000-present

Journal Reviewer

Regular Reviewer, Biochemistry
Regular Reviewer, Cancer Research
Regular Reviewer, Biophysical Chemistry
Regular Reviewer, Biopolymers
Regular Reviewer, Nucleic Acids Research
Regular Reviewer, Proceedings of the National Academy of Science, USA
Regular Reviewer, Chemico-Biological Interactions
Regular Reviewer, Journal of Biological Chemistry
Regular Reviewer, Chemistry & Biology
Regular Reviewer, Journal of the American Chemical Society
Regular Reviewer, J. Natural Products
Regular Reviewer, J. Med. Chemistry

Granting Agency Reviewer

National Institutes of Health
National Science Foundation
American Cancer Society
American Chemical Society
Cottrell Research Corporation

TEACHING RESPONSIBILITIES:

Research Methods in Chemistry and Biochemistry	(Chem 201)
Biochemistry	(Chem 460)
Biophysical Chemistry	(Chem 463)
Advanced Biochemistry (I & II)	(Chem 461, 462)
Biochemistry Laboratory	(Chem 464)
Biochemical Techniques	(Chem 564)
Graduate Biochemistry (I & II)	(Chem 561, 562)
Physical Biochemistry	(Chem 563)
Priniciples & Applications of 1- & 2-D NMR Spectroscopy	(Chem 639, 739)

PROFESSIONAL SERVICE

Symposium Organizer

2005, Symposium entitled "Frontiers in Nucleic Structure and Energetics", Southeast/Southwest Joint Regional meeting of the American Chemical Society, Memphis, Tennessee, Nov. 1-4, 2005.

2004, Symposium entitled "Nucleic Acids: Structural Motifs and Applications", Southeast Regional meeting of the American Chemical Society, Durham, North Carolina, Nov. 10-13, 2004.

2001, Symposium entitled "Topoisomerase Targeting Agents: Chemistry to Chemotherapy II", (National Cancer Institute funded), held on the University of Mississippi campus, August 25-28, 2001.

2000, Symposium entitled "Physical Chemistry of Nucleic Acids", Southeast – Southwest Regional Meeting of the American Chemical Society, New Orleans, Louisiana, December 6-8, 2000.

1998, Symposium entitled Sequence Selective Binding to DNA: Southwest Regional Meeting of the American Chemical Society, Baton Rouge, Louisiana, Nov. 1-3, 1998.

1998, Symposium entitled "Topoisomerase II Targeting Agents: Chemistry to Chemotherapy I (National Cancer Institute funded), held on the University of Mississippi campus, August 29-September 1, 1998.

1995, Southeastern DNA Symposium at the Southeast/Southwest Regional Meeting of the American Chemical Society, Memphis, Tennessee, Nov. 29-Dec. 1, 1995.

1994, Southeastern DNA Symposium at the Southeast Regional Meeting of the American Chemical Society, Birmingham, Alabama, October 7-9, 1994.

1990 Southeastern DNA Symposium, University of Mississippi, October 26-28, 1990.

PUBLICATIONS:

Pitts, S., Jablonsky, M., Duca, M., Dauzonne, D., Monneret, C., Arimondo, P., Anklin, C., Graves, D.E., and Osheroff, N. (2011) "Contributions of the D-Ring to the Activity of Etoposide Against Human Topoisomerase II: Potential Interactions with DNA in the Ternary Enzyme-Drug-DNA Complex" Biochemistry (accepted May 6, 2011).

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Gilbert, P.L., Graves, D.E. and Chaires, J.B. (1991) "Inhibition of the "B-Z" Transition in Poly(dGdC)·poly(dGdC) by Covalent Attachment of Ethidium: Equilibrium Studies" Biochemistry 30, 10925-10931.

Gilbert, P.L., Graves, D.E., Mark Britt, and Chaires, J.B. (1991) "Inhibition of the "B-Z" Transition in Poly(dGdC)·poly(dGdC) by Covalent Attachment of Ethidium: Kinetic Studies" Biochemistry 30, 10931-10937.

Wadkins, R. M. and Graves, D.E. (1991) "Interactions of Anilinoacridines with Nucleic Acids: Effects of Substituent Modifications on DNA Binding Properties"
Biochemistry 30, 4278-4283.

Graves, D.E.. and Wadkins, R.M. (1990) "Thermodynamic Studies of Amsacrine Antitumor Agents with Nucleic Acids" in Molecular Basis of Specificity in Nucleic Acid-Drug Interactions" (ed. Bernard Pullman), Kluwer Academic Publishers, Dordreicht, Holland.

Wadkins, R. M. and Graves, D.E. (1989) "Thermodynamics of the Interactions of m-AMSA and o-AMSA with Nucleic Acids: Influence of Ionic Strength and DNA Base Composition", Nucleic Acids Research 17, 9933-9946.

Rill, R.L., Marsch, G.A., and Graves, D.E. (1989) "7-Azido Actinomycin D: A Photoaffinity Probe of the Sequence Specificity of DNA Binding by Actinomycin D" J. Biomolecular Structure and Dynamics 7, 591-605.

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Eftink, M.R., Jia, Y-W., and Graves, D.E. (1989) "Intramolecular Fluorescence
Quenching in an Acrylamide-Indole Adduct" Photochem. Photobiol. 49, 725-729.

Graves, D.E. and Wadkins, R. M. (1989) "7-Azido Actinomycin D: A Novel Probe for Examining Actinomycin D-DNA Interactions" J. Biological Chemistry 264. 7262-7266.

Elmore, R.H., Wadkins, R.M. and Graves, D.E. "Cooperative Binding of m-AMSA to Nucleic Acids" (1988) Nucleic Acids Research 16, 9707-9719.

Stone, M.P., Gopalakrishnan, S., Harris, T.M., and Graves, D.E. (1988) "Carcinogen-Nucleic Acid Interactions: Equilibrium Binding Studies of Aflatoxins B_1 and B_2 with DNA and the Oligodeoxyribonucleotide $d(ATGCAT)_2$" J. Biomolecular Structure and Dynamics 5, 1025-1043.

Graves, D.E., Stone, M.P., and Krugh, T.R. (1985) "Structure of the Anthramycin-$d(ATGCAT)_2$ Adduct from One- and Two-Dimensional Proton Nuclear Magnetic Resonance Experiments in Solution." Biochemistry 24, 7573-7581.

Graves, D.E., Stone, M.P., and Krugh, T.R. (1985) "NMR Analysis of an Oligodeoxy- ribonucleotide-Drug Adduct." in Molecular Basis of Cancer. Part B: Macromolecular Recognition, Chemotherapy, and Immunology. Interdiciplinary Discussion on the Basic and Applied Aspects of Cancer. (ed. Rein, R.) Alan R. Liss, Inc., New York, New York.

Graves, D.E., Pattaroni, C., Krishnan, B.S., Ostrander, J.M., Hurley, L.H., and Krugh, T.R. (1984) "The Reaction of Anthramycin with DNA: Proton and Carbon Nuclear Magnetic Resonance Studies on the Structure of the Anthramycin-DNA Adduct". Journal of Biological Chemistry 259, 8202-8209.

Graves, D.E. and Krugh, T.R. (1983) "Single-Cell Partition Analysis: A Direct Fluorescence Technique for Examining Drug-DNA Interactions." Analytical Biochemistry 134, 73-81.

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Rosenberg, L.S., Balakrishnan, M.S., Graves, D.E., Lee, K.R., Winkle, S.A., and Krugh, T.R. (1982) "Evidence of Cooperativity and Allosterism in the Binding of Various Antibiotics and Carcinogens to DNA." in Biological Activities of Polymers, (ed. Carraher, C.E., Jr. and Gebelein, C.G.), American Chemical Society Symposia in Biophysical Sciences, Washington, D.C., pp 269-285.

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Garland, F., Graves, D.E., Yielding, L.W., and Cheung, H.C. (1980) "Comparative Studies of the Binding of Ethidium Bromide and its Photoreactive Analogs to Nucleic Acids by Fluorescence and Rapid Kinetics." Biochemistry 19, 3321-3326.

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