

# TARGETING G-QUADRUPLEXES WITHIN THE ADAM-15 PROMOTER: A NOVEL THERAPEUTIC APPROACH FOR BREAST CANCER

by  
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A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of  
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## **DEDICATION**

This thesis is dedicated to the memory of my mother, Brenda Jenkins. Throughout her eleven-year battle with breast cancer, she lived with courage, strength, humility, and grace. The impact she made on my life and on many others will not be forgotten.

## ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. Tracy Brooks, for her invaluable guidance and support throughout the researching and writing process. She willingly welcomed me into her laboratory, taught me how to conduct research, answered my countless questions, and supported and encouraged me along the way. I could not have completed this project without her, and I am extremely grateful for all of her assistance.

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I am extremely appreciative to my family and friends for their support and encouragement while completing this project. They have always believed in me and pushed me to be the best that I can be.

Finally, I would like to acknowledge my Lord and Savior, Jesus Christ. It is because of His grace, love, and providence that I have reached the place I am in today. My strength, hope, talents, and joy come from Him.

## **ABSTRACT**

**RACHEL LEIGH JENKINS: Targeting G-Quadruplexes within the ADAM-15 Promoter: A Novel Therapeutic Approach for Breast Cancer**  
(Under the direction of Dr. Tracy A. Brooks)

ADAM-15 is a protein that is up-regulated in many diseases, particularly breast cancer; its over-expression is correlated with more aggressive and invasive phenotypes. The critical core promoter region of ADAM-15 is capable of forming a secondary DNA structure known as a G-quadruplex. The stabilization of this G-quadruplex has the potential to decrease the transcription of the over-expressed ADAM-15 protein. Six hundred forty compounds were screened for their ability to cause a shift in the melting temperature of an ADAM-15 oligonucleotide using FRET melt. Two compounds, NSC 146771 and NSC 260594, produced a significant shift in the melting temperature; further experimentation, such as circular dichroism, cytotoxicity MTS assays, and RT-qPCR, was performed to confirm the ability of these small molecules to stabilize the G-quadruplexes within the ADAM-15 promoter. Neither compound showed cytotoxicity, and NSC 260594 showed an increased capacity for reducing the transcription of ADAM-15. Further pharmacokinetic and pharmacodynamic experimentation needs to be completed, but NSC 260594 shows potential to significantly decrease ADAM-15 expression and therefore improve the prognosis of breast cancer patients.

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## LIST OF ABBREVIATIONS

ADAM	a disintegrin and metalloproteinase
Bcl-2	B-cell lymphoma 2
CD	circular dichroism
C <sub>q</sub>	quantification cycle
ddH <sub>2</sub> O	double-distilled water
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
E-cadherin	epidermal cadherin
EGFR	epidermal growth factor receptor
Erk	extracellular signal-regulated kinase
FAM	6-carboxyfluorescein
FBS	fetal bovine serum
FRET	fluorescence resonance energy transfer
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
Grb2	growth factor receptor-bound protein 2
HER2	human epidermal growth factor receptor



HER3	human epidermal growth factor receptor 3
hTERT	human telomerase reverse transcriptase
IC <sub>50</sub>	half maximal inhibitory concentration
IUPAC	International Union of Pure and Applied Chemistry
kRAS	Kirsten rat sarcoma viral oncogene homolog
MCF-7	Michigan Cancer Foundation-7 breast cancer cell line
mRNA	messenger ribonucleic acid
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)- 2-(4-sulfophenyl)-2H-tetrazolium
MW	molecular weight
NCI	National Cancer Institute
NHE	nuclease hypersensitive element
NSC	National Service Center
PBS	phosphate-buffered saline
PDGF-A	platelet-derived growth factor subunit A
PIPER	<i>N,N'</i> -bis-[2-(1-piperidino)ethyl]-3,4,9,10- perylene-tetracarboxylic diimide
PMS	phenazine methosulfate
pRb	phosphorylated retinoblastoma protein
qPCR	quantitative real-time polymerase chain reaction
RFU	relative fluorescence units
RGD	arginylglycylaspartic acid

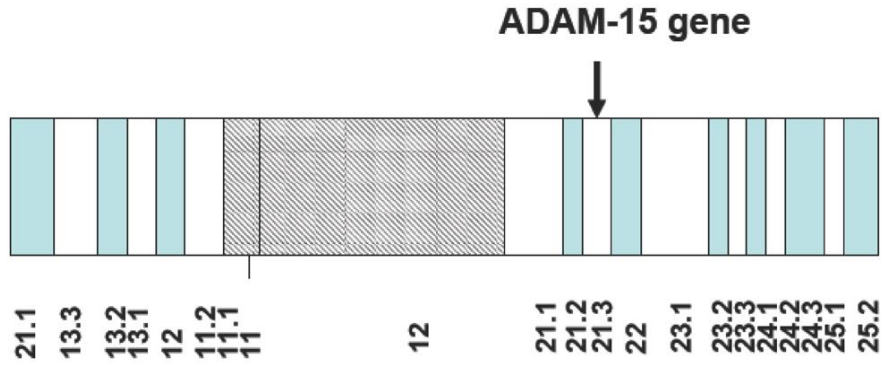
RHPS4	3,11-difluoro-6,8,13-trimethyl-8H-quinol(4,3,2-kl)acridinium methosulfate
rpm	revolutions per minute
sE-cad	soluble epidermal cadherin fragment
SVD	single value decomposition
TAMRA	tetramethylrhodamine
T <sub>M</sub>	melting temperature
TMPyP4	tetra-( <i>N</i> -methyl-4-pyridyl)porphyrin
VEGF-A	vascular endothelial growth factor

## INTRODUCTION

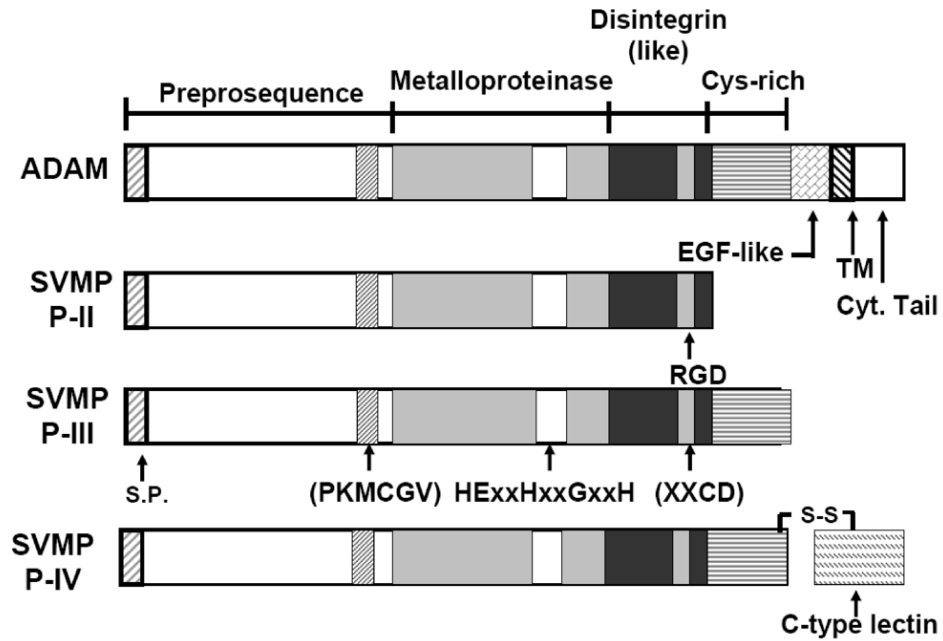
ADAM (A Disintegrin and Metalloproteinase) proteins are membrane-bound cell surface glycoproteins that have a variety of functions in cells, including growth, differentiation, and motility (Lendeckel, 2005). ADAM's basic structure is comprised of modular metalloproteinase motifs, an integrin-binding domain (disintegrin), and a cysteine-rich epidermal growth factor-like domain in the extracellular region (Kuefer, 2006). ADAMs include more than 30 different family members, and these members' functions encompass neurogenesis and angiogenesis, signaling through the epidermal growth factor receptor (EGFR), activating the Notch receptor, and shedding various membrane-bound proteins such as cytokines, growth factors, and adhesion molecules (Lendeckel, 2005; Maretzky, 2009). Members of the ADAM family are normally expressed in early embryonic development, where they play a role in contributing to the homeostasis of the extracellular matrix, organogenesis, transduction of signals, tissue remodeling, adhesion, inflammation, and cell migration (Brown, 2013; Lendeckel, 2005). However, some ADAM family members can become abnormally expressed in several types of diseases, including inflammatory disorders, atherosclerosis and various cancers (Lu, 2010).

The focus of this study is on human ADAM-15, which is known to be up-regulated in many adenocarcinomas, specifically metastatic prostate cancer, breast

cancer, and melanoma (Maretzky, 2009; Kuefer, 2006). ADAM-15 is located on chromosome 1 at position 1q21.3, as shown in **Figure 1** (Lu, 2010). The gene encoding ADAM-15 contains 23 exons and 22 introns; its extracellular domains include a pro-domain, metalloproteinase domain, disintegrin-like domain, cysteine-rich domain, and EGF-like domain, demonstrated in **Figure 2** (Lu, 2010). The cytosolic C-terminal tail of ADAM-15 contains Src homology 2 and 3 recognition sequences, which can bind to adapter proteins such as Grb2, SH3PX1, and endophilin I; thus, this cytoplasmic portion could play a role in signal transduction and protein localization (Najy, 2008). The disintegrin domain of ADAM-15 detaches cells from the extracellular matrix, and the metalloproteinase domain degrades cells from the extracellular matrix (Kuefer, 2006). Notably, ADAM-15 is the only ADAM family member that has the integrin binding motif Arg-Gly-Asp (RGD) in its disintegrin-like domain, which is analogous to a sequence found in snake venom disintegrins (Lu, 2010; Kuefer, 2006). These disintegrins are known to be potent inhibitors of various integrins; therefore, this domain is thought to interfere with cell-cell and cell-matrix interactions (Lu, 2010). In addition to the degradation of collagens I and IV in the extracellular matrix, the metalloproteinase domain of ADAM-15 also plays a role in the ectodomain shedding of growth factors, growth factor receptors, and adhesion molecules (Najy, 2008). The shedding of these molecules can render a tumor cell resistant to growth inhibitory or differentiating signals, which can contribute to the progression of metastasis (Lendeckel, 2005).



**Figure 1.** Location of the ADAM-15 gene on chromosome 1.  
 From Lu D, Scully M, Kakkar V, Lu X. ADAM-15 disintegrin-like domain structure and function. *Toxins*. 2010 October 19; 2(10): 2411-2427.



**Figure 2.** Domain structures of ADAMs compared to snake venom metalloproteinases (SVMP).  
 From Lu D, Scully M, Kakkar V, Lu X. ADAM-15 disintegrin-like domain structure and function. *Toxins*. 2010 October 19; 2(10): 2411-2427.

One of ADAM-15's most significant activities in its role in breast cancer is its promotion of the extracellular shedding of epidermal cadherin (E-cadherin), a type I transmembrane glycoprotein (Brown, 2013; Najy, 2008). E-cadherin is important in cell-cell interactions, and its role is well established in embryonic development, organ morphogenesis, tissue integrity, and wound healing (Najy, 2008). However, the disruption of E-cadherin by proteolytic cleavage leads to the loss of cell-cell integrity, promoting cell migration and invasion (Najy, 2008). Correspondingly, the soluble E-cadherin fragment (sE-cad) produced by this ectodomain shedding is increased in the serum and urine of patients with breast, prostate, ovarian, gastric, melanoma, and bladder cancers and is a marker of poor prognosis (Najy, 2008). Interestingly, this sE-cad fragment binds to and stabilizes the ErbB receptor HER2 and HER3 heterodimerization, leading to Erk-dependent signaling, which causes activation, increased motility, and proliferation in these cancer cells (Najy, 2008; Brown, 2013).

Because of these activities, ADAM-15 is thought to play a significant role in the metastasis of cancers. In fact, higher levels of amplification of the region of chromosome 1 in which ADAM-15 is located are associated with more advanced metastatic cancers, as opposed to lower levels of amplification in primary diseases (Kuefer, 2006). Therefore, there is an increased expression of ADAM-15 as the cancer progresses from low-grade to more advanced and metastatic grades (Kuefer, 2006). ADAM-15 is significantly up-regulated during breast cancer progression, and increased sE-cad levels are also correlated with advanced breast cancer (Najy, 2008). Additionally, the HER2 receptor, to which the sE-cad produced by ADAM-15's activity binds, is over-expressed in 20 - 30% of breast cancers and is also a marker of poor prognosis (Najy, 2008). It has been

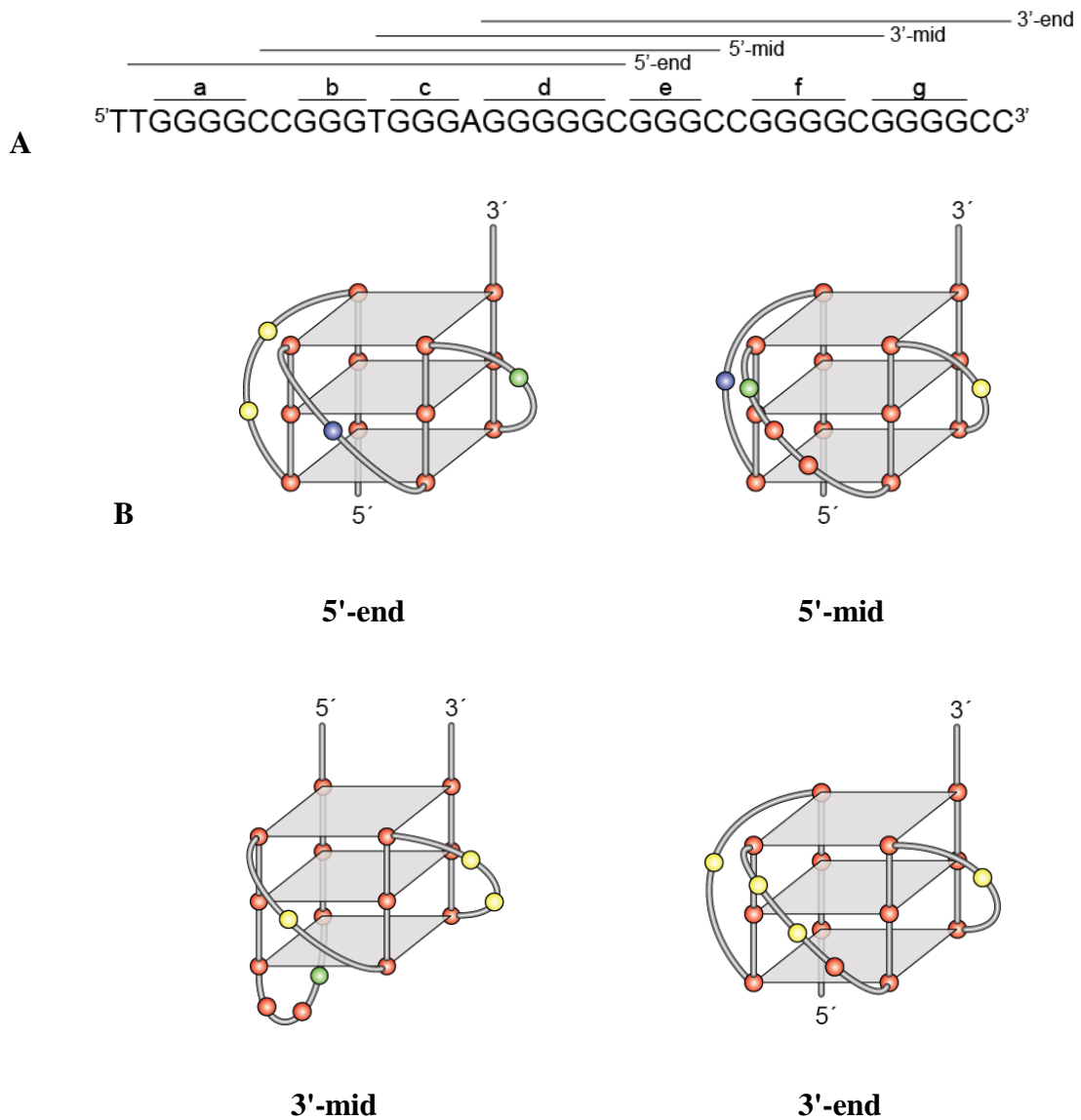
demonstrated that ADAM-15 and HER2 are up-regulated simultaneously during breast cancer progression, and that targeted down-regulation of both of ADAM-15 and HER2 function to synergistically kill breast cancer cells (Najy, 2008; Brown, 2013). However, to date there are no therapeutic options for decreasing ADAM-15 function or expression (Brown, 2013). Thus, the focus of this study was to elucidate novel therapeutic strategies for targeting ADAM-15 in breast cancer cells.

The critical core promoter of ADAM-15 contains a unique string of guanine-rich DNA that consists of seven neighboring runs of three or more consecutive guanines. Under superhelical strain, this region can relax from the double-helical form of DNA and can form an intrastrand secondary-DNA structure known as a G-quadruplex (Brown, 2013). G-quadruplexes were first described by Davies and co-workers in 1962, and since have been found in telomeres, gene promoters, ribosomal DNA, mini-satellites, and the immunoglobulin heavy chain switch region, demonstrating their widely varied biological roles. These structures form through an association of four guanines into a cyclic Hoogsteen hydrogen bonding motif, where each guanine nucleotide base forms two hydrogen bonds with its neighbors; this basic structure is known as a G-quartet (Ou, 2008). Two or more of these G-quartets can then stack on top of each other and are connected by intervening variable-length sequences that form loops on the exterior of the core. These loops are analogous to amino acid side chains, and they are the major elements that define the structural variability in G-quadruplexes (Balasubramanian, 2011). The loops can vary in strand stoichiometry, strand polarity, glycosidic torsion angle, and in the location in which they link the guanine strands (Ou, 2008). Though G-quadruplexes are widespread, the diversity conferred by the loops suggests that a high

level of selectivity could be possible when targeting these structures (Balasubramanian, 2011). It has been shown that many gene promoter G-quadruplex sequences are capable of forming more than one discrete arrangement and that these arrangements may be in dynamic equilibrium with each other (Balasubramanian, 2011). The sequence of contiguous runs of guanines and the major G-quadruplex species formed in the ADAM-15 promoter sequence are shown in **Figure 3**.

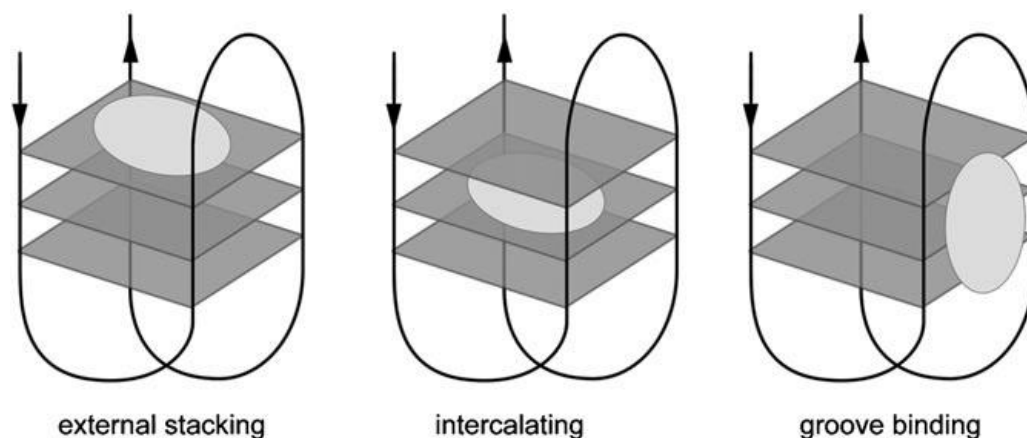
The significance of G-quadruplex formation in the ADAM-15 promoter is its putative ability to control transcription of this gene. G-quadruplexes have been found to form in the promoters of many other oncogenes, including c-MYC, c-Kit, kRAS, pRb, Bcl-2, VEGF-A, hTERT, and PDGF-A. Significantly, altered expression of these oncogenes are recognized as the hallmarks of cancer because they have the capacity for self-sufficiency of growth signals, insensitivity to anti-growth signals, evasion of apoptosis, sustained angiogenesis, limitless replicative potential, and tissue invasion and metastasis (Brooks, 2010). Generally, stabilization of these G-quadruplexes suppresses the genes' expression; for example, stabilization of the G-quadruplex in the NHE III<sub>1</sub> region of the promoter of c-MYC turns down transcriptional activation, down-regulates mRNA expression, inhibits cell proliferation, and induces delayed apoptosis of lymphoma cells (Brown, 2011). Likewise, G-quadruplex formation within the ADAM-15 promoter affects the rate of transcription and leads to a down-regulation of protein expression (Brown, 2013).





**Figure 3.** Guanine sequence and major G-quadruplex species in ADAM-15 promoter sequence. (A) Contiguous runs of guanine in the 5'-3' ADAM-15 promoter sequence (assigned a through g). (B) Models of the different G-quadruplex species formed within the ADAM-15 promoter. From Brown RV, Gaerig VC, Simmons T, Brooks TA. Helping Eve overcome ADAM: G-quadruplexes in the ADAM-15 promoter as new molecular targets for breast cancer therapeutics. *Molecules*. 2013 December 5; 18(12): 15019-15034.

A promising novel anticancer strategy is the stabilization of these G-quadruplexes within gene promoters by small molecules. Traditional DNA-targeted cancer therapies such as alkylating agents are dependent on duplex DNA; such therapies can be very non-specific across the genome and cytotoxic to normal cells (Ou, 2008). However, ideal ligands that bind quadruplexes should have minimal affinity for duplex DNA, would have oncogene-selective binding, and should therefore have a lower toxicity to normal cells (Neidle, 2010). The conformation of G-quadruplexes provides selective recognition sites for small molecules; variations in the sequences and sizes of the loops in the G-quadruplex are likely potential sites for drug binding (Ou, 2008). There are various potential binding modes for small molecules to interact with G-quadruplexes, as illustrated in **Figure 4**. These binding modes include external stacking on the surface of the terminal quartet, intercalating between the stacks of the G-quartets, and groove binding between the loops (Ou, 2008). External stacking and groove binding are thought to be the major mechanisms in which small molecules interact with G-quadruplexes, because intercalator binding between quartets requires a very high energy cost to disrupt the structure of the G-quadruplex (Ou, 2008).



**Figure 4.** Small-molecule-G-quadruplex complex binding modes.  
 From Ou T, Lu Y, Tan J, Huan Z, Wong K, Gu L. G-quadruplexes: targets in anticancer drug design. *ChemMedChem*. 2008 May 19; 3(5): 690-713.

Several groups of small molecules have been identified as having the capability to specifically interact with G-quadruplexes; these compounds include cationic porphyrin (TMPyP4), acridine (BSU6039), polycyclic acridine (RHPS4), and *N,N'*-bis-[2-(1-piperidino)ethyl]-3,4,9,10-perylenetetracarboxylic diimide (PIPER) (Ou, 2008). Many of these compounds target a structural feature common to all G-quadruplexes: the large planar surface of the G-quartet (Balasubramanian, 2011). While the targeting of this structure does allow differentiation from duplex DNA, this feature by itself is insufficient for high affinity and unlikely to confer selectivity (Balasubramanian, 2011). For example, quindoline and berberine are planar aromatic chromophores with one or two aminoalkyl side chains that down-regulate c-MYC expression in several cancer lines. However, their derivatives can also act as telomerase inhibitors and inducers of telomerase damage; therefore, selectivity for one particular type of G-quadruplex is modest. More selective approaches target the ligand side chain binding pockets in the grooves and loops of G-quadruplexes (Balasubramanian, 2011). A first-in-class G-

quadruplex-interacting drug, Quarfloxin, progressed to Phase II clinical trials before the trial was halted due to difficulties with delivery and excessive albumin binding (Brown, 2013). Although bioavailability issues prevented the trial from moving forward, Quarfloxin's toxicity profile was very low, the compound was well tolerated by patients, and objective responses were observed during the Phase I clinical trials (Balasubramanian, 2011). Thus, the future is promising for G-quadruplex-interacting compounds.

The main objective of this study was to identify small molecules capable of stabilizing G-quadruplexes in the promoter sequence of ADAM-15, to determine the effects of these small molecules on the transcriptional control of ADAM-15, and to discern any cytotoxic effects *in vitro*. Because of ADAM-15's significance in the metastasis of breast cancer, and because down-regulation of this gene along with a HER2/neu-targeted agent has the potential to synergistically kill breast cancer cells, this study focused on molecules that facilitated the down-regulation ADAM-15 expression in breast cancer.

## MATERIALS AND METHODS

### I. Compound Identification

#### *FRET Melt*

Twenty 96-well plates were received from the National Cancer Institute/National Institute of Health's Developmental Therapeutics Program, several of which were screened for their ability to stabilize the G-quadruplex within the ADAM-15 promoter. NCI plates 4721, 4722, 4724, 4725, 4727, 4728, 4729, and 4730 were screened; 80 distinct compounds were in each plate, for a total of 640 compounds examined. A master mix of 0.4  $\mu$ M FRET probe (with a G-rich ADAM-15 promoter sequence [5'-TTGGGGCCGGGTGGGAGGGGGCCGGGCCGGGGCC] sandwiched between a 5'-FAM and a 3'-TAMRA moiety), 10 mM sodium cacodylate buffer, 100 mM lithium chloride, and double-distilled water (ddH<sub>2</sub>O) was made, based on a final volume of 25  $\mu$ L/well and 100 wells. This master mix was heated to 95°C for 5 minutes, then allowed to slowly cool to room temperature. While this was cooling, 2.5  $\mu$ L of each compound from the 10x compound stock (100  $\mu$ M, for a final incubation with DNA at 10  $\mu$ M) NCI plates were added to 96-well plates, along with ddH<sub>2</sub>O or potassium chloride to serve as negative and positive controls, respectively. Next, 22.5  $\mu$ L of the room-temperature master mix was aliquoted to each well. The 96-well plate was then vortexed and centrifuged. The fluorescence was read on the CFX Connect Real-Time PCR detection

system in the FAM channel using the FRET MELT program. This program heated the plate from 25 - 95°C at a rate of 2°C/minute; fluorescence was measured every 1°C.

### *Circular Dichroism (CD)*

Circular dichroism was used to validate the hypothesis that the binding of specific small molecules to the ADAM-15 promoter and subsequently stabilized the formed G-quadruplex. A master mix of 5  $\mu$ M ADAM-15 oligonucleotide (or ddH<sub>2</sub>O), 50 mM tris-acetate, 10 mM potassium chloride, and ddH<sub>2</sub>O was made, with enough to have 300  $\mu$ L per spectra. Two hundred seventy microliters of master mix was aliquoted to individual tubes, and 30  $\mu$ L compound from 10x compound stock (100  $\mu$ M) NCI plate was added to each tube (ddH<sub>2</sub>O or 10 mM KCl were used to serve as oligonucleotide controls). These mixtures were heated to 95°C for 10 minutes, then allowed to cool to room temperature overnight. Matching blanks with all contents but lacking DNA were made to subtract background data from each sample. The spectra (225-350 nm) and T<sub>M</sub> (20 - 100°C, measured at every 10°C after holding for 1 minute at temperature before spectra were collected) were run on an Olis CD machine, coupled to a Peltier heat block. Data were converted to a single value decomposition (SVD), which was then used to calculate the T<sub>M</sub> using GraphPad Prism<sup>®</sup>.

## II. Mechanistic Confirmation

### *Cytotoxicity MTS Assay*

A cytotoxicity assay using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was used to assess any cytotoxic effects the identified small molecules had on breast cancer cells (Mosmann, 1983). The breast cancer cell line MCF-7 was grown in Dulbecco's Modified Eagle Medium (DMEM, purchased from Life Technologies) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich) and 1% penicillin/streptomycin mixture. For the MTS assay, the cells were trypsinized with 0.5 - 2 mL of TrypLE (Life Technologies) at 37°C for 3 - 5 minutes until they were detached from the flask surface. The cells were then suspended in 5 mL of media and centrifuged at 4000 rpm for 5 minutes. The supernatant was discarded and the cells were resuspended in 3 - 5 mL of fresh DMEM. A mixture of 10  $\mu$ L of cultured cells and 90  $\mu$ L of Trypan Blue Solution (0.4%, Life Technologies), was made; 10  $\mu$ L of this mixture was placed on a hemocytometer and the cells were counted to determine the stock concentration. Cells were added to a 96-well plate, at  $1.5 \times 10^4$  cells per well for the 24 hour plate and  $5 \times 10^3$  cells per well for the 72 hour plate; each well was plated with a volume of 90  $\mu$ L. These cell plates were incubated overnight. The next day, a 10x drug stock plate was made, with compounds serially diluted over a 5 - 6 log range, starting from a high dose of 1 mM (for a high dose incubating with cells of 100  $\mu$ M). Ten microliters from each well of the 10x drug plate were added to corresponding columns on the cell plate. These plates were incubated for 24 and 72 hours. At the appropriate time point (after 24 and 72 hours), 20 $\mu$ L of 2 mg/mL MTS and 5% phenazine methosulfate (PMS) was added to each well. The plates were

then incubated for 2-4 hours, and the absorbance at 490 nm was read with a Bio-Tek spectrophotometer. Finally, calculations were performed to compute the percentage of viable cells remaining; this was done by subtracting the number of drug blanks from the number of cells, then dividing by the number of control cells and multiplying by 100. The half maximal inhibitory concentration ( $IC_{50}$ ) was then determined by non-linear regression using GraphPad Prism<sup>®</sup>.

### *RT-qPCR*

To determine if the binding of the identified small molecules to the ADAM-15 promoter knocked out the transcription, real-time quantitative polymerase chain reaction (RT-qPCR) was performed. In order to do this, the mRNA had to first be extracted from the MCF-7 breast cancer cell line. For this protocol,  $2 \times 10^5$  cells were plated per well in 1 mL of supplemented DMEM in a 12-well plate; these plates were incubated overnight. The next day, variable amounts (25, 50, or 100  $\mu$ M) of the identified small molecules, or TMPyP4 (a known stabilizer of G-quadruplexes), were added to the cell plates. DMSO and TMPyP4 were used as vehicle and positive controls, respectively. The plates were incubated for 48 hours.

To extract the mRNA from the cell cultures, the GeneJET RNA Purification Kit (Life Technologies) was used, following the "Mammalian Cultured Cells Total RNA Purification" protocol. Briefly, the media was poured off of the cell plates, and the cells were washed twice with PBS to remove residual medium. The cells were detached from the plate and lysed by 350  $\mu$ L of the Lysis Buffer supplemented with 1%  $\beta$ -



mercaptoethanol; the samples were then vortexed for 10 seconds to thoroughly homogenize the mixture. Next, 360  $\mu\text{L}$  of 100% ethanol was added to the samples, which were mixed by pipetting. Up to 700  $\mu\text{L}$  of the lysates was transferred to the GeneJET RNA Purification Column, which was placed in a collection tube and centrifuged at 12000 rpm for 1 minute. The flow-through was discarded, and the purification column was placed in a new collection tube. Seven hundred microliters of Wash Buffer 1, supplemented with ethanol, were added to the purification column, and the column was then centrifuged at 12000 rpm for 1 minute. The flow-through was again discarded. Next, 600  $\mu\text{L}$  of Wash Buffer 2, supplemented with ethanol, was added to the purification column, and the column was again centrifuged at 12000 rpm for 1 minute. The flow-through was discarded, and an additional 250  $\mu\text{L}$  of Wash Buffer 2 was added to the purification column, which was centrifuged at 12000 rpm for 2 minutes. Flow-through was discarded, and the purification column was placed in a sterile, RNase-free microcentrifuge tube. Finally, 100  $\mu\text{L}$  of nuclease-free water was added to the center of the purification column membrane, and the tubes were centrifuged at 12000 rpm for 1 minute to elute the RNA. The concentrations of the eluted mRNA were measured using a NanoDrop 2000 UV-Vis Spectrophotometer.

After the mRNA was extracted, it was used as a template to make complementary DNA through reverse transcription. This was accomplished using the Maxima H Minus First Strand cDNA Synthesis Kit, with dsDNase (Life Technologies). To eliminate the genomic DNA, a mixture of 1  $\mu\text{L}$  10X dsDNase Buffer, 1  $\mu\text{L}$  dsDNase, 500 ng of template RNA, and variable amounts of nuclease-free water was placed on ice. This mixture was then incubated for 2 minutes at 37°C, chilled on ice, centrifuged, and placed

again on ice. Next, 25 pmol of both oligo (dT)<sub>18</sub> primer and a random hexamer primer supplied with the kit were added to the tubes, along with 1 µL of 10 mM dNTP Mix and variable amounts of nuclease-free water. The tubes were centrifuged, and 4 of µL 5X RT Buffer and 1 µL Maxima H Minus Enzyme Mix was added. The samples were centrifuged again, and incubated for 10 minutes at 25°C, followed by 22 minutes at 50°C. The reaction was terminated by heating the samples at 85°C for 5 minutes.

The products from this reverse transcription reaction were then used for RT-qPCR. A master mix of Taq buffer (SSoFast Mix, Bio-Rad), FAM-labeled ADAM-15 TaqMan primers (ABI), VIC-labeled GAPDH TaqMan primers (Bio-Rad), and water was made. Sixteen microliters of the master mix was placed into each well on a PCR plate, and 4 µL of the synthesized cDNA was then added in each well. The PCR plate was then placed in a CFX Connect Real-Time PCR Detection System, and a two channel, two-step fast amplification was run. Expression was calculated by normalizing the quantification concentration ( $C_q$ ) of ADAM-15 to the  $C_q$  of the control, GAPDH, using the equation  $\Delta C_{q\ S1} = C_{q\ FAM} - C_{q\ VIC}$ . Next, the sample effect was normalized to the control using the equation  $\Delta\Delta C_{q\ S1} = \Delta C_{q\ S1} - \Delta C_{q\ control}$ . Finally, the fold change over expression was calculated by  $2^{-\Delta\Delta C_q}$  (Haimes, 2010).

## RESULTS AND DISCUSSION

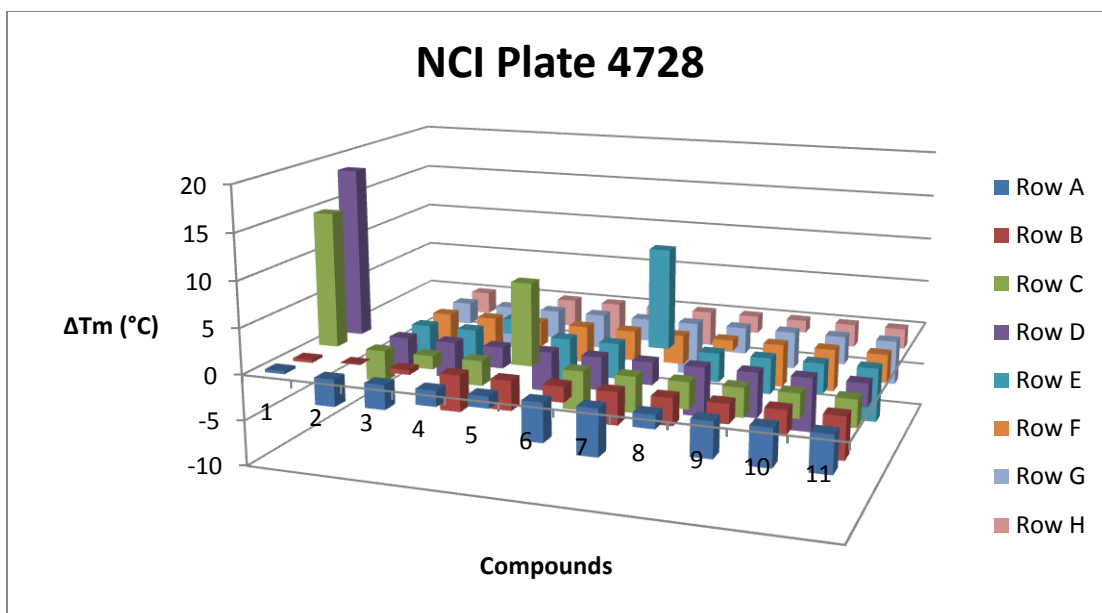
### I. Compound Identification

#### *FRET Melt*

Because fluorescence resonance energy transfers are distance- and orientation-dependent, they can give valuable information about the structure of nucleic acids (Ou, 2008). A fluorescence donor and acceptor were attached to opposite ends of the ADAM-15 oligonucleotide. When screened with various small molecules, if the ligand stabilized the G-quadruplex formed within the ADAM-15 promoter, the melting temperature increased. Six hundred forty compounds were screened, and two distinct compounds gave consistent increases in the melting temperature of ADAM-15, as shown in **Table 1** and **Figure 5**.

**Table 1.** Shift in thermal stability of ADAM-15 oligonucleotide with compounds from NCI Plate 4728. Compounds in wells C5 and E7 increased the melting temperature.

	1	2	3	4	5	6	7	8	9	10	11
A	0.33	-2.96	-2.66	-1.7	-1.26	-4.21	-5.03	-1.46	-3.82	-4.05	-4
B	-0.33	0.08	-0.51	-3.98	-3.23	-1.72	-3.51	-2.93	-2.08	-2.52	-4.49
C	14.86	-3.42	-1.48	-2.75	8.97	-4.3	-3.94	-2.91	-3.17	-2.65	-2.97
D	18.66	-3.76	-4.03	-2.37	-4.41	-3.69	-2.53	-5.45	-4.96	-5.86	-2.52
E		-4.24	-4.28	1.73	-5.45	-3.99	11.05	-3.2	-3.99	-3.31	-5.85
F		-3.9	-4.98	-2.85	-4.19	-3.41	-3.28	-1.2	-4.78	-4.71	-3.08
G		-2.48	-4.97	-3.22	-3.93	-2.52	-6.06	-3	-4.2	-3.17	-4.9
H		-2.51	-2.49	-3.16	-4.26	-2.92	-3.93	-1.97	-1.41	-2.57	-2.28



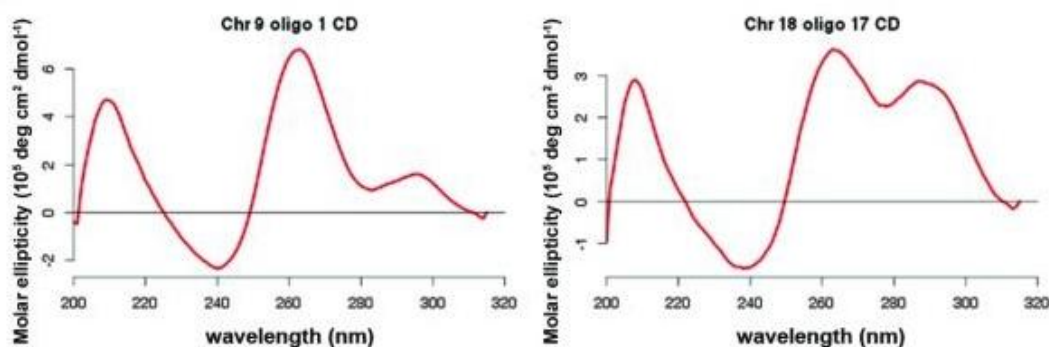
**Figure 5.** ADAM-15's shift in melting temperature when bound with ligands from NCI Plate 4728. Column 1, Rows C and D, contained KCl, a known potent G-quadruplex stabilizer, to serve as a reference.

The two compounds that showed increased melting temperatures, C5 and E7 on NCI plate 4728, correspond to NSC compounds 146771 and 260594, respectively. With repeated screenings, NSC 146771 had an average increase in melting temperature of  $11.88 \pm 2.52$  °C. NSC 260594 had an average increase in melting temperature of  $11.97 \pm 1.35$  °C. These increases in melting temperature suggest the ability of the small molecules to stabilize the G-quadruplex within the ADAM-15 promoter region.

### *Circular Dichroism*

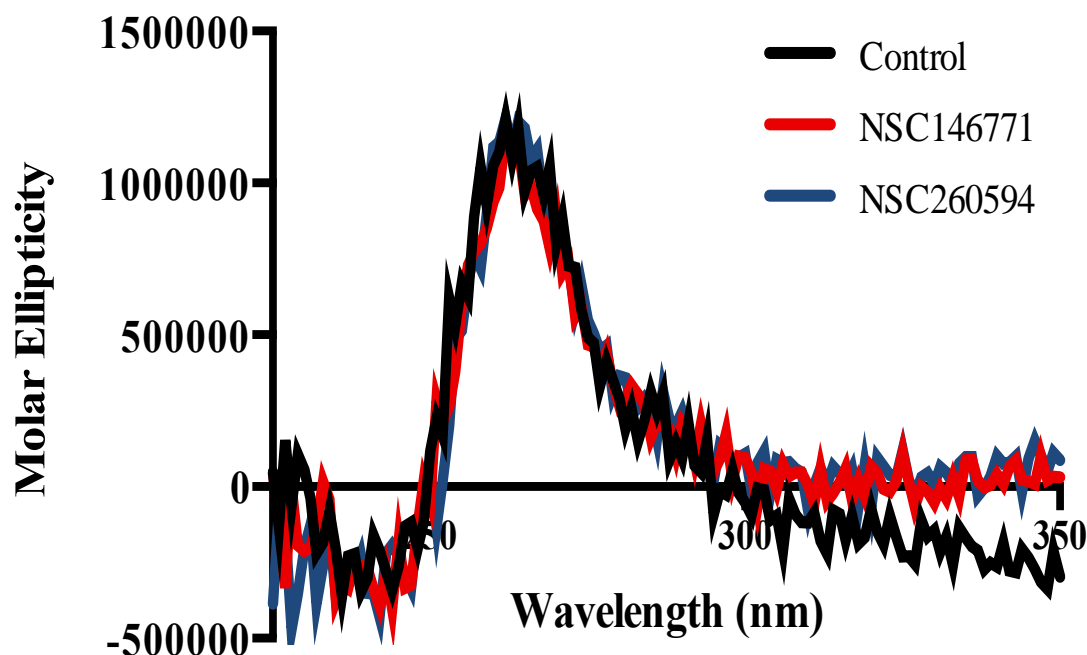
To confirm that these two ligands could stabilize the formation of a G-quadruplex within the ADAM-15 promoter, electronic circular dichroism (CD) was used to

determine asymmetrical structure formation – e.g. the G-quadruplex – and its thermal stability. In general, non-chiral molecules such as the NSC compounds are CD-inactive; however, DNA within the G-quadruplexes give characteristic spectral maxima and minima, as shown in **Figure 6**. The negative minima of a G-quadruplex will be at about 240 nm; for a parallel G-quadruplex, the positive maxima will be between 260 - 265 nm, and for an anti-parallel G-quadruplex, there will be a positive maxima at about 290 nm.



**Figure 6.** Characteristic circular dichroism spectra for parallel and anti-parallel G-quadruplexes. The spectra for "Chr 9 oligo 1" shows the characteristic peak and trough for a parallel G-quadruplex. The spectra for "Chr 18 oligo 17" shows the characteristic peaks and trough for a mixed parallel (260 nm maxima) and an anti-parallel (290 nm maxima) G-quadruplex. From Lam EY, Beraldi D, Tannahill D, Balasubramanian S. G-quadruplex structures are stable and detectable in human genomic DNA. *Nat Commun.* 2013 April; 4(1796). **Figure 3:** Motif and CD analyses substantiate G-quadruplex identification.

To verify that the NSC compounds 146771 and 260594 stabilized the G-quadruplexes, the CD spectra for the ADAM-15 oligonucleotide were measured with and without the NSC compounds. If the spectra with the compounds gave off similar signals as what are characteristic for G-quadruplexes, then the hypothesis that these compounds stabilized the G-quadruplex could be supported. The spectra, in units of molar ellipticity, for the control, NSC compound 146771, and NSC compound 260594 are shown in **Figure 7**.



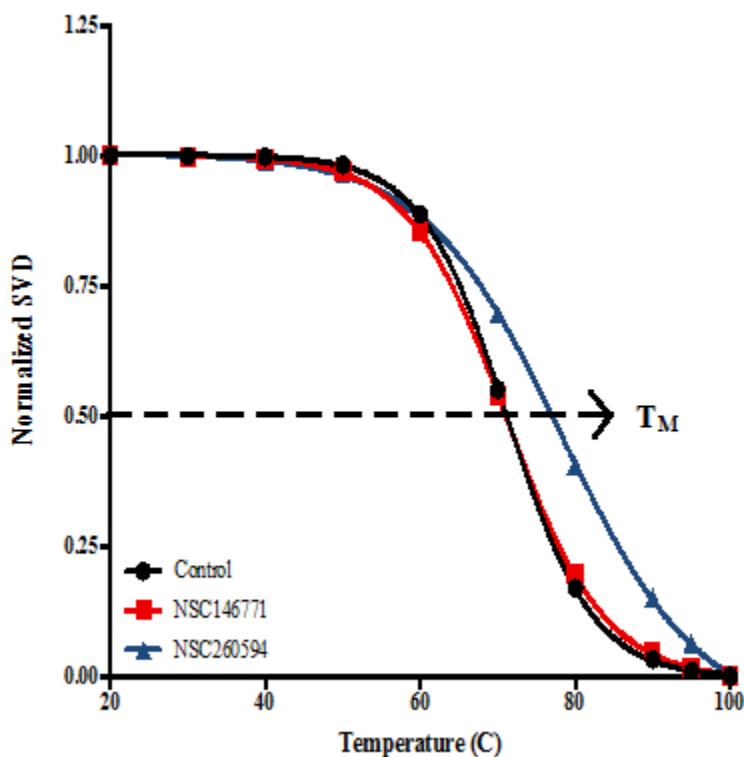
**Figure 7.** Circular dichroism spectra for NSC compounds 146771 and 260594.

For NSC compound 146771, the negative minima was at 244 nm, and the positive maxima was at 262 nm. For NSC compound 260594, the negative minima was at 242 nm, and the positive maxima was at 262 nm. Both of these spectra are indicative of parallel G-quadruplex formation within the ADAM-15 promoter, supporting the hypothesis that these compounds stabilize this predominant secondary DNA structure.

From the thermal circular dichroism experiment, the melting temperature ( $T_M$ ) of the DNA, or the temperature at which half of the DNA is present as an intramolecular G-quadruplex, and half of the DNA is present in single-stranded form, was calculated. If the melting temperature for the ADAM-15 oligonucleotide, in combination with one of the NSC compounds, increased, it could be surmised that the compound improved the

stability of the G-quadruplex, and thus improved the overall stability of the nucleic acid.

The results from the  $T_M$  calculations are shown in **Figure 8**.



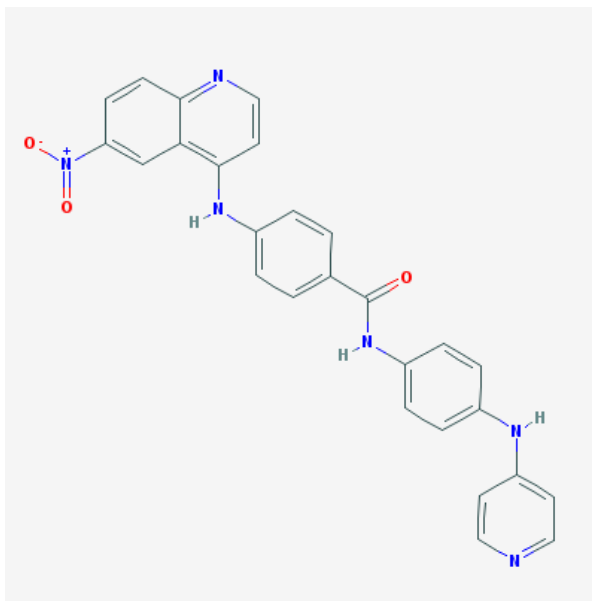
**Figure 8.** Melting temperatures ( $T_M$ ) of ADAM-15 oligonucleotide with NSC compounds 146771 and 260594.

As shown above, NSC compound 146771 did not notably affect the melting temperature of the ADAM-15 oligonucleotide. However, NSC compound 260594 did increase the ADAM-15 oligonucleotide's melting temperature by an average of 7.1 °C. Thus, it can be concluded that NSC compound 260594 can bind to the G-quadruplex within the ADAM-15 promoter and improve its stability.

## II. Compound Characterization

### NSC 146771

The first compound to show affinity for the ADAM-15 G-quadruplex was NSC 146771, found in well C5 of NCI Plate 4728. This compound has a chemical formula of  $C_{27}H_{20}N_6O_3$ , and its IUPAC name is 4-[(6-nitroquinolin-4-yl)amino]-N-[4-(pyridin-4-ylamino)phenyl]benzamide (PubChem: CID 4532204). The 2D structure and 3D conformer are shown in **Figure 9** and **Figure 10**, respectively. NSC 146771 has a molecular weight of 476.4861 g/mol, its  $\log P$  value is 4.9, and it has 3 hydrogen-bond donors and 7 hydrogen-bond acceptors (PubChem: CID 4532204). Therefore, according to Lipinski's Rule of Five, this compound is predicted to be membrane permeable and easily absorbed across the body (Leeson, 2012).



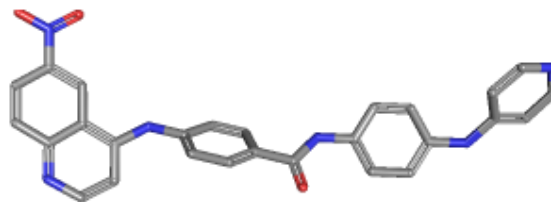
**Figure 9.** 2D structure of NSC compound 146771.

From PubChem Compound [Internet]. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information. Compound Summary for: CID 4532204.

Available from:

<http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?sid=51987431&viewopt=PubChem#itabs-2d>





**Figure 10.** 3D conformation of NSC compound 146771.

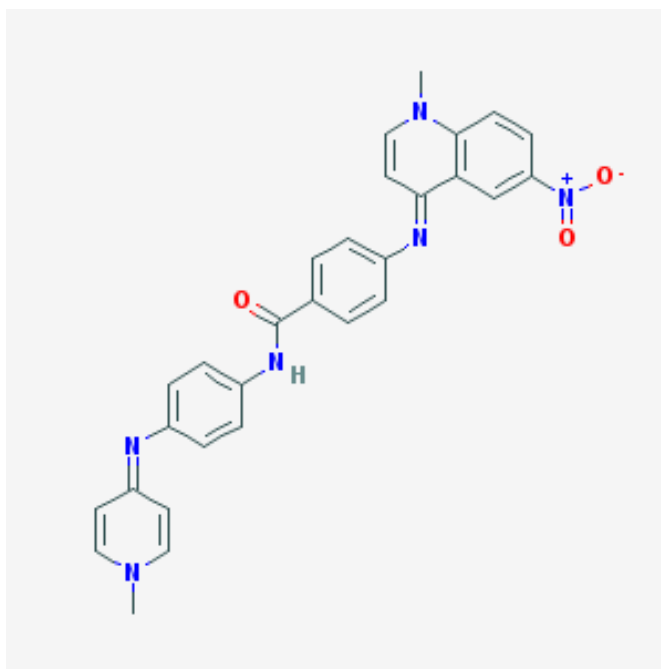
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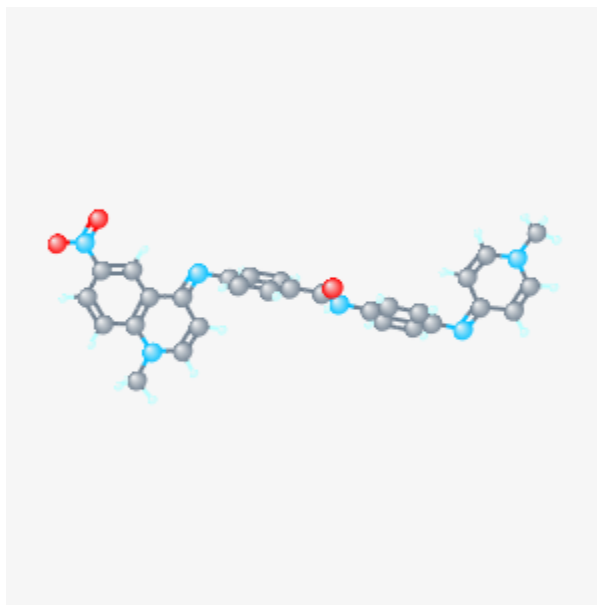
<http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?sid=51987431&viewopt=PubChem#itabs-2d>

#### *NSC 260594*

The second compound to show affinity for the G-quadruplex within the ADAM-15 promoter was NSC 260594, found in well E7 of NCI Plate 4728. This compound has a chemical formula of  $C_{29}H_{24}N_6O_3$ , and its IUPAC name is 4-[(1-methyl-6-nitroquinolin-4-ylidene)amino]-N-[4-[(1-methylpyridin-4-ylidene)amino]phenyl]benzamide (PubChem: CID 319089). The 2D structure and 3D conformer of this compound are shown in **Figure 11** and **Figure 12**, respectively. NSC 260594 has a molecular weight of 504.53926 g/mol, its  $\log P$  value is 4.4, and it has 1 hydrogen-bond donor and 7 hydrogen-bond acceptors (PubChem: CID 319089). Although its molecular weight is at the upper end of the MW guidelines, this compound would still be predicted to easily cross membranes and be absorbed according to Lipinski's Rule of Five.



**Figure 11.** 2D structure of NSC compound 260594.  
From PubChem Compound [Internet]. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information. Compound Summary for: CID 319089.  
Available from: <http://pubchem.ncbi.nlm.nih.gov/compound/319089#section=Top>

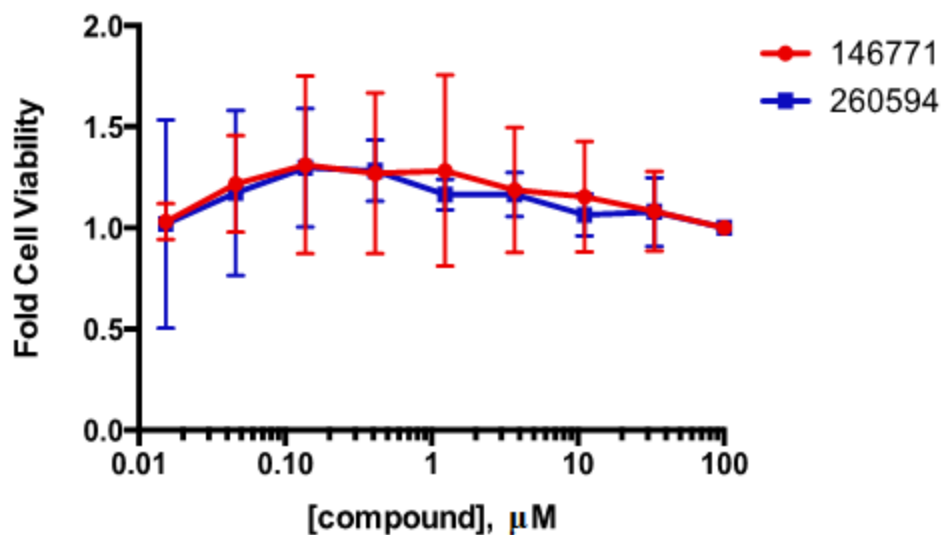


**Figure 12.** 3D conformation of NSC compound 260594.  
From PubChem Compound [Internet]. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information. Compound Summary for: CID 319089.  
Available from: <http://pubchem.ncbi.nlm.nih.gov/compound/319089#section=Top>

### III. Mechanistic Confirmation

#### *Cytotoxicity MTS Assay*

An MTS assay was done to investigate if the NSC compounds 146771 and 260594 had any cytotoxic effects on breast cancer cells. If the compounds were cytotoxic to the cells, they could cause apoptosis or necrosis, leading to loss of membrane integrity and release of the cells' contents. The MTS assay measures the cells' ability to reduce 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), in the presence of phenazine methosulfate (PMS), to a colored formazan product. Viable, or alive, cells are able to reduce the tetrazolium salt in their mitochondria, whereas non-viable cells cannot form the formazan product. The results of this assay showed that these two compounds did not cause cytotoxicity within the MCF-7 breast cancer cell line (**Figure 13**). As ADAM-15 is a zymogen involved in metastasis and invasion, but not cell growth and proliferation, this result was anticipated if the ADAM-15 promoter G-quadruplex is the primary structure being stabilized by these compounds. The lack of cytotoxicity may also indicate that they were not modulating any genes at all; thus, the effects of the NSC compounds on gene expression need to be evaluated by measuring changes in mRNA.



**Figure 13.** MCF-7 cell viability after MTS assay done with NSC compounds 146771 and 260594.

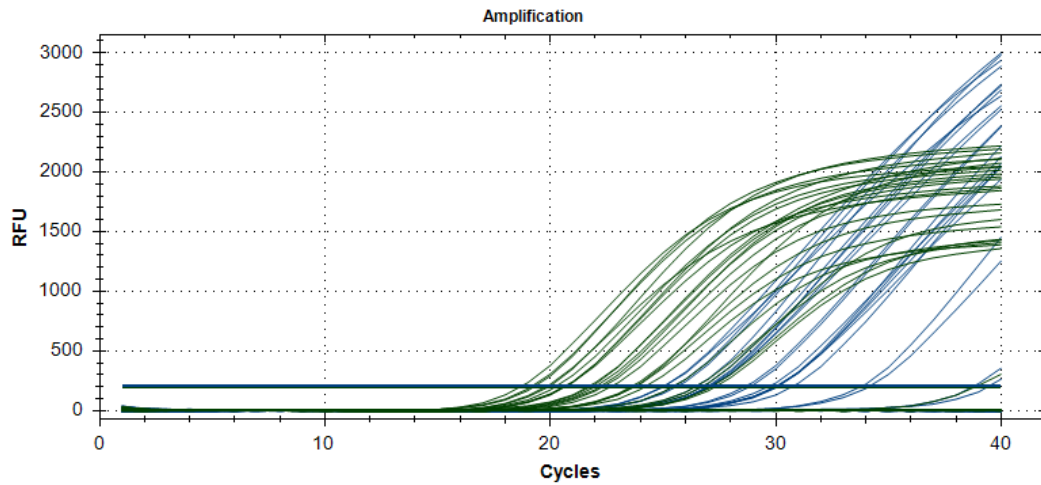
### *RT-qPCR*

To validate the hypothesis that small molecule stabilization of G-quadruplexes within the ADAM-15 promoter could reduce transcription, quantitative polymerase chain reaction (qPCR) was performed. A TaqMan<sup>®</sup> probe-based assay was employed, which allowed for specific hybridization between the primer-probe pair and the gene target. Two particular primer-probe pairs were used: a FAM-labeled ADAM-15 probe-primer pair, to measure the gene of interest, and a VIC-labeled GAPDH probe-primer pair, to measure the housekeeping gene and normalization control. When the probes are intact, the quencher dye on the 3'-end greatly reduces the fluorescence emitted by the reporter dye on the 5'-end because of their close proximity. However, as cDNA of the target sequence is amplified, the annealed probe is displaced by extension from elongation of an upstream primer, and is cleaved by the 5' nuclease activity of Taq DNA polymerase.

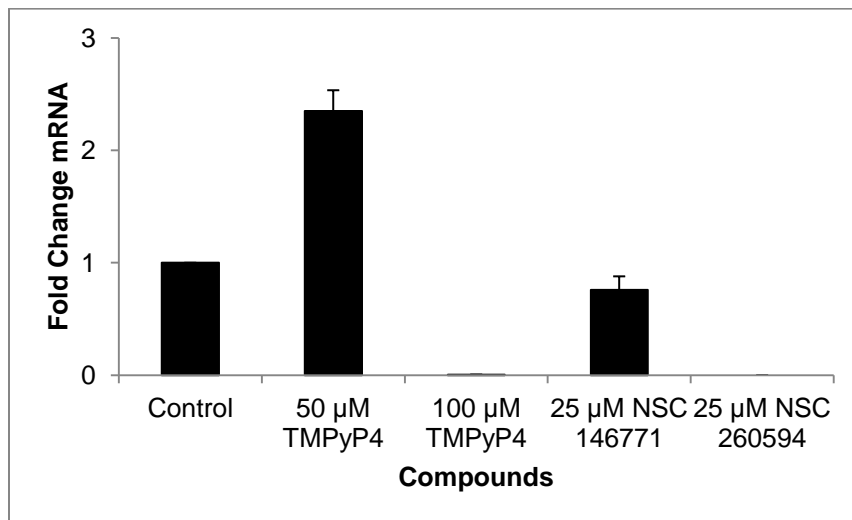
This cleavage separates the reporter dye from the quencher dye; therefore, the emission from the reporter dye is increased. Additionally, since the probe has been removed from the target strand, the primer can continue to extend to the end of the template strand. With each cycle, additional reporter dyes are cleaved from their respective probes, which results in an increase in fluorescence intensity proportional to the amount of mRNA that is amplified (Life Technologies, 2015). **Figure 14** shows the amplification of VIC-labeled GAPDH in green and of FAM-labeled ADAM-15 in blue. GAPDH served as a control because of its low variability within a tissue; as expected, this amplified during the polymerase chain reaction. ADAM-15 was also amplified from untreated and vehicle-controlled wells; when NSC compounds 146771 and 260594 were added, ADAM-15's amplification curve showed dose-dependent message knockdown, as evidenced by a right-hand shift in the fluorescence detection curves. The higher C<sub>q</sub> values attained when the NSC compounds were added corresponded to lower expression; therefore, these compounds showed effectiveness in silencing the ADAM-15 transcript.

To assess the NSC compounds' ability to silence the ADAM-15 gene, relative gene expressions were calculated with the  $\Delta\Delta C_q$  method, using GAPDH as a reference gene, and normalizing to vehicle control treated cells (**Figure 15**). One hundred micromolars of TMPyP4 (a known G-quadruplex stabilizer) significantly decreased expression of ADAM-15 by over 99%. Twenty-five micromolars of NSC compound 146771 trended toward decreased expression of ADAM-15, but did not reach statistical significance (p=0.06). However, 25  $\mu$ M of NSC compound 260594 completely knocked out the expression of ADAM-15, relative to control. Together, these data support that two compounds identified *ex vivo* show potential to regulate the ADAM-15 promoter G-

quadruplex *in vitro* and to silence transcription. In particular, the more stabilizing compound NSC 260594 significantly decreased ADAM-15 expression. This compound warrants further testing in a dose-dependent manner, and more pre-clinical testing in an expanded cell line panel and *in vivo*.



**Figure 14.** Amplification of GAPDH (control) and ADAM-15. VIC-labeled GAPDH amplification is indicated in green, and FAM-labeled ADAM-15 amplification is indicated in blue.



**Figure 15.** Expressivity of ADAM-15 mRNA in MCF-7 cells after treatment with NSC compounds 146771 and 260594.

## CONCLUSION

Metastatic breast cancer is a very serious disease that affects an estimated 162,000 women in the United States per year (MBCN, 2015). While the survival rates for many cancers have improved in recent years, the 5-year relative survival rate for metastatic (Stage IV) breast cancer remains at only 22% (American Cancer Society, 2014). Because metastatic breast cancer can be so deadly, there is a great need for improved therapeutic options. A novel approach is the targeting of ADAM-15, a protein whose expression is up-regulated in the more aggressive and invasive breast cancer phenotypes. The critical promoter region of ADAM-15 contains a guanine-rich sequence that is capable of forming alternate DNA structures known as G-quadruplexes; the stabilization of these G-quadruplexes has the potential to reduce transcription of this over-expressed ADAM-15 protein (Brown, 2013). In the current study, 640 compounds were screened for their ability to stabilize ADAM-15 promoter's G-quadruplexes. Two compounds, NSC 146771 and NSC 260594, demonstrated putative ability to stabilize the G-quadruplexes by causing an increase in the melting temperature. These compounds did not cause cytotoxicity, and NSC 260594 was able to knock out the transcription of ADAM-15. Future areas of study include conducting cell migration and invasion assays to assess if these compounds, particularly NSC 260594, are capable of preventing metastasis of breast cancer cells. Other future areas of research include assessing the ability of these small molecules to be optimized through analog development, preclinical testing, and

clinical testing into FDA approved drugs. In particular, their selectivity for the ADAM-15 promoter G-quadruplex, their cancer-versus-normal specificity, and their pharmacokinetic properties such their absorption, distribution, metabolism, and excretion (ADME) need to be enhanced.

Overall, G-quadruplex stabilizers have the potential to revolutionize the treatment of many cancers. G-quadruplexes can be found in telomeres and various oncogene promoters; when these structures are stabilized, transcription is decreased and cancer progression putatively stops. One of ADAM-15's most significant activities is the cleavage of E-cadherin; this cleavage disrupts cell-cell contact and considerably contributes to metastasis. Additionally, the solubilized E-cadherin fragment stabilizes the heterodimerization of HER2 and HER3, leading to increased intracellular signaling and further contributing to metastasis and proliferation. Furthermore, ADAM-15's over-expression in breast cancer is coincidental with the up-regulation of HER2; thus, these small molecules targeted towards ADAM-15 promoter's G-quadruplexes have the potential to be used in combination with HER2-targeted drugs such as Trastuzumab to improve its efficacy and increase the duration and quality of life in thousands of breast cancer patients a year.



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Figure 3: Motif and CD analyses substantiate G-quadruplex identification.

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