

Exploring the Immunogenicity and Therapeutic Applications of Boranophosphate-
modified RNA: siRNA and RNA Aptamers

by

Mariam Lucila Sharaf

Department of Chemistry
Duke University

Date: _____

Approved:

Barbara Ramsay Shaw, Supervisor

Katherine J. Franz

Thom LaBean

Bruce Sullenger

Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
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ABSTRACT

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Abstract

Borane (BH_3) chemistry offers unique chemical characteristics that enable boranophosphate (BP) oligonucleotides with potential to enhance RNA therapeutic applications such as RNA interference (RNAi) and RNA aptamers. Further, BP nucleotides are substrates for RNA polymerases which allow the enzymatic synthesis of stereoregular boranophosphate (BP)-RNA molecules of different lengths and properties. We expect that these BP-RNAs will interact in a novel way with the desired target molecules because they can coordinate with a diverse array of ligand sites in proteins or other RNA molecules. This is due to the distinct hydrophobicity, stereospecificity, and polarity properties imparted by the phosphorus-boron (P-B) chemical bond compared to the natural phosphorus-oxygen (P-O) bond.

The object of this dissertation is to explore the therapeutic applications of the BP-RNA such as siRNA, RNA aptamers, and in addition investigate the immunogenicity of this modification. We used mouse cells to determine if BP-RNA would activate toll-like receptor (TLR 7), which is involved in innate immune response to foreign single stranded RNA (ssRNA). This response is undesired when applied to oligonucleotide therapeutics such as siRNA and RNA aptamers. In terms of RNAi, it would be an advantage to have low immunogenicity and high downregulation activity by the siRNA. To determine the innate immune activation of the BP-RNA through the TLR 7 we used a known activator, the human immunodeficiency virus (HIV) derived single-stranded

RNA (ssRNA40) and measured the production of cytokines as a function of the number of modified BP-linkages. The production of cytokines IL-6 and TNF α was quantified after the boranophosphate (BP), phosphorothioate (PS) or natural ssRNA40 were transfected into murine macrophage Raw264.7 cells. Natural and phosphorothioate RNA (PS-RNA) have been shown to be activators of TLR 7 receptors. In contrast, we found that fully modified BP- ssRNA40 did not activate TLR 7. This is relevant in oligonucleotide applications such as siRNA and RNA aptamers where off-target effects such as immune activation after administration are not desired.

Subsequently, the low immune activation would be an advantage when coupled to RNAi activity of the oligonucleotide. Thus, we explored whether BP modified siRNA molecules would modulate gene expression and if there was an effect on downregulation activity when increasing the number of BH₃ modifications on the phosphate backbone. Our therapeutic model was the multi-drug resistance 1 (MDR1) gene that expresses *P*-glycoprotein (*P*-gp), which has been notoriously difficult to modulate. The aberrant regulation of genes such as MDR1 in cancer cells are a major cause of chemotherapeutic treatment failure against human cancers. Hence, controlling the expression of cancer genes with antisense technology is a possible cancer therapy. Specifically, correcting the overexpression of *p*-glycoprotein using modified siRNAs that target and degrade the *P*-glycoprotein mRNA produced by the MDR1 gene. We found that there is a reduction of siRNA activity with an increasing number of BP-

modifications. It appears that there is a fine balance between lack of immune response and gene downregulation when applied to BP-siRNA.

Finally, we compared the enrichment during the Systematic Evolution of Ligands by EXponential enrichment (SELEX) method of two libraries, one BP-RNA (U α B) compared to a doubly-modified RNA (2'FC & U α B), against a human thrombin. Aptamers modulate protein activity and interfere with protein signaling by binding to the desired protein with high affinity and specificity leading to their use in therapeutic applications where protein activity needs to be controlled or it is anomalous. In the case of blood coagulation, thrombin plays a central role in coagulation signaling cascade and it is a good target to use to control blood coagulation in clinical settings. We attempted to optimize the selection of BP- RNA aptamers through 4-8 rounds of SELEX against the protein thrombin. We found that the selection conditions were not optimal for BP-RNA SELEX possibly due to non-specific binding to a bovine serum albumin (BSA) in the selection buffer.

This dissertation is dedicated to my family: my husband Jesse Hastings, my parents Nematullah Sharaf and Rocio Palma, my siblings Amina, Naima and Nemat, and my nephew Julius. Without their endless love, motivation and support, during graduate school and always, this would not have been possible.

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List of Abbreviations

a-P-borano Nucleotides and oligonucleotides modified with BH_3 on the α -phosphorus atom

A260 UV Absorbance at 260 nanometers

AP Antarctic phosphatase

AMV RT Avian Myeloblastosis Virus Reverse Transcriptase

bp Base pairs

BP Boranophosphate

BP-RNA Boranophosphate-modified RNA

BP-DNA Boranophosphate-modified DNA

BSA Bovine serum albumin

CD Circular Dichroism

cDNA complementary DNA

CHAPS 3[(3-Cholamidopropyl)dimethylammonio]-propanesulfonic acid

dATP 2'-Deoxyadenosine 5'-triphosphate

dCTP 2'-Deoxycytidine 5'-triphosphate

dGTP 2'-Deoxyguanosine 5'-triphosphate

dsDNA Double-stranded DNA

dsRNA Double-stranded RNA

dTTP Thymidine 5'-triphosphate

dNTP dATP + dCTP + dGTP + dTTP

DEPC Diethylpyrocarbonate ($C_6H_{10}O_5$)

DI Water Deionized water

DNA Deoxyribonucleic acid

DNase Deoxyribonuclease

DTT Dithiothreitol ($C_4H_{10}O_2S_2$)

E₂₆₀ Molar absorptivity at 260 nanometers

EDTA Ethylenediaminetetraacetic acid ($C_{10}H_{16}N_2O_8$)

FBS Fetal Bovine Serum

HPLC High performance liquid chromatography

mRNA Messenger RNA

NMR Nuclear magnetic resonance spectroscopy

NMP Nucleotide monophosphate

nt. Nucleotide

NTP Nucleotide triphosphate

ODN Oligodeoxynucleotide (DNA backbone)

PAGE Polyacrylamide gel electrophoresis

PBS Phosphate Buffered Saline

PCR Polymerase Chain Reaction

PNK Polynucleotide kinase

PS Phosphorothioate

RISC RNA interference silencing complex

RNA Ribonucleic acid

RNase Ribonuclease

RNAi RNA interference

rt. Room temperature

rNTP UTP, CTP, GTP, ATP

rNTP α B rUTP α B, rCTP α B, rGTP α B, rATP α B

SELEX Systematic Evolution of Ligands by EXponential enrichment

SD Standard deviation

siRNA Small interfering RNA

ssRNA Single-stranded RNA

TEA Triethylamine (C₆H₁₅N)

TEAA Triethylamine acetate (C₈H₁₉NO₂)

TEAB Triethylammonium bicarbonate (C₇H₁₇NO₃)

TEMED N,N,N',N'-Tetramethylethylenediamine (C₆H₁₆N₂)

TLR Toll-like receptor

T_m Melting temperature

Tris Tris(hydroxymethyl)aminomethane (C₄H₁₁NO₃)

UV Ultraviolet

wt wild type

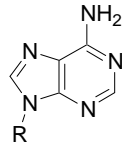
2'-F 2' Fluoro modification on the 2' position of the ribose

2'-OMe 2'-O-methyl modification

Chapter 1 - Introduction

Nucleic acids are the fundamental building blocks of living cells and have the potential for self-duplication. Nucleic acids store and transmit genetic information for every cell, tissue and organism. There are two types of nucleic acids: Ribonucleic acid (RNA) and deoxyribonucleic acid (DNA). RNA and DNA are long chains of building blocks called nucleotides, each of which consists of a nucleobase, a sugar (ribose for RNA or deoxyribose for DNA), and a phosphate group. The difference between RNA and DNA is that RNA contains a 2'-OH on the sugar moiety and uses uracil instead of thymine for base pairing to adenosine (**Figure 1**). The nucleobase is arranged in a particular sequence that is capable of storing and transmitting genetic information; these bases are linked to the phosphodiester-linked sugar residues which form the backbone of RNA or DNA.

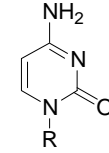
DNA/RNA



Adenine

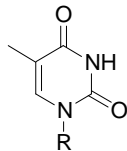


Guanine



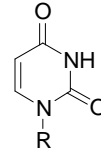
Cytosine

DNA



Thymine

RNA



Uracil

Figure 1 - Nucleobase structures found in DNA or RNA. Adenine, guanine and cytosine base structures are found in both RNA and DNA. However, RNA contains uracil and DNA has thymine. Thymine is simply 5-methyluracil.

The flow of genetic information, from DNA to RNA to protein is referred to as the central dogma of biology. Messenger RNA (mRNA) is used to transfer information from DNA to the cellular machinery that directs the synthesis of proteins. This transfer is regulated by small RNAs such as microRNAs (miRNAs) and small interfering RNAs (siRNAs). Like proteins, some RNA molecules play an active role in cells by catalyzing biological reactions, controlling gene expression, and/or participating in cell signaling.

Systemic administration *in-vivo* of RNA/DNA oligonucleotides can allow the control of gene regulation and protein signaling modification. Oligonucleotides can be

used to modulate aberrant gene expression found in many types of cancers and inhibit protein signaling. However, some drawbacks of oligonucleotide therapies are their instability against degradation by endonucleases and their activation of the innate immune response. The incorporation of chemically modified nucleotides into RNA and DNA may solve a few problems associated with the systemic administration of oligonucleotides, such as stability against nucleases, activation of the immune system, and pharmacokinetics.

Modified nucleotides can be incorporated into synthetic RNA or DNA to modulate the molecule's stability, conformation, and activity in cells. **Figure 2** illustrates a few modifications that are used to stabilize RNA and DNA in therapeutic applications such as RNA interference (RNAi) (Chiu and Rana 2003) and aptamers (Gold 1995; Fire, Xu et al. 1998). Modification can be made to the nucleobase, the sugar, or the phosphodiester backbone.

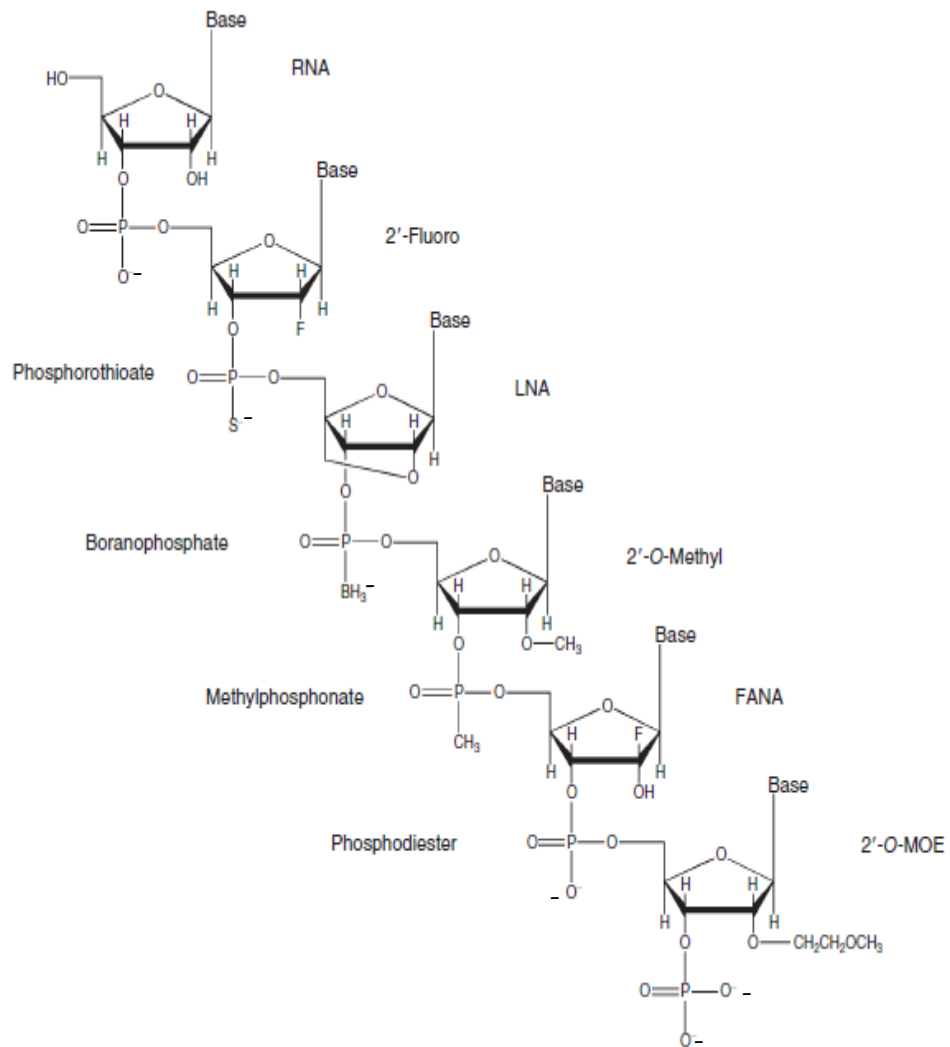


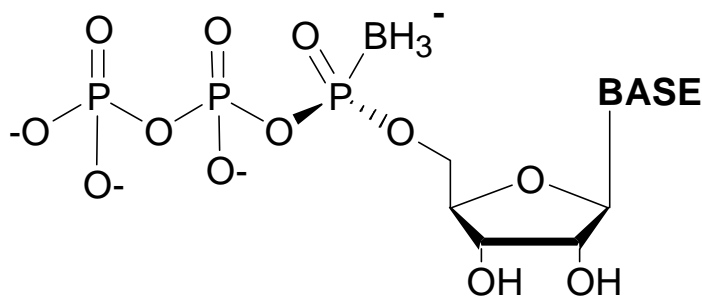
Figure 2 – Chemical modifications that are commonly used for therapeutic applications such as siRNA and aptamer therapies. Shown are some examples of sugar (left side) and backbone (right side) modifications in oligonucleotides (Chiu and Rana 2003).

Two examples of sugar modifications are the 2'-O-methyl (2'-OMe) and 2'-fluoro (2'-F) modifications. Some examples of backbone modifications are phosphorothioate (**PS**), methylphosphonate (**MP**) and boranophosphate (**BP**). This dissertation will focus

on the investigation of boranophosphate RNA (BP-RNA) for applications in the design of siRNA and RNA aptamers using cell-based assays.

1.1 Boranophosphate (BP)-Nucleic Acids

In boranophosphate (BP)-modified nucleoside triphosphates (NTP α Bs), the non-bridging oxygen (O) in the phosphodiester bond (P-O), e.i., phosphoryl, is replaced by an isoelectronic borane (BH₃) moiety (**Figure 3**) (Sood 1990; Summers and Shaw 2001; Li, Sergueeva et al. 2007). Within the P-B bond, some of the negative charge is distributed away from the boron towards the phosphorus (as compared to the P-O bond) because of the decreased electronegativity of boron (B) compared to oxygen (O), thus changing the polarity of the bond (Summers and Shaw 2001; Shaw, Dobrikov et al. 2003).



Boranophosphate (BP) R_p -NTP

Figure 3 - Structure of R_p ribo- nucleoside 5'-(α -P-borano) triphosphate.

The BH₃ group replaces the non-bridging oxygen found in the natural triphosphate. The molecule maintains a net negative charge but the chemical properties change significantly because of the reduced electronegativity of the B atom and its lack of non-binding electrons available for hydrogen bonding. The base can be uracil, adenine, guanine, or cytosine.

The BP modification is isosteric with the neutral MP group; however, like the PS and natural PO, it retains some negative charge (**Figure 4**). In addition, the BH₃ group is isoelectronic with oxygen occurring in the natural PO group and pseudoisoelectronic with the sulfur group in a PS modification (**Figure 4**) (Shaw, Sergueev et al. 2000; Summers and Shaw 2001; Li, Sergueeva et al. 2007).

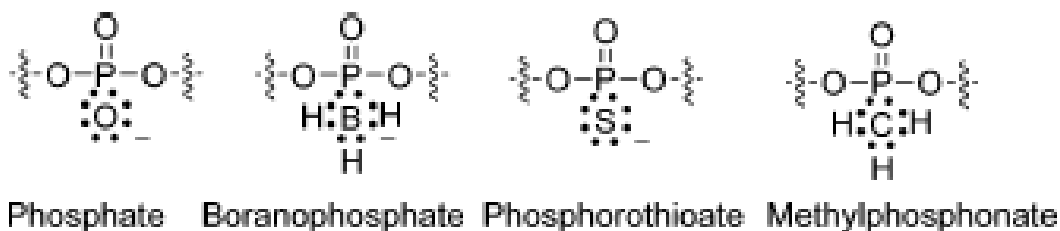


Figure 4 - Phosphate and its isolobal analogues (Shaw, Sergueev et al. 2000)

In crystal structure studies of PO and BP dimethylesters, the bonding geometries around the phosphorus were found to be tetrahedral, although, the lengths of the P-O versus the P-B bond differs by 0.4 Å (Summers, Roe et al. 1998; Summers and Shaw 2001; Li, Sergueeva et al. 2007). A comparison of the bond lengths and charge distribution of anionic phosphate and boranophosphate diesters is shown in **Figure 5**. The charge distribution is not evenly distributed between the P-B bond and the P-O in BP modification compared to the natural P-O bonds because of the change in electronegativity and the absence of unpaired electrons of the B atom compared to the lone pairs of electrons on the O atom. Accordingly, the phosphorus atomic charge changes from +2.78 (oxygen) to +2.38 (BH₃) after the substitution with a borane, resulting in the reduction of the positive charge on the phosphorus atom from +2.78 to +2.38

(Figure 5) (Summers, Roe et al. 1998; Summers and Shaw 2001; Li, Sergueeva et al. 2007).

The BH₃ group has a -0.87 negative charge as compared to -1.26 negative charge on the oxygen atom (Summers, Roe et al. 1998). We hypothesize that this change of the surface charge distribution leads to a change in surface interactions with other molecules, such as proteins and metal ions.

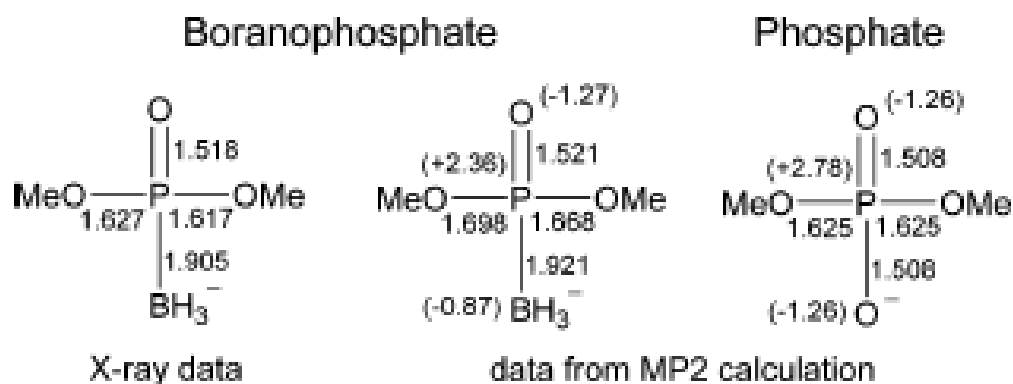


Figure 5 - Bond lengths and charge distribution of anionic phosphate and boranophosphate diesters determined from crystal structures. Values in parenthesis are atomic charge and those without represent bond length (Summers, Roe et al. 1998; Li, Sergueeva et al).

Although we are trying to compare a single atom substitution with a molecular substitution, we can compare the overall resulting surface charge change that occurs when the backbone is fully modified. This follows that the surface charge that results from the non-bonding electrons on an oxygen or sulfur, in natural or phosphorothioates (PS) respectively, is reduced in the case of borane group substitution. Similarly to the phosphoryl oxygen in natural RNA, often denoted as the non-bridging oxygen, the sulfur has available electrons in its outer shell to form bonds and coordinate to cationic

regions of proteins as well as water. In contrast, the BH_3 group does not have available hydrogen bonding electrons; hence replacing the non-bridging oxygen with BH_3 on the RNA decreases its localized negative charge (Summers, Roe et al. 1998; Summers and Shaw 2001). Consequently, the overall surface charge of the BP-modified RNA is less negative compared to oxygen and sulfur substitutions. This precludes the fully modified boranophosphate backbone from interacting in the same manner as the natural or PS backbones. We hypothesize that the decreased negative charge density in the molecular surface of BP-modified RNA may lead to a change in structure, affinity and specificity of the RNA and thus change RNA/protein interactions. Furthermore, BP-modified RNA may interact with a new diverse array of ligand sites in proteins; for example, the modification may change the interaction of BP-modified siRNA and aptamers with the RNA-induced silencing complex (RISC) or enhance the ability of the modified RNA aptamers to bind and interact with therapeutic protein targets.

It has been shown by our lab that 5'-(α -P-borano) nucleoside triphosphates ($\text{NTP}\alpha\text{B}$) are substrates for DNA and RNA polymerases, which allows the enzymatic synthesis of DNA and RNA oligonucleotides of varying lengths (Shaw, Madison et al. 1993; Sergueeva, Sergueev et al. 2001; Shaw, Dobrikov et al. 2003; Wan and Shaw 2005; Shaw 2007). Therefore, a wide range of oligonucleotide applications are possible.

The chemical synthesis of $\text{NTP}\alpha\text{Bs}$ yields a mixture of R_p and S_p stereoisomers. The isomers can be separated by reverse-phase high performance liquid

chromatography (HPLC) using a C₁₈ column. The *R_p* isomers of NTP α B are preferentially used by RNA polymerase, resulting in RNA with *S_p* BP linkages (Wan and Shaw 2005) (**Figure 6**).

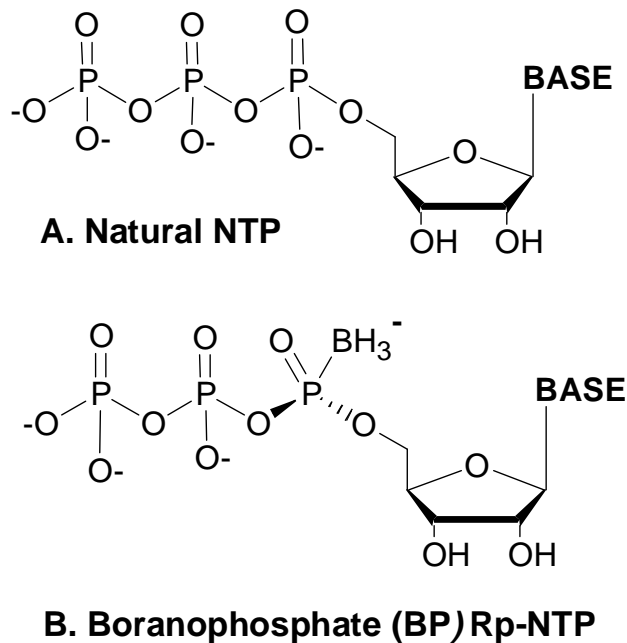


Figure 6 – Natural and boranophosphate NTPs

(A) Natural NTP; (B) BP-NTP *R_p* isomer is incorporated into the RNA strand by T7 RNA polymerase to yield the *S_p* linkage in the newly synthesized BP-RNA. (BASE = A, C, U, or G)

1.2 RNA interference (RNAi) with modified siRNA

The phenomenon called RNA interference (RNAi) was discovered by Fire and Mello et. al. (1998) in *C. elegans* (Fire, Xu et al. 1998). RNAi was shown to modulate gene expression by sequence-specifically binding to the homologous mRNA of the desired gene. This effect is currently being harnessed to develop a new class of drugs that interfere with disease-causing or disease-promoting genes. In addition, siRNA can have

genome study applications by systematically turning genes off, allowing investigators to study the resulting phenotype. It was found that injecting double-stranded (dsRNA) into the nematode modulated the translation of the targeted gene. Fire et al. also found that some amount of interference was observed by injecting either the sense strand or the anti-sense strand (Fire, Xu et al. 1998). The antisense strand leads to the degradation of the mRNA target since their sequences can base pair and form a duplex inside the RNAi machinery. However, the activity seen by injecting only the sense strand, which has the same sequence and cannot base pair to the target mRNA, leads to the activation of an off-target mechanism that is not part of the RNAi pathway. This phenomenon seen early on elucidates another function of RNA that the literature now calls immune activation by oligonucleotides through the activation of TLR receptors (Hashimoto, Hudson et al. 1988; Diebold, Kaisho et al. 2004; Diebold, Massacrier et al. 2006; Robbins, Judge et al. 2007; Diebold 2008; Gantier, Tong et al. 2008; Judge and MacLachlan 2008; Robbins, Judge et al. 2008; Robbins, Judge et al. 2009).

1.3 Off-target effects of RNA and toll-like receptor activation of modified oligonucleotides

Non-self RNA and DNA are recognized by the host immune system and activate an innate immune response via Toll-like receptors (TLRs). This response leads to the activation of interferon and cytokine genes. TLRs 7, 8 and 9 recognize viral RNA, siRNA, and guanosine analogs (**Figure 7**) (Robbins, Judge et al. 2007; Judge and MacLachlan 2008; MacLachlan 2008; Robbins, Judge et al. 2008; Judge, Robbins et al. 2009; Robbins,

Judge et al. 2009). TLR3 recognizes dsRNA, siRNA, polyI:C, and un-methylated bacterial mRNA (Robbins, Judge et al. 2009). This recognition can be advantageous, for example, when using adjuvants for vaccine therapies. However, it is not advantageous for certain oligonucleotide therapies, such as siRNA and RNA/DNA aptamers. Chemical modifications can modulate immunoactivation by oligonucleotides and reduce off-target effects. The addition of chemical modifications, such as PS, 2'-F, or 2'-OMe to the RNA molecule is widely used to increase stability of the oligonucleotide against nuclease degradation, allowing for the development of RNA therapeutics. These can also modulate the off-target effects of the RNA. Understanding how chemical modifications modulate this immune activation is important when developing oligonucleotide therapies.

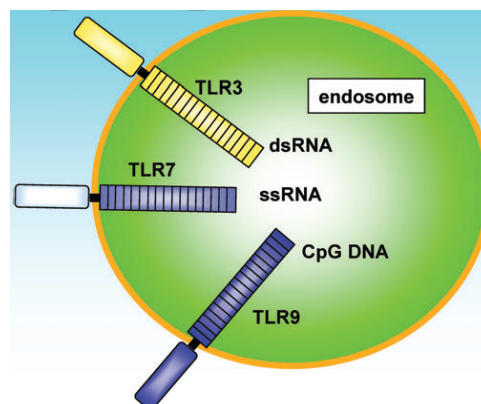


Figure 7 - TLRs involved in oligonucleotide recognition after internalization of the foreign molecules into the endosomes.

Endosomes are membrane compartments found inside eukaryotic cells and contain a family of receptors. TLR 3 recognizes dsRNA, TLR 7 recognizes ssRNA, and TLR 9 recognize CpG DNA after internalization into the endosome. Figure adapted from Robbins, et. al. 2009 (Robbins, Judge et al. 2009)

1.4 Aptamers

An aptamer is a 50-83 mer oligonucleotide sequence (RNA or DNA) that binds a protein with high affinity and specificity and may impair protein function (Tuerk and Gold 1990; Gold 1995). Aptamers are so highly specific that they are able to distinguish between chiral molecules and distinct epitopes in proteins, allowing even closely related molecules such as theophylline and caffeine to be distinguished (Tuerk and Gold 1990; Gold 1995; Gold 1995). These RNA or DNA molecules can be chemically modified to increase stability *in vivo* by the incorporation of modified NTPs. The ability to incorporate NTP α Bs into RNA by RNA polymerases creates the tremendous potential to use *in vitro* combinatorial selection techniques to rapidly and specifically select unique RNA sequences. In addition, the borane also imparts a unique chemical functionality because of its unique chemistry. Furthermore, the BH₃ group increases the lipophilicity and imparts nuclease resistance to the RNA molecule (Shaw, Dobrikov et al. 2003; Hall, Wan et al. 2006; Shaw 2007).

In vitro combinatorial selection techniques, such as SELEX, use DNA and RNA polymerases to amplify the selected ligands. These techniques are by far the most rapid way to select molecules with high affinity and high selectivity to a wide variety of targets (Gold 1995; Gold, Polisky et al. 1995). The initial oligonucleotide library is has a large variation in sequences: 10¹⁴ - 10¹⁵ individual molecules; this is due to the random region (20-60 nt) in the aptamer libraries (Gold 1995; Gold, Polisky et al. 1995). The

aptamer library is incubated with the protein target and the bound RNA is partitioned. The partitioned RNA is reverse transcribed followed by PCR to amplify the surviving selected RNA molecules; thus enriching the library pool with high affinity ligands after every round. The large library and ease of selection and amplification means a greater diversity of binding sites is likely to be targeted.

The goal of this project was to optimize the SELEX method with borano-modified RNA and select an aptamer to thrombin. Thrombin plays a central role in coagulation during the activation and propagation phases of homeostasis and it is a good target to control blood coagulation. Aptamers modulate protein activity and interfere with protein signaling by binding to the desired protein with high specificity. We attempted to optimize the selection of BP- RNA aptamers by the SELEX (Systematic Evolution of Ligands by EXponential enrichment) against the protein thrombin.

1.5 Project Definition

Most oligonucleotides used for research in cell or animal models today are chemically modified. Chemical modifications allow RNA molecules to have different patterns of immune activation, activity in RNA interference and change its pharmacokinetic properties.

This dissertation is organized in seven chapters and an appendix. **Chapter 1** provides the relevant background on boranophosphate (BP)-nucleic acids, possible

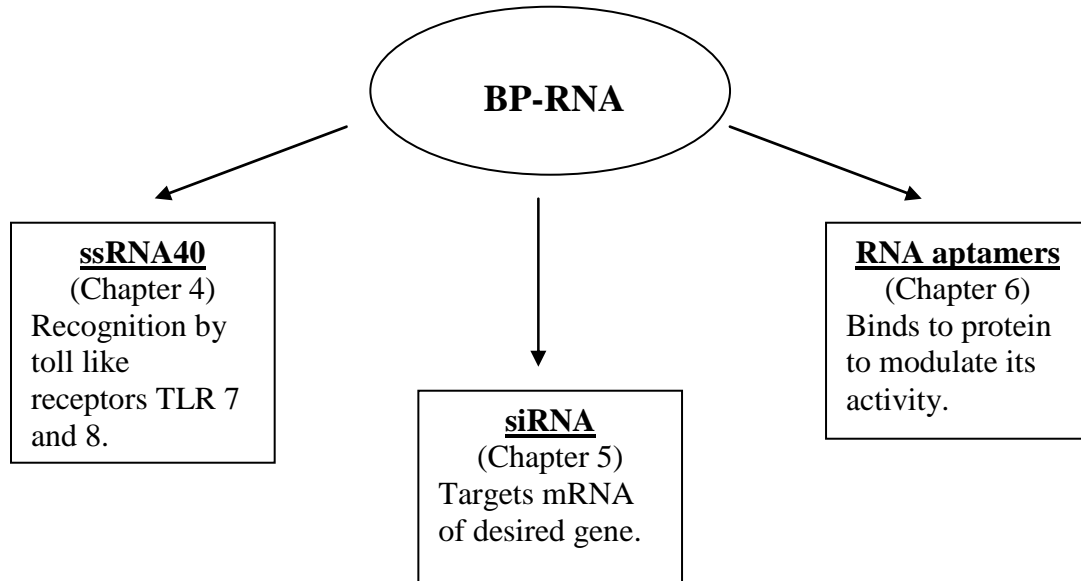
applications in RNA interference (RNAi), immunogenicity of BP-modified RNA and the utility of RNA aptamers. In **Chapter 2**, the general instruments, materials and methods relevant to the experiments presented in the dissertation were discussed. **Chapter 3** focuses on the enzymatic synthesis of BP- RNA of various lengths (ssRNA40, siRNA and RNA aptamers) and the experiments that were carried out to improve the yield of the transcription reactions. We tested how the incubation time and NTP concentration translated to transcription yields. In addition, we used two different T7 RNA polymerase promoter regions to test whether incorporation of the first two nucleotides, after the T7 promoter region (+1 or +2), was affected when modified NTPs were used. Further, we tested the yields of different template sequences and compared the RNA transcription yields when these were either natural or modified. We found that the yields were not affected by the type of promoter used but by the NTP concentration, incubation time and sequence.

Chapter 4 focuses on studying the BP- RNA for immune activation in mouse cells. Specifically, we wanted to know whether the BP modified single-stranded RNA (ssRNA40, derived from the HIV sequence), activated TLR 7 in mouse cells. This sequence has been extensively studied for its activation of TLR 7 and TLR 8 in cell based and *in-vivo* assays (Heil, Hemmi et al. 2004; Gantier, Tong et al. 2008). We found that fully modified BP- ssRNA40 did not activate TLR 7 in Raw264.7 cells in contrast with the very immunogenic phosphorothioate (PS) RNA. Immune activation after administration

of RNA is a general drawback to oligonucleotide therapies. The BP-RNA effect on the innate immune response initiated by the toll like receptors (TLR) found in endosomes had not previously been examined. This knowledge may allow the design of prototypic therapeutically relevant molecules (siRNA and RNA aptamers) without the undesired off-target effects.

For therapeutic applications such as small-interfering RNAs (siRNA) (**Chapter 5**) or RNA aptamers (**Chapter 6**), we predicted that the BP- RNA may have less off-target effects compared to unmodified RNA, and in addition maintain its activity. In **Chapter 5** we investigated whether BP-RNA could downregulate the multi-drug resistance gene (MDR1) and whether the activity of siRNA was modulated by increasing the number of BP linkages per molecule. We used an siRNA sequence shown to regulate gene expression by targeting the mRNA of multi-drug resistant gene 1 (MDR1) that encodes for P-glycoprotein (P-gp) (Fisher, Abramov et al. 2007). Using this sequence, we incorporated different amounts of BP modifications during transcription and transfected the modified RNA into cells. We found that there is a fine line between the numbers of BP-modified linkages and the molecules' activity and that this effect is target specific. In the MDR1 assay, the activity of BP-RNA to downregulate *P-gp* was significantly reduced with an increasing number of BP linkages. In **Chapter 7**, future directions of these projects are discussed.

Brief graphical outline of BP-RNA projects



Appendix I focuses on the polymerization of the cytidine analogue Gemcitabine (gem), into RNA (gem-RNA). The gem-RNA polymer was synthesized with a mutant T7 RNA polymerase and annealed to an Epidermal Growth Factor Receptor (EGFR) aptamer. In preliminary studies, the aptamer-gem-RNA molecule was added to pancreatic cancer cells and was found to be active leading to cancer cell death.

Chapter 2 - Materials and Methods

2.1 Instruments and methods

Gel Electrophoresis

Agarose Gels

Agarose gels were used to analyze the purity of the DNA templates and the dsDNA templates after annealing and Klenow extension. The Horizontal Gel Rig Model MPH (International Biotechnology, Inc.) was used to run the agarose gels (10 cm (w) x 15 cm (l) x 0.5 cm (H)). The casting tray was taped at both sides with general lab tape to prevent the liquid agarose from leaking while pouring the solution before polymerization. A 10 cm 10-well comb was used to form the wells. Each well was 0.6 cm wide and the optimal sample volume was 2-6 μ L. A PS 3000 DC power supply (Hoefer, Inc.) was used to supply the necessary voltage (7 V/cm).

A 3% agarose gel slurry of NuSieve® GTG® Agarose (Cambrex Bio Science Rockland, Inc.) in chilled 1X TBE (Tris/Borate/EDTA) was made in a heat-proof container. The mixture was heated in a microwave for 2 min on medium power and gently swirled the flask to re-suspend any settled powder. Hot distilled water was added to obtain the original weight and the solution was cooled to 50-60° C before casting.

Polyacrylamide gel electrophoresis (PAGE)

PAGE was used to qualify and purify ssRNA. Two types of vertical gel rigs were used for PAGE analysis: sequencing gels and Hoefer gels (half the size of a sequencing gel). The GIBCO BRLSA-32 sequencing system (Life Technologies) was used for sequencing gels: 20 cm (w) x 32 cm (l) x 0.2 cm (H). The sequencing gel comb created 16 wells that were 1 cm wide x 0.5 cm (l) and 0.75 mm (H). The second rig was the Hoefer apparatus SE600 Standard Vertical Electrophoresis Unit (Hoefer, Inc.), which was used for short gels with dimensions 18 cm (w) x 16 cm (l). Both systems used a PS 3000 DC power supply (Hoefer, Inc.). Hoefer glass plates were cleaned thoroughly with DI water and detergent. Every six months, the plates were coated with "Glass free" reagent. Before casting the gel, the plates were further cleaned with 200-proof ethanol and two glass plates were placed on top of each other with a 0.75-mm spacer on either side of the plates. The plates were then secured to each other with a plastic bolts. The glass pair was secured on the Hoefer casting stand before the gel solution was poured with a syringe. Gel solution consisted of 40 mL of PAGE solution (20% PAGE stock solution with 7 M urea plus the appropriate amount of 7 M urea dilution solution). The stock solution was diluted to the desired % by adding the 7 M urea solution i.e., for a 10% PAGE, 15 mL of 20% Stock was diluted with 15 mL urea dilution solution. After dilution, we added 80 μ L 20% ammonium persulfate (APS), and 40 μ L TEMED to polymerize the solution.

Sequencing gels were cast using plate sets consisting of one short plate (19.5 cm wide x 33.5 cm long) and one long plate (19.5 cm wide x 36.5 cm long). Plates were cleaned as described above. The plates were clipped together using 0.35-mm spacers and black binder clips. A plug (2 mL PAGE solution, 10 μ L 10% APS, 5 μ L TEMED) was poured at the underside of the plate pair and allowed to solidify to prevent the gel from flowing out of the bottom. Then, the main gel solution was made and poured, carefully avoiding bubbles and tapping any bubbles formed, into the top of the plates with a 25-mL syringe and a 22-gauge needle. A 0.4 mm 16-well comb was used to create 15 μ L wells.

All gels were allowed to polymerize for at least one hour prior to running. Gels that set overnight were stored wrapped in Saran wrap at room temperature. In each case, the wells were rinsed 3-15 times before loading the 2X formamide loading buffer (Formamide 9.75ml, 250 μ L 10 X TBE, toothpick amount of Bromophenol blue and Xylene cyanol) for the pre-run. The gels were pre-run for 1 -3 hours. Samples were loaded in 1X formamide loading buffer using flat-tipped gel loading pipettes after cleaning the wells again 3-15 times. The gels were run for 1- 2 hours at 300 V in 1X TBE running buffer.

Gels that were stained using 0.5 mg/mL ethidium bromide were immersed for 10 minutes in the ethidium bromide solution and then de-stained using de-ionized water for 5 minutes and immediately imaged using the UVP imager. Gels that contained

radioactive samples were removed from the plate sandwich, wrapped tightly in Saran wrap, and exposed to a low-energy storage phosphor screen 2-4 hours in a light-tight screen cassette (Molecular Dynamics) at RT to prevent sample diffusion.

Imaging Systems

Typhoon 9410 Variable Mode Imager (Amersham Biosciences)

The Typhoon 9410 phosphoroimager/ fluorescence imager (Jones building, 126) was used to quantify nitrocellulose membrane-bound RNA end- labeled with ^{32}P in filter binding experiments and to image gels (agarose or PAGE) containing RNA end- labeled with ^{32}P . The filter or gel was exposed to a low-energy autoradiography screen inside a light-blocking cassette for four hours. The screen was then imaged on the Typhoon using the storage phosphorescence setting; this instrument is available in the immunology department at Duke University. This instrument used the Typhoon Scanner Control Software, V 4.0 (Amersham) and intensities of the dots or bands were quantified using ImageQuant 5.2 software. The Typhoon has a dynamic range of five orders of magnitude and has spatial resolution of two line pairs per millimeter. The pixel accuracy is $\pm 0.15\%$ and the uniformity is $\pm 5\%$ over the entire area scanned. The digital images were saved as .gel or .pdf files.

UVP Bioimaging System

The instrument was used to image nucleic acids present in agarose and polyacrylamide gels that had been stained with ethidium bromide (FFSC building,

chemistry instrument room). This system consisted of an Epi Chem II Darkroom (imaging box), a CoolSNAP-ProCF Monochrome camera, and a 2UV Transilluminator. The instrument was controlled by the Labworks Imaging and Analysis Software V 4.0. Gels were stained using ethidium bromide and scanned directly on the instrument with an orange filter to monitor the fluorescence. The digital images were saved as JPEG files and bands were quantified using the Labworks software.

Liquid Scintillation Counter

Radiation in liquid samples containing ^{32}P -labeled RNA used in filter binding assays was determined using a Beckman Coulter LS-6000 Scintillation Counter (Beckman Coulter). The radioactive samples were dissolved in a counting cocktail solution, ScintiSafe 30% (Fisher Scientific). Winterm computer supported by LS WinConnection Suite was used to collect readings. The resulting reading was reported in counts per minutes. The resolution of the instrument is 0.06 keV.

Bio-Dot Apparatus (Bio-Rad)

The Bio-Rad apparatus is a microfiltration blotting device that was used for filter binding assays. Its dimensions are 9" x 6.5" x 12" (W x H x D) and it consists of an 8 x 12 format 96 well-sample template with attached sealing screws, vacuum manifold base, gasket support plate, and sealing gasket. The wells are 3 mm in diameter, the apparatus utilizes two membranes (nylon and nitrocellulose) size 12 cm x 9 cm (W x L), and the sample volume that can be loaded in each well is 50–600 μL .

2.2 Chemicals and Nucleic Acids

Chemicals

In this section, the chemicals used are listed alphabetically by name with their abbreviations and sources.

Acrylamide:bis-acrylamide 19:1, 40% solution, electrophoresis grade, EMD

Agarose, NuSieve GTG, Lonza

Ammonium persulfate (APS), electrophoresis purity reagent, Across

Borane-dimethyl sulfide complex, 2.0 M in THF (BMS), Aldrich

Bromophenol blue (BB), electrophoresis purity reagent, Bio-Rad

2-Chloro-4H-1,3,2-benzodioxaphosphorin-4-one (Sal-p-Cl), Aldrich

Chloroform, Acros Organics

Coomassie Blue, Bio-Rad

DEPC diethylpyrocabonate (C₆H₁₀O₅)

DEPC treated Water, Ambion

Diammonium citrate, Sigma Aldrich

Dichloromethane (DCM, CH₂Cl₂), HPLC grade, Fisher

Diethyl ether, Fisher

Dichlorodimethylsilane in hydrocarbon solvent (Glass Free)

Dithiothreitol, DL (DTT), 98%, Aldrich

Ethanol, 200-proof (EtOH), EMD Chemicals

Ethidium bromide, 10 mg/ml (EtBr), Promega

FBS Fetal Bovine Serum, Gibco®, Invitogen

FITC Fluorescein isothiocyanate

Formamide, Ambion

Methanol (MeOH), HPLC grade, J.T. Baker

Potassium phosphate, monobasic (KH₂PO₄), Sigma

Potassium phosphate buffer, 0.1 M, pH 7.5, Sigma

Phenol:chloroform:isoamyl alcohol, 25:24:1, pH 6.6, Ambion

RNase-away, Molecular Bioproducts

Sodium hydroxide, 1 N solution, Fisher

TEMED N,N,N',N'-Tetramethylethylenediamine, molecular grade, Molecular
Bioproducts

Tributylamine, anhydrous (TBA), Aldrich

Triethylamine (TEA), Glen Research

Triethylammonium acetate (TEAA), HPLC grade, Glen Research

Tris(hydroxymethyl)aminomethane (Tris base), Thomas Scientific

Urea, RNase- and DNase-free, Promega

Xylene cyanol FF (XC), electrophoresis purity reagent, Bio-Rad

Buffers

Buffers obtained RNase-free unless otherwise stated.

5X siRNA buffer, Thermo Scientific

Calcium Chloride, 1M (CaCl₂)

DMEM Dulbecco's modified eagle medium, Gibco®, Invitrogen

Ethylenediaminetetraacetic acid, 0.5 M (EDTA), disodium salt, Ambion

Magnesium acetate, 1M, Optimize

Magnesium chloride, 1M (MgCl₂), Ambion

Potassium acetate, 3M, Ambion

Potassium chloride, 2M (KCl), Ambion

Sodium Acetate, 3M (NaC₂H₃O₂), Promega

Sodium chloride, 5 M, Ambion

Tris-borate-EDTA, 10X (10X TBE), electrophoresis grade, Sigma

Tris-HCL, 1 M, pH 7.0 and pH 8.0, Ambion

Water, deionized, purified by Hydro filtration system and available on tap

Water, DEPC-treated, Ambion

Water, HPLC grade, Fisher

Water, RNase-Free, Ambion

Commercially available Kits and Enzymes

Ribomax T7 RNA transcription kits

The Ribomax® Large Scale RNA Production System – T7 contained T7 RNA

Polymerase Enzyme Mix (RNA polymerase, recombinant RNasin® Ribonuclease

Inhibitor, and recombinant inorganic pyrophosphatase), 5X transcription buffer (where the 1X transcription buffer is 40 mM Tris-HCL pH7.5, 6 mM MgCl₂, 5 mM NaCl and 10 mM spermidine), 100 mM ATP, CTP, GTP, and UTP, linear control DNA (1 mg/mL), RQ1 RNase-free DNase (1 U/μL), 3 M sodium acetate, and RNase-free water.

DuraScribe T7 Transcription kits

DuraScribe® kit (Epicentre) included a mutant T7 RNA polymerase with a single Y639F point mutation which facilitates the incorporation 2'-F modified NTPs into RNA (Padilla and Sousa 1999). The synthesis of mixed 2'-F and boranophosphate RNA was performed with the DuraScribe T7 Transcription kit, which contained DuraScribe 10X Reaction Buffer, 50 mM ATP and GTP, 50 mM 2'-F-dCTP and 2'-F-dUTP, 100 mM DTT, sterile, DI water, DuraScript RNA Control DNA Template (0.5 μg/μL), and RNase-free DNase 1 (1 U/μL).

ELISA Kits

Mouse TNFα ELISA set II and mouse IL-6 ELISA kits (BD Bioscience).

Enzymes

(All enzymes were stored at -20° C, obtained from Promega)

Klenow Fragment (exo⁻) in storage buffer (25 mM Tris-HCL, pH 7.4, 1 mM DTT, 0.1 mM EDTA and 50% glycerol)

Shrimp Alkaline Phosphatase (SAP) in storage buffer (25 mM Tris-HCL, pH 7.6, 1 mM MgCl₂, 0.1 mM ZnCl₂ and 50% glycerol).

T4 Polynucleotide Kinase (PNK) in storage buffer (25 mM Tris-HCL, pH 7.5, 25 mM KCl, 2 mM DTT, 0.1 mM EDTA and 50% glycerol)

T7 RNA Polymerase mixture (RNA polymerase, recombinant RNasin® Ribonuclease Inhibitor, and recombinant inorganic pyrophosphatase) storage buffer information is proprietary.

Cell lines and Cultures

Murine macrophage cells **Raw264.7** (ATCC) were cultured at 37° C in 5% CO₂ incubator and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS (fetal calf serum), 100 units/mL penicillin and 100 µg/mL streptomycin.

MDR-3T3 cells were obtained from Michael Gottesman (National Cancer Institute). The 3T3 cells contain a plasmid with the human MDR1 gene (Kane, Reinhard et al. 1989).

Nucleotide triphosphate and oligonucleotide concentration

The concentrations of nucleotides and nucleic acids used in this work were determined by measuring their UV absorbance at base-specific wavelengths (A = 259 nm, C = 271 nm, G = 253 nm, U = 262 nm) for nucleotides and at 260 nm for oligonucleotides (RNA/DNA). Measurements were performed using a NanoDrop ND-100 Spectrometer. The absorbance of a blank sample was measured before loading the 2 µL desired sample onto the pedestal. After measurement, the sample was removed with a Kimwipe and the pedestal was cleaned 2-3 times with 2 µL of DI water.

The concentrations of the nucleotides were calculated using the Beer-Lambert Law (Equation 1), which uses absorbance (A), path length (B), and molar absorptivity (ϵ) to determine concentration (C).

$$A_{\max} = \epsilon_{\max} \times B \times C \quad (\text{Equation 1})$$

Substitution of one of the non-bridging oxygen with a borane group has no effect on the UV absorbance properties of the nitrogenous base (He, Porter et al. 1999). Thus the concentrations of both unmodified and modified NTPs and oligonucleotides were determined in the same way. The ϵ_{\max} for each base: A = 15 400 M⁻¹cm⁻¹, C = 9 000 M⁻¹cm⁻¹, G = 13 700 M⁻¹cm⁻¹, U = 10 000 M⁻¹cm⁻¹, was used in the calculations. The ϵ_{\max} for oligonucleotide molar absorptivity for each sequence was calculated using the Ambion oligonucleotide extinction coefficient calculation tool (Ambion). The MW calculation assumes that the oligonucleotide is not phosphorylated and has no counter ions. The appropriate amount of atomic mass was added to the number displayed if phosphates or/and counter ions are present. This calculator uses the nearest-neighbor method, which has been shown to give the most accurate approximation for oligonucleotides sequences less than 100 nucleotides in length (Breslauer, Frank et al. 1986; Sugimoto, Nakano et al. 1986).

DNA template preparation

Annealing of complementary strands for short RNA synthesis (siRNA or ssRNA40)

Each 107 mer DNA template strand was added in equimolar amounts (6 nmol each) in annealing buffer with final concentration of (10 mM Tris-HCl, pH 7.5; 10 mM MgCl₂) in a total volume of 90 µL. The mixture was heated to 95° C for 5 min and allowed to cool to RT temperature for 2 h to allow base pairing. The dsDNA was run in a 3% agarose gel with the ssDNA strands to detect that there was no excess of ssDNA since the dsDNA migrates slower than the ssDNA.

Klenow extension for SELEX library synthesis

The DNA template strand was mixed with the DNA primer at a 1:2 ratio in annealing buffer (10 mM Tris-HCl pH 7.5, 10 mM MgCl₂). This reaction mixture was heated to 95° C for 2 min and allowed to cool to RT. The Klenow reaction was assembled on ice with 0.5 mM deoxyribonucleotides (dNTPs), 1X Klenow buffer (50 mM Tris-HCl (pH 7.2 at 25° C), 10 mM MgSO₄, 0.1 mM DTT. and 30 units of Klenow enzyme (Promega). one unit of enzyme is defined as the amount of enzyme required to convert 10 nmol of dNTPs to an acid insoluble form in 30 mins at 37° C (Defined by manufacturer protocol, Promega). The dsDNA was run in a 3% agarose gel to analyze for full length product.

Dephosphorylation of RNA

Shrimp alkaline phosphatase (SAP) from *P. borealis* was used to remove the 5'-phosphate of ssRNA before labeling the 5'-end of the RNA with [³²P] ATP and T4 polynucleotide kinase. SAP (1 U/μL, Promega) was received in storage buffer containing 25 mM Tris-HCl, pH 7.6 at 4° C, 1 mM MgCl₂, 0.1 mM ZnCl₂, and 50% glycerol, this enzyme was stored at -20° C. One unit of SAP was defined as the amount of enzyme required to catalyze the hydrolysis of 1 μmole of 4-nitrophenyl phosphate per minute at 37° C in 1 M diethanolamine, 10.9 mM 4-nitrophenyl phosphate, 0.5 mM MgCl₂, and pH 9.8. The SAP 10X reaction buffer was composed of 500 mM Tris-HCl, pH 9.0 at 37° C and 100 mM MgCl₂. The reaction was incubated at 37° C for 15 minutes and then heat inactivated at 65° C for 15 minutes. Before extraction, 300 μL TE buffer [10 mM Tris-HCl pH 8.0, 0.1 mM EDTA] was added to increase the total volume. The reaction mixture was mixed with 400 μL phenol: chloroform: isoamyl alcohol (25:24:1). The sample was vortexed 1 min, spun at 15,000 rpm for 5 min at 4° C, and transferred supernatant to a new tube. The aqueous layer was mixed with 400 μL chloroform: isoamyl alcohol (24:1) (2X), vortexed 1 min, and spun at 15,000 rpms for 5 min in a centrifuge. The sample **ethanol precipitated** as follows: The aqueous layer was transferred to a new tube and 0.1 volume (30-40 μL) of 3M sodium acetate pH5.2 was added and as well as 5 μL Linear acrylamide. Then, 2.5 volumes of cold ethanol (-20° C) were added. The sample was placed on powdered dry ice for 30 min (-78° C) until

frozen. The sample was centrifuged at 15,000 rpm for 30 min at 4° C, the supernatant was removed with a pipette, and 500-1000 µL cold 70% ethanol (-20° C) was added. The sample was centrifuged, the ethanol was removed, and the sample was air-dried at rt. for 15 min. The pellet was re-suspended in TE buffer (10mM Tris HCl pH8.0, 0.1mM EDTA) or 1X siRNA buffer.

5'-end labeling of RNA

RNA with a 5'-phosphate was dephosphorylated with SAP prior to labeling with T4 Polynucleotide Kinase (PNK) and [γ -³²P]ATP. PNK (5-10 U/µL) purified from *E. coli* cells expressing a recombinant clone was supplied in storage buffer containing 20 mM Tris-HCl pH 7.5, 25 mM KCl, 2 mM DTT, 0.1 mM EDTA, 0.1 µM ATP, and 50% (v/v) glycerol, this enzyme was stored at -20° C. One unit was defined as the amount of PNK required to catalyze the transfer of 1 nanomole of phosphate from [γ -³²P] ATP to the 5'-OH end of a polynucleotide in 30 minutes at 37° C in 40 mM Tris-HCl pH 7.5, 10 mM DTT, 0.1 mM [γ -³²P] ATP, and 0.5 µg/µL 5'-OH polynucleotide end concentration. The 10X PNK exchange reaction buffer was supplied with the enzyme and contained 500 mM imidazole-HCl pH 6.6, 100 mM MgCl₂, 50 mM DTT, 1 mM spermidine, and 1 mM EDTA. The reaction was run through a G25 column to remove excess hot ATP. The labeled RNA was stored at -20 in micro-centrifuge tubes (for high G-force tubes) in the PNK reaction buffer.

Chapter 3 - Enzymatic synthesis of natural and modified RNA with T7 RNA polymerase

3.1 Introduction

Transcription of genetic sequences in cells is mediated by DNA-dependent RNA polymerases that use the genome DNA as a template and ribonucleoside triphosphates (rNTPs) to make the required RNA. One such polymerase is T7 RNA polymerase, which was obtained from the T7 bacteriophage, and catalyzes initiation and synthesis of RNA in the 5'→3' direction using a DNA template (Chamberlin and Ring 1972; Melton, Krieg et al. 1984). A T7 RNA polymerase expressed in *E. coli*, is used *in-vitro* with a double-stranded DNA (dsDNA) template and a mixture of four NTPs(U, G, C, A) to synthesize RNA (Chamberlin and Ring 1972; Chamberlin 1974). The enzyme requires a double-stranded promoter region to initiate the synthesis (**Figure 8**) (Chamberlin 1974; Milligan, Groebe et al. 1987). T7 RNA Polymerase exhibits extremely high affinity and specificity for T7 promoter sequences (Chamberlin and Ring 1972; Rosa 1979). Two commonly used DNA promoter regions are the T7 **class II** promoter ϕ 2.5 and the T7 **class III** promoter ϕ 6.5, which differ by a single nucleotide change on the DNA promoter sequence (**Figure 8**) (Rosa 1979; Huang, He et al. 2008). **Class II** has a T and **Class III** has an A as the first (+1) nucleotide added to the desired sequence. To improve transcription yields with T7 RNA polymerase, Milligant et al. (1987) varied the concentration of natural

NTPs. They found that too low (0.01 mM) NTP concentration decreased the yield of the reaction and too high (20 mM) inhibited the enzyme (Milligan, Groebe et al. 1987).

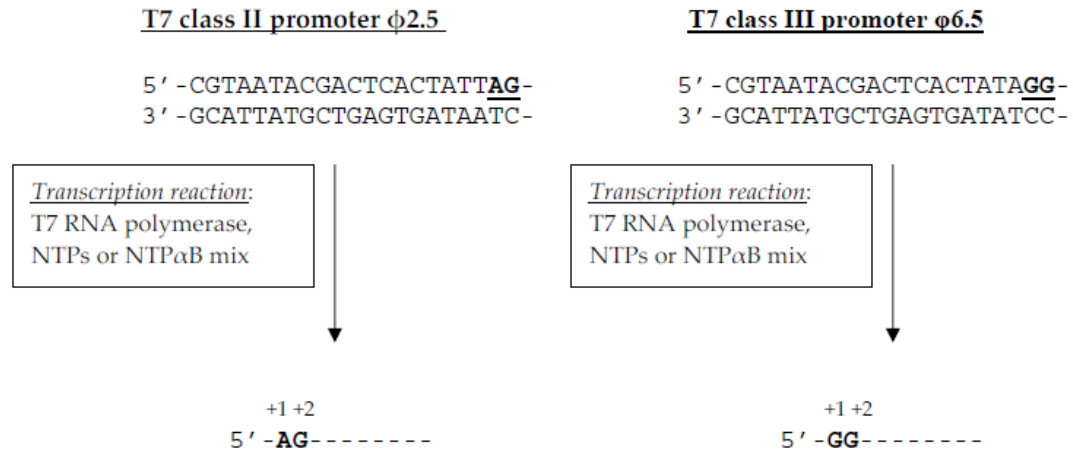


Figure 8 – Two types of DNA promoter sequences for T7 RNA polymerase (Huang, He et al. 2008).

Class II starts the transcription with 5'-AG incorporated into the RNA strand. Class III on the other hand, incorporated 5'-GG at the start of the RNA synthesis. The first 2 nucleotides are referred to positions +1, +2 after the promoter region which is -19 to 0 starting at the 5'-end.

The transcription by T7 RNA polymerase can be broken down into the following steps: initiation, elongation, and termination (Chamberlin and Ring 1972; Chamberlin 1974; Milligan, Groebe et al. 1987). The change between initiation and elongation relies on crucial interactions between the template, triphosphates, and the enzyme. This step determines if the polymerase will continue transcription or starts the transcription again (Milligan, Groebe et al. 1987). During synthesis, transcripts that are shorter than the desired length are seen because the polymerase cycles between the promoter region to

+1 to +8 and releases short RNAs from the initial transcription complex (ITC) (Mukherjee, Briebe et al. 2002). If the polymerase keeps going past nucleotide +9, this moves the polymerase from the promoter region to the rest of the DNA template and forms a stable elongation complex (Mukherjee, Briebe et al. 2002). This elongation step gives the full-length product and often a longer transcript. Longer than desired length RNAs are seen because the T7 RNA polymerase may add one to three non-templated nucleotides at the end of the transcript before termination giving a range of sized RNA (Milligan, Groebe et al. 1987).

To prepare chemically modified RNAs with modifications such as 2'F or 2'-OMe, the enzyme was mutated at specific regions to allow the incorporation of modified this NTPs more readily. When the wt type enzyme was mutated at tyrosine 639 and changed to phenylalanine, the mutated enzyme was unable to discriminate between hydrogen and non-hydrogen bonding 2'-ribose substitutions on NTPs (Padilla and Sousa 2002; Sousa and Mukherjee 2003). The Y639F mutant T7 RNA polymerase was found to incorporate 2'-sugar modified NTPs like 2'F and 2'-NH₂, but not bulky substituents like 2'-OMe (Padilla and Sousa 1999).

This chapter focuses on examining the enzymatic synthesis of BP- RNA of various lengths (ssRNA40 (21 mer), siRNA (21 mer) and RNA aptamers (83 mer)) and the experiments carried out to improve the yield of the transcription reactions. To study the applications of BP-RNA, we first have to obtain sufficient quantity of full length

RNA material to test in cells. To attain this goal, we varied the incubation time and NTP concentration, and calculated how that translated to transcription yields. Many types of modified NTPs are not incorporated by wild type (wt) T7 RNA polymerase. However, wt T7 RNA polymerase can incorporate a few types of modified NTPs including NTP α Bs (Wan and Shaw 2005) and phosphorothioate triphosphates (NTP α S) (Eckstein 1985). However, in previous studies only the class III type of T7 promoter was used where the first two nucleotides incorporated are GG. We hypothesized that the transcript quality and yield may change using a different promoter that allowed the first NTP (+1) incorporated to be a natural A instead of BP-modified G. We found that the yields were not affected by the type of promoter used, but were affected by the NTP concentration, the reaction incubation time and nucleotide sequence.

3.2 Experimental

5'-(α -P-borano) nucleoside triphosphate (NTP α B) synthesis

R_p 5'-(α -P-borano) nucleoside triphosphates were chemically synthesized in our lab by Marcus Cheek (He, Porter et al. 1999). These were delivered as a lyophilized mixture of *R_p* and *S_p* NTP α Bs and also included the naturally occurring degradation products such as NDP α B and NMP α B.

High Performance Liquid Chromatography (HPLC) Systems

A Waters Delta 600 HPLC system in the Shaw lab was used to separate the NTP α B stereoisomers and degradation products. The HPLC consisted of a Waters 996

Photodiode Array (range of 190-800 \pm 1 nm), and a Waters 600E Multisolvent Delivery system (including a 600s controller and a Rheodyne 7725i manual injector). The HPLC was controlled by a Dell Dimension computer equipped with Millennium32 software (Version 3.20). For milligram scale separation, a semi-preparative C-18 Waters Delta Pak™ (300X7.8 mm, 15 μ m, 300 Å) column. The column was placed in a Prep LC Universal Base (Waters Corp) that was attached to the solvent delivery module. The detector flow cell had a 10-mm path length and 0.009-inch tubing. The chromatographic profiles were obtained and the desired NTP fraction (5-8 mL) was collected in a conical tube. The NTP α B R_p isomer was lyophilized two times with DEPC water and stored at -20° C.

The Varian Prostar HPLC in the Shaw lab was used to analyze the purity of the R_p -NTP α B stereoisomers, which consisted of a 330.71 Photodiode Array detector, a solvent delivery module, and a 430 Autosampler (Varian). For analysis, a C-18 DeltaPak column (Waters Corp) was used with dimensions as follows: 3.9 mm (d) x 300 mm (l), 15 μ m column diameter and 100 Å particle size.

All solvents were filtered through a 0.2-micron filter (Millipore) and included methanol, deionized water, and 50 mM tetraethyl ammonium acetate (TEAA). The samples were eluted with a buffer consisting of triethylammonium acetate (TEAA, pH 6.8) and methanol at a flow rate of 3.0 mL/min. The elution conditions were as follows:

for R_p -UTP and R_p -CTP the buffer had a gradient of 3-13% methanol; R_p -ATP α B and R_p -GTP α B eluted using a 5-17% methanol gradient.

DNA templates

DNA was obtained from Integrated DNA technologies (IDT)

Class II or Class III promoter study

Class II promoter ϕ 2.5 MDR1 siRNA ORF 1 with AG at 5'-end

ORF1 (**ORF 1-AG**) 22 mer

5'-TAATACGACTCACTATTAG TTCGAATAGCTGTCAATACTT-3'
3'-ATTATGCTGAGTGATATTC AAGCTTATCGACAGTTATGAA-5'

Mismatched (**Mis - AG**)

5'-TAATACGACTCACTATTAG TTCGTATAGGTCTCTATACTT-3'
3'-ATTATGCTGAGTGATATTC AAGCATATCCAGAGATATGAA-5'

Class II promoter - MDR1 siRNA ORF 1 with GG at 5'-end

ORF1 (**ORF 1-GG**) 22 mer

5'-TAATACGACTCACTATAGG TTCGAATAGCTGTCAATACTT-3'
3'-ATTATGCTGAGTGATATCC AAGCTTATCGACAGTTATGAA-5'

Mismatched (**Mis-GG**) 22 mer

5'-TAATACGACTCACTATAGGTTCGTATAGGTCTCTATACTT-3'
3'-ATTATGCTGAGTGATATCCAAGCATATCCAGAGATATGAA-5'

DNA templates used for transcription with different sequences and U α B

DNA (**A1/B1**) 21 mer

A1 5'-TAATACGACTCACTATA GGC TCA CTG AGA CTA CAT CTT-3'
B1 3'-ATTATGCTGAGTGATAT CCG AGT GAC TCT GAT GTA GAA-5'

DNA (**E1/F1**) 21 mer

E1 5'-TAATACGACTCACTATA GGA CGA AGA CGA ACA CTT CTT-3'
F1 3'-ATTATGCTGAGTGATAT CCT GCT TCT GCT TGT GAA GAA-5'

DNA (A2/B2) 21 mer

A2 5'-TAATACGACTCACTATA GGAT GTA GTC TCA GTG AGC CTT-3'

B2 3'-ATTATGCTGAGTGATAT CCTA CAT CAG AGT CAC TCG GAA-5'

DNA (C2/D2) 21 mer

C2 5'-TAATACGACTCACTATA GGAA GTG TTC GTC TTC GTC CTT-3'

D2 3'-ATTATGCTGAGTGATAT CCTT CAC AAG CAG AAG CAG GAA-5'

Transcription with T7 RNA polymerase

The dsDNA template (10 µg), prepared as described in **Chapter 2**, was incubated with a T7 RNA Polymerase mixture (RNA polymerase, recombinant RNasin® Ribonuclease Inhibitor, and recombinant inorganic pyrophosphatase) provided in the Ribomax Large Scale RNA Production System (Promega, Madison, WI) and the corresponding natural and/or modified triphosphates (PS, 2'F, BP) at a 2.0 mM concentration in 100 µL of 1X buffer (80mM HEPES-KOH pH 7.5, 24 mM MgCl₂, 2 mM spermidine, 40 mM DTT). The sources of the NTPs were as follows: Natural NTPs (Promega, Madison, WI), alpha-phosphate thio-modified nucleoside triphosphates (PS) mixture of *R_p* and *S_p* (Trilink, San Diego, CA) and 2'F UTP/ 2'F CTP (Epicentre). The *R_p* 5'-(α-P-borano) nucleoside triphosphates (NTP_{αB}) were chemically synthesized in our lab and HPLC purified as described previously (He, Porter et al. 1999). The transcription reaction mixture was incubated for 2 h, 4 h, or overnight at 37° C. The DNA template was digested using RQ1 RNase-free DNase (1 U/µL) (Promega, Madison, WI) by incubation at 37° C for 15 min. The unincorporated NTPs were removed by G25 spin columns (GE Healthcare,

Buckinghamshire, United Kingdom). The final product was ethanol precipitated and re-suspended in DEPC treated water and quantified by UV. The sample was **ethanol precipitated** as follows: The aqueous layer was transferred to a new tube and 0.1 volume (30-40 μL) of 3M sodium acetate pH5.2 was added and as well as 5 μL Linear acrylamide. Then, 2.5 volumes of cold ethanol (-20°C) were added. The sample was placed on powdered dry ice for 30 min (-78°C) until frozen. The sample was centrifuged at 15,000 rpm for 30 min at 4°C , the supernatant was removed with a pipette, and 500-1000 μL cold 70% ethanol (-20°C) was added. The sample was centrifuged, the ethanol was removed, and the sample was air-dried at rt. for 15 min. The quality and size of the RNA was analyzed using a 7 M urea 10% (19:1) polyacrylamide gel electrophoresis (PAGE) and stained with ethidium bromide solution (0.5 $\mu\text{g}/\text{ml}$).

3.3 Results

Purification and analysis of BP-RNA

Purity of R_p -NTP α Bs analyzed with HPLC

Chemical synthesis of NTP α Bs was performed by Marcus Cheek according to a published method by He et al. (1998) with some modifications [manuscript in preparation] (He, Hasan et al. 1998). The chemical synthesis produces two stereoisomers, R_p and S_p , purification of the isomers are necessary because enzymes are stereospecific to only one isomer. For enzymatic synthesis of RNA, with T7 RNA polymerase we use only the R_p isomer since it is incorporated at >98-fold better than the

S_p isomer (He, Hasan et al. 1998). We separate the mixtures to assure complete stereospecificity of the RNA backbone and better the yields of the transcription reaction. Purification of the stereoisomers was carried out by ion-pairing chromatography using the Varian HPLC as described above in the material section of this chapter. Samples were dissolved in DEPC-treated water. The isomers were separated using isocratic elution, where the mobile phase consisted of methanol and 25 mM TEAA. The fractions corresponding to all the desired peaks were collected and analyzed by analytical HPLC. **Figure 9** shows the analytical HPLC profiles of mixture and purified R_p -UTP α B and S_p -UTP α B under the same buffer conditions (3-13% methanol / 0.25 mM TEAA) as our standard which was the natural UTP.

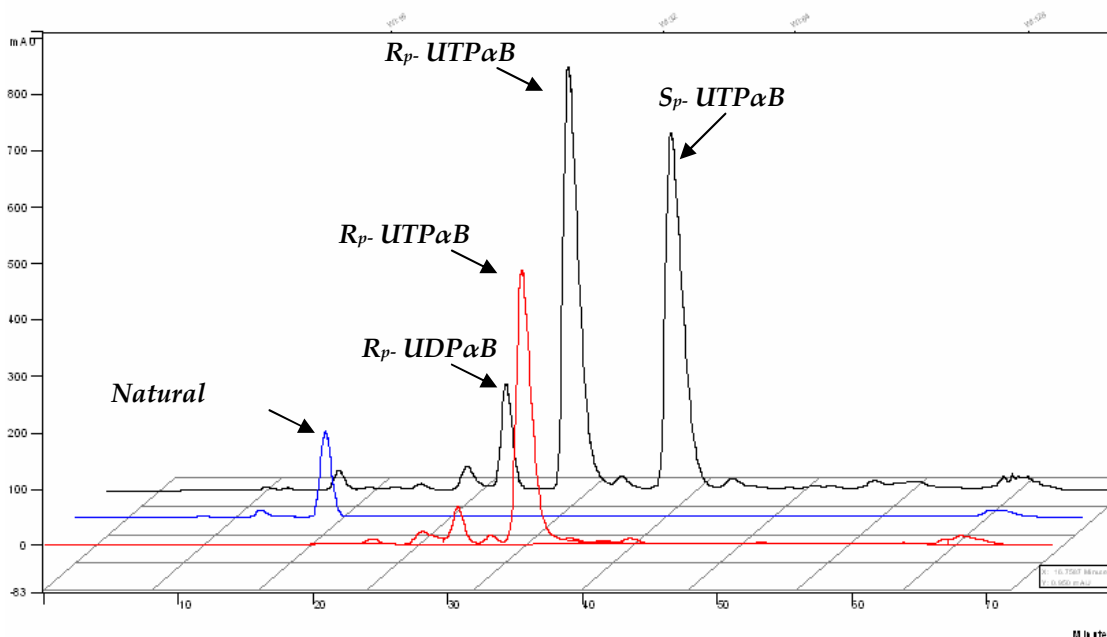


Figure 9 – HPLC profile for UTP and UTP α B HPLC.

Red; Purified UTP α B, Blue: Natural UTP, Black: R_p UTP α B and S_p UTP α B. The isomers are base line separated under this buffer conditions and there is no corresponding peak for natural NTP contamination. The R_p-NTP α B isomer was used during transcription reactions with T7 RNA polymerase.

The R_p-UTP α B isomer was used to transcribe boranophosphate RNA. All four NTP α Bs were HPLC purified and their profiles compared to the natural NTPs to ensure the quality of the triphosphates prior to enzymatic synthesis.

T7 RNA polymerase transcription conditions: Effects of Reaction Time and NTP concentration

It was first necessary to establish optimal conditions for transcription reactions to obtain the necessary amount for testing the BP-RNA properties future studies. The Shaw lab already showed that NTP α B are incorporated into RNA for 21 mer transcript (Wan and Shaw 2005). We tested a longer RNA, 83 mer, using two 100-mer templates

(template #1 or template #2), and the reaction times at 2 h, 4 h and overnight (O) and at high (7.5 mM) and low (2.0 mM) NTPs concentrations were compared. In addition, we used a linearized DNA template control supplied with the transcription kit which was 1,800 bases long (linearized control template, Promega).

The enzymatic synthesis reaction conditions were varied and included high (7.5 mM) and low (2 mM) NTPs (natural and modified) concentration; the reaction time was either two hours (2 h), four hours (4 h) and overnight (O). The RNA was quantified using nanodrop UV at 260 nm, the yields are reported in μg of RNA obtained using 10 μg of dsDNA template. For the natural NTPs, there was a clear trend; the highest yield was observed when high NTP concentrations and overnight incubation time was used (**Figure 10**). The full-length transcripts were analyzed using PAGE (**Figure 11**) along with a DNA marker. With template #1 and template #2 had higher transcription yields at high NTP concentration and either 2 h or 4 h (**Figure 10 template #1 and template #2**).

Normal NTPs

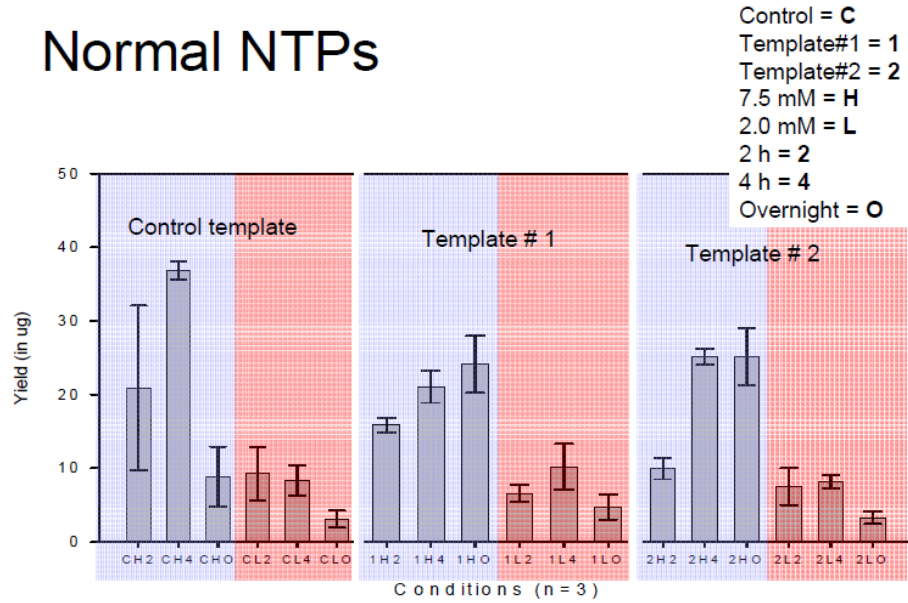


Figure 10 - Normal RNA yields were determined by UV at 260 nm (using the nanodrop spectrometer).

Using three different templates: Control (1,800 mer), template #1 (100 mer) and template #2 (100 mer) to transcribed with natural NTPs at high (7.5 mM) or low (2.0 mM) NTPs and three incubation times (2 h, 4 h, Overnight). Standard error calculated from 3 separate experiments (n=3). Highest yields were obtained with 7.5 mM NTP concentration and longer incubation times.

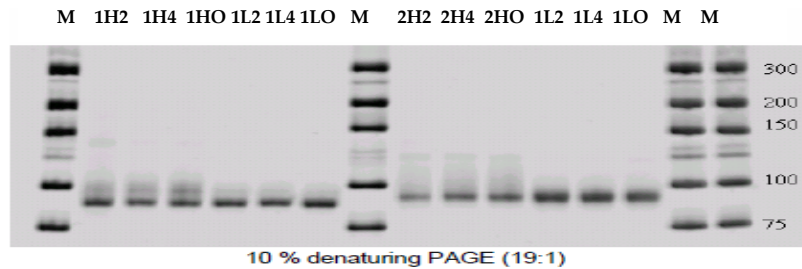


Figure 11 - Analysis of natural RNA products obtained after transcription by 10% PAGE/ 7 M Urea and stained with ethidium bromide solution.

Full length products were observed for the RNA transcribed with template #1 and template #2 with natural NTPs at various time points and NTP concentrations. The DNA marker lengths are marked on the left hand side of the gel and the RNA transcripts migrate slightly lower than the 100 mer DNA marker band. Full length

RNA products (83 mer) are observed under all reaction conditions tested. (Sample name based on name key from figure 12)

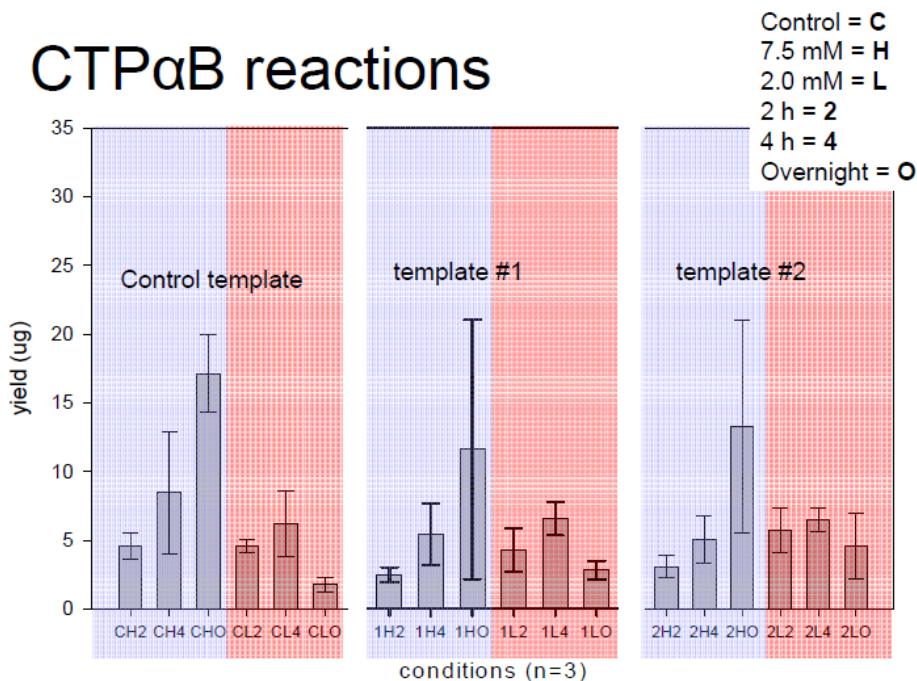


Figure 12 – Modified RNA yields were determined by UV at 260 nm (using the nanodrop spectrometer).

Three different templates were used: Control (1,800 mer), template #1 (100 mer) and template #2 (100 mer). The three different control templates were transcribed with T7 RNA polymerase using CTP α B instead of natural CTP at different NTP concentrations and varied incubation times (2h, 4h, overnight). The yields were calculated with the nanodrop UV at 260 nm. Error bars were calculated as the standard error of 3 separate experiments (n=3). We hypothesize that the error for the 1HO or 2HO is due to the increasing and random degradation of the modified RNA during overnight incubation.

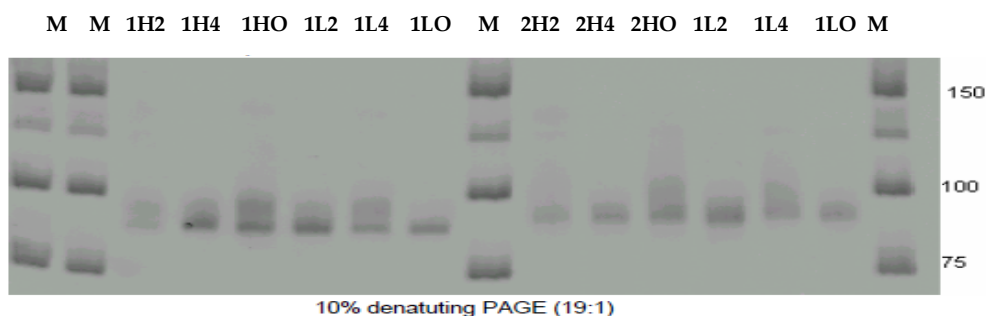


Figure 13 – Analysis of BP-modified RNA library on 10% PAGE/ 7 M Urea. Template #1 and template #2 transcribed with T7 RNA polymerase and CTP α B instead of natural CTP at varied incubation times and NTP concentration. The DNA marker was run with the samples and their size shown on the left-hand side of the gel. Full-length RNA gel band products are seen at expected close to the 100 mer DNA marker. (Sample name based on name key from figure 12)

The control template included in the transcription kit is the dsDNA template (1,800 nt) the company has used to optimize the transcription conditions with natural NTPs. We measured the yield using the nanodrop UV at 260 nm, assuming that the extinction coefficient was the same for natural and modified RNA, and calculated the μ g obtained when 10 μ g of dsDNA template was transcribed. We contrasted the yield obtained with this sequence with natural and CTP α B in the different reaction conditions. Using this control template there is the expected higher yield with natural NTPs, at high concentration (7.5 mM) and 4 hour incubation compared to 2 h or overnight or low NTP (2.0 mM) concentration (**Figure 10 control template**). In contrast, when using CTP α B instead of CTP, with the same control template, a higher yield is obtained with an overnight incubation and high CTP α B concentration (**Figure 12 control template**). However when comparing template #1 or template #2 no clear trend was observed for

the modified CTP α B experiments (**Figure 12 template # 1 and template # 2**). Full length products (83 mer) were observed for all the three templates using PAGE (**Figure 13**). The RNA obtained under all the reaction conditions had the same mobility to each other and slightly more than the 100 mer DNA marker. This suggests that the RNA is the expected full length product of approximately 83 nucleotides long.

We conclude that the standard conditions, applied by the supplier used to determine optimal yields for the Ribomax[®] transcription kit are not optimized for transcriptions with modified NTP α Bs.

Enzymatic synthesis of BP-modified RNA: Comparison of Yield with Different T7 Promoters and Different Sequences

Comparison of transcription yields with promoter class II or Class III

To further optimize the yields of the transcription reaction, the enzymatic synthesis of RNA with natural and modified NTPs was compared using two different templates that contained two different promoter regions. The T7 RNA polymerase has high affinity and specificity to its promoter sequences (Rosa 1979). The promoter region is critical for successful transcription reaction (Chamberlin 1974; Rosa 1979). We examined two different classes of promoters, class II and class III. The class II promoter starts the transcript with 5'-AG and the class III promoter, which is more commonly used, starts with 5'-GG (**Figure 8**). **Table 1** compares the yields of each sequence with

either GG or GA as the first two nucleotides (+1, +2) incorporated by the T7 RNA polymerase. In addition, we compared the yields when using either natural CTP or CTP α B.

Table 1- Yields for Class II and Class III T7 RNA pol promoters using the nanodrop UV spectrometer at 260 nm.

Name	Natural (μ g)	G α B RNA (μ g)	Promoter
ORF1-GG	10.5	6.8	class III
Mis-GG	22.1	15.5	class III
ORF1-AG	15.4	14	class II
Mis-AG	19.1	15	class II

yields in μ g per 10 μ g of dsDNA template used

Name	Sequence	Promoter
ORF1-GG	GG UUC GAA UAG CUG UCA AUA CUU	class III
Mis-GG	GG UUC GUA UAG GUC UCU AUA CUU	class III
ORF1-AG	AG UUC GAA UAG CUG UCA AUA CUU	class II
Mis-AG	AG UUC GUA UAG GUC UCU AUA CUU	class II

Using GTP α B instead of GTP reduced the yields by 5 to 30% using both types of promoters. It did not matter that the transcription reactions started with GG or AG where the modification was at either the first or second position. Thus, the GTP α B incorporation, in either +1 or +2 position gave comparable yields to natural GTP.

Comparison of transcription with four different sequences

We next compared the transcription yields with four different sequences and UTP α B. The dsDNA was analyzed on an agarose gel to ascertain that the template was double stranded (**Figure 14**). The dsDNA shows slower migration on the agarose gel because of its greater mass to charge ratio compared to ssDNA.

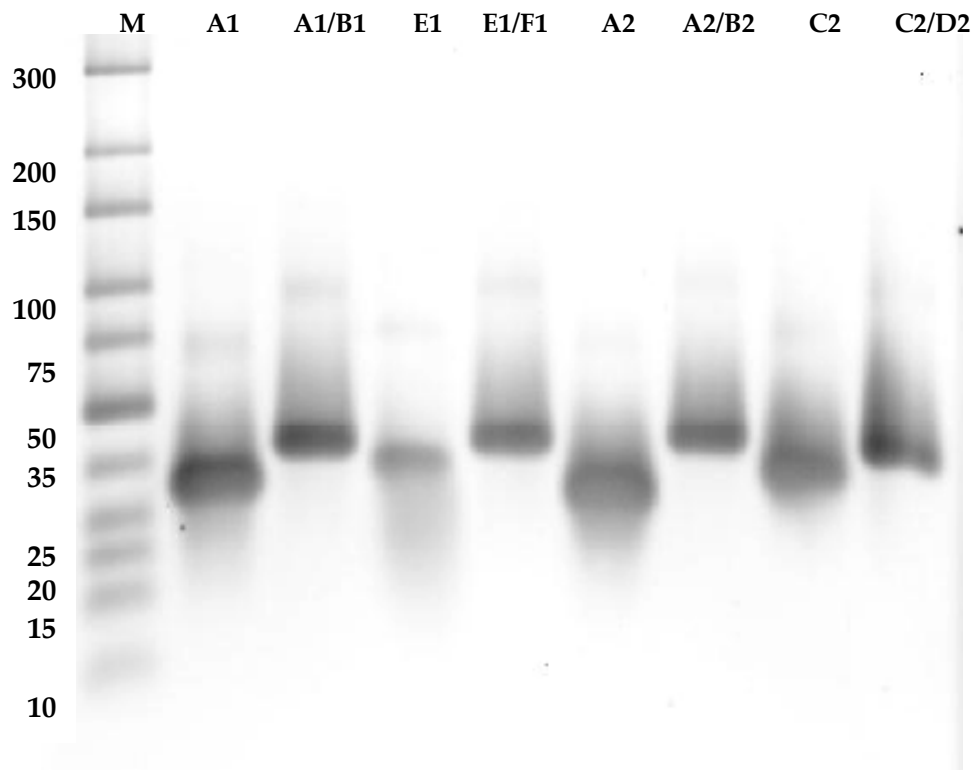


Figure 14 – Double-stranded DNA (dsDNA) templates (A1/B1, E1/F1, A2/B2) analyzed by 3% agarose gel.

The dsDNA shows a slower migration compared to the single-stranded DNA (A1, E1, and A2). All samples were loaded at a concentration of 1-2 $\mu\text{g}/\mu\text{L}$. The enzymatic synthesis of RNA with T7 RNA polymerase requires that the template be double-stranded. The dsDNA templates were used as templates to synthesize the modified RNA.

Table 2 – dsDNA (38 mer) templates used to transcribe short (21-22 mer) modified RNA.

Name	DNA template Sequence
A1/B1	A1 5'-TAATACGACTCACTATA GGC TCA CTG AGA CTA CAT CTT -3' B1 3'-ATTATGCTGAGTGATAT CCG AGT GAC TCT GAT GTA GAA-5'
A2/B2	A2 5'-TAATACGACTCACTATA G GAT GTA GTC TCA GTG AGC CTT-3' B2 3'-ATTATGCTGAGTGATAT C CTA CAT CAG AGT CAC TCG GAA-5'
E1/F1	E1 5'-TAATACGACTCACTATA GGA CGA AGA CGA ACA CTT CTT-3' F1 3'-ATTATGCTGAGTGATAT CCT GCT TCT GCT TGT GAA GAA-5'
C2/D2	C2 5'-TAATACGACTCACTATA G GAA GTG TTC GTC TTC GTC CTT-3' D2 3'-ATTATGCTGAGTGATAT C CTT CAC AAG CAG AAG CAG GAA-5'

Sequences shown in the 5' to 3' direction

This dsDNA template was transcribed with T7 RNA polymerase with either natural UTP or UTP α B to give the RNA sequences shown in **Table 3**. This allowed us to compare four different sequences to determine how the yields were affected after replacing UTP with UTP α B in the transcription reaction.

Table 3 - RNA sequence for each short transcript (modification not shown)

Name	RNA Transcript Sequence
A1/B1	GGC UCA CUG AGA CUA CAU CUU
A2/B2	GGA UGU AGU CUC AGU GAG CCU U
E1/F1	GGA CGA AGA CGA ACA CUU CUU
C2/D2	GGA AGU GUU CGU CUU CGU CCU U

Sequences shown in the 5' to 3' direction

The transcription with natural NTPs gave 4-10 times more RNA than BP modified NTPs depending on the sequence (**Table 4**). Transcription yields were low for the A1/B1 template using either modified (B) or natural (N) triphosphates compared to

the other sequences. This suggests that yields are template-dependent and not only modification-dependent.

Table 4 – Comparing the transcription yields of short (21-22 mer) natural or BP modified RNA using different sequences. Yields calculated from 10 µg of dsDNA template (38 mer), using the nanodrop UV spectrometer at 260 nm.

Name	yield (µg)
N-A1/B1	40
B-A1/B1	7.6
N-A2/B2	88
B-A2/B2	16
N-E1/F1	64
B-E1/F1	15
N-C2/D2	80
B-C2/D2	10

Yields in µg from 10 µg dsDNA template

N = natural RNA, B = borano

The RNA transcripts were run on a denaturing 7 M urea/ 10%PAGE gel to analyze for length and the range of lengths. Usually, T7 RNA polymerase adds a few non-templated nucleotides at the end of the transcript in addition to the full length product (**Figure 15**) with both natural (**N**) and modified (**B**) UTP α Bs. In our experiments there are three visible product bands in N-A2 compared to the modified RNA B- A2, which only has two visible product bands (**Figure 15**). Looking at another sequence, the natural transcript has two distinct bands N-A1 compared to the modified transcript B-A1, which presents one major band and one minor band (**Figure 15**). Thus, the range of lengths of the RNA transcript, and their relative concentration depends on the sequence and whether there are modifications present.

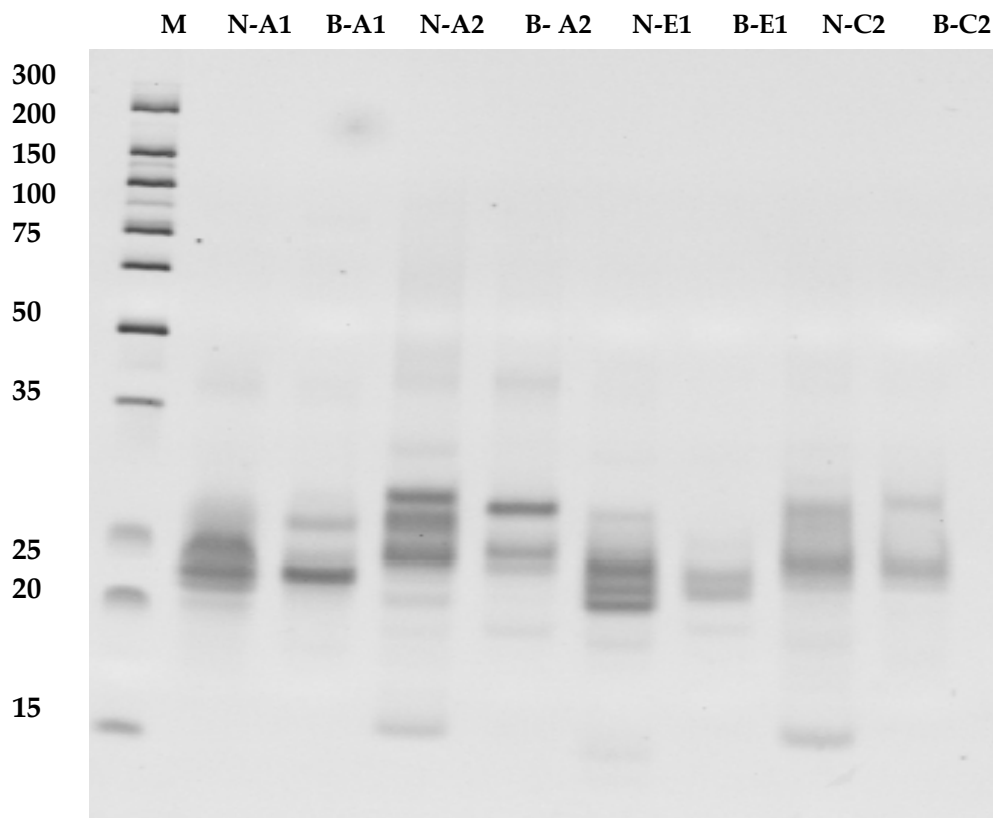


Figure 15 – Analysis of natural and modified RNA products in a 10% PAGE/ 7 M urea with ethidium bromine staining.

Full-length natural (N) or BP-modified (B) RNA of different sequences (A1, A2, E1, C2). Both N and B have full length product bands that cluster around 20-30 mer DNA marker band as expected, multiple RNA product bands are also seen and this are sequence specific as well as modification specific (Yields are shown in table 4).

3.4 Discussion

The transcription yields depend on the sequence, incubation time, and number of modifications. We cannot generalize the effects on the yields from these experiments.

However, we can infer that the incubation time and triphosphate concentrations will make a difference in modified enzymatic synthesis. Two clear patterns emerged from

the experiments using three templates: transcription with high concentration of natural

NTPs, with the longer incubation time, gave higher yields. This was not such a clear case for CTP α B-modified transcripts; where the control sequence (1,800 mer) gave higher yields after overnight incubation with high NTPs concentration but template #1 (107 mer) and template #2 (107 mer) did not have a clear optimal conditions. We hypothesize that the large error for the **1HO** or **2HO** is due to the increasing and random degradation of the modified RNA during overnight incubation (**Figure 12**).

It has been reported that the yield of the short boranophosphate RNA (5'-GGG AGA CCA CAA CCU CUC GU-3') was more sensitive to whether the nucleotide at the fourth position was modified or not (Wan and Shaw 2005). Thus we wanted to see if changing the first two incorporated nucleotides (AG or GG) would make a difference in the yield. We compared two promoter regions that allowed the G to be incorporated at the first two nucleotides (GG) or at the second position only (AG) (**Figure 8**). We found that the addition of a modification at the first NTP and second positions or the second NTP position only did not make a difference in the yield of this sequence (data not shown).

Further, we wanted to test our hypothesis that the enzymatic synthesis yield is template-dependent. We tested four sequences and then compared the yields between the sequences and using either UTP or UTP α B. The overall yields were lower for the UTP α B-modified RNA but there was also a decrease in yield with natural NTPs with the

A1/A2 template. We conclude that the yield is sequence-dependent as well as modification-dependent.

Both natural and mutant T7 RNA polymerase enzymes were found to incorporate boranophosphate NTPs (NTP α Bs) but with a decrease in yield compared to natural NTPs. This reduction in yield is likely due to the kinetics of incorporation of the NTP α B. NTP α Bs have higher incorporation efficiency than the natural NTPs but they also have a tighter apparent binding affinity to the enzyme (Wan Jing, Dissertation Duke 2005). We hypothesize that the tighter binding of the NTP α B stalls the enzyme, especially during the early stages of transcription and it slows the enzyme down, thus reducing the transcription yields with NTP α B.

In addition, using the model suggested by Steitz et al. (1994) in **Figure 16** where the transition complex of the natural NTP and the polymerase, shows the non-bridging phosphorus in the α phosphorus interacting with two Mg⁺² ions: the BH₃ group is not able to coordinate metal ions; this might prevent the efficient elongation of the complex and possibly there is a stall during the transition state.

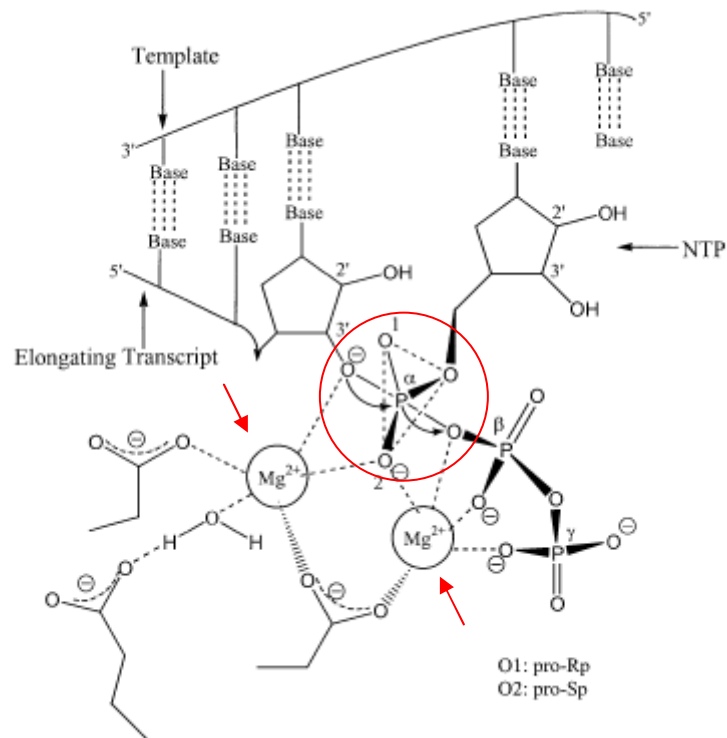


Figure 16 - Transition complex for Y7 RNA polymerase with NTP α Bs. Highlighted in the α -phosphorus (red circle) where the BH₃ modification would be located. The non-bridging oxygen in this phosphorus interacts was proposed to interact with the two Mg⁺² ions (red arrows) in the transition complex. (Figure adapted from (Steitz 1997; Brautigam and Steitz 1998; Steitz 1999).

Chapter 4: BP-modified ssRNA40 does not activate toll Like Receptor 7 (TLR)

4.1 Introduction (TLR Activation)

Innate immune activation by non-self RNA is an undesirable side effect associated with RNA therapies such as siRNA (Kariko, Buckstein et al. 2005; Diebold 2008; Gantier, Tong et al. 2008; Robbins, Judge et al. 2009). Understanding how chemical modifications modulate this immune activation is advantageous when developing oligonucleotide therapies. In siRNA therapies, a short RNA is systemically administered and it targets the mRNA of a target gene (Fire, Xu et al. 1998; Hannon 2002). The RNA sensing Toll-like receptors (TLR 3, TLR 7, and TLR 8) recognize viral and foreign RNA as well as antiviral compounds such as imidazoquinolines (Diebold, Kaisho et al. 2004; Heil, Hemmi et al. 2004). Activation of these receptors leads to the secretion of inflammatory cytokines such as tumor necrosis factor alpha (TNF α), interleukin-6 (IL-6), and interferon (INF) (Robbins, Li et al. 2006; Robbins, Judge et al. 2009).

TLRs are a conserved set of pattern-recognition receptors and form the first line of defense against invading pathogens (Takeda, Kaisho et al. 2003). Of these, TLR 7 and 8 recognize ssRNA, as well as polyuridine- and GU-rich RNA that lead to the activation of the host innate immune system (**Figure 17**) (Heil, Hemmi et al. 2004; Gantier, Tong et al. 2008). TLR 3 recognizes dsRNA, PolyIC and unmethylated mRNA (Robbins, Judge et al. 2009).

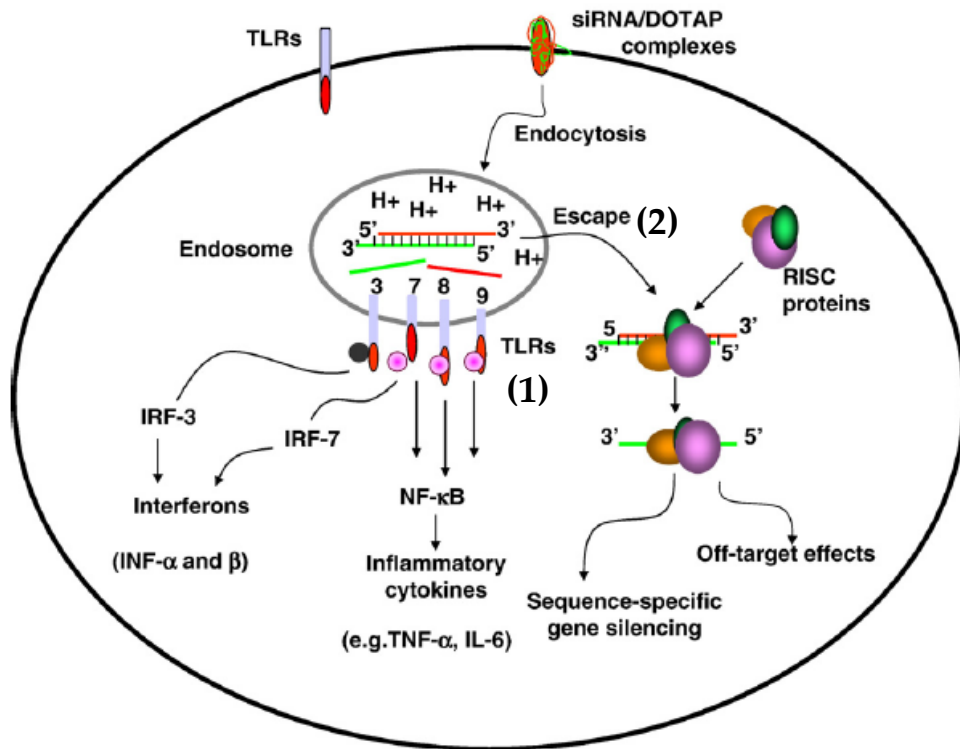


Figure 17: TLR activation of siRNA.

The siRNA/DOTAP complexes are internalized into an endosomal compartment where **(1)** they activate TLRs mediated immune reaction leading to the production of interferons and/or inflammatory cytokines (TNF α or IL-6) or **(2)** escape the endosome and get incorporated into the RISC complex leading to gene silencing of the desired gene or genes with enough homology. The unintended gene silencing is termed off-target effects. (Figure adapted from (Sioud 2007))

TLR 7/8 are expressed in endosomal structures by plasmacytoid dendritic cells (pDC) cells and B cells. After stimulation with RNA complexed with Lipofectamine, inflammatory cytokines are produced (Robbins, Judge et al. 2009). It has been shown that endosomal activation of the TLRs is needed to activate cytokine production (**Figure 17**) (Sioud 2007). TLRs 7, 8, and 9 are located in endosomal compartments inside the cell and their ectodomains (EDCs) are responsible for ligand binding (Choe, Kelker et al.

2005). The Toll-interleukin-1 receptor domain (TIR) recruits adaptor molecules to initiate cytokine-producing signaling cascades (Choe, Kelker et al. 2005). There is no direct experimental evidence that RNA binds directly to the TLRs; it has been suggested that the RNA binds to the receptor via a co-receptor protein (Choe, Kelker et al. 2005). Chemical modifications of the sugar or phosphate backbone can modulate the innate immune response through TLR pathways. For example, 2'-F- and 2'-OMe-modified siRNAs can selectively inhibit activation of TLRs 7 and 8 as seen in **Figure 18** (Robbins, Judge et al. 2007; Sioud, Furset et al. 2007).

Interestingly, these sugar-modified siRNAs have been shown to antagonize other TLR 7 and 8 agonists (Robbins, Judge et al. 2007; Sioud 2007; Robbins, Judge et al. 2009). While these sugar modifications reduce the immunogenicity of siRNA, they may also inhibit the anti-viral activity of TLR7 and facilitate microbial invasion. Since TLR stimulation promotes host defense against intracellular organisms, inhibition may lead to immune suppression. This is exemplified by studies showing that mice that are genetically deficient in TLR signaling are susceptible to infection (Tjarnlund, Guirado et al. 2006; Bhan, Lukacs et al. 2007; Sheahan, Morrison et al. 2008).

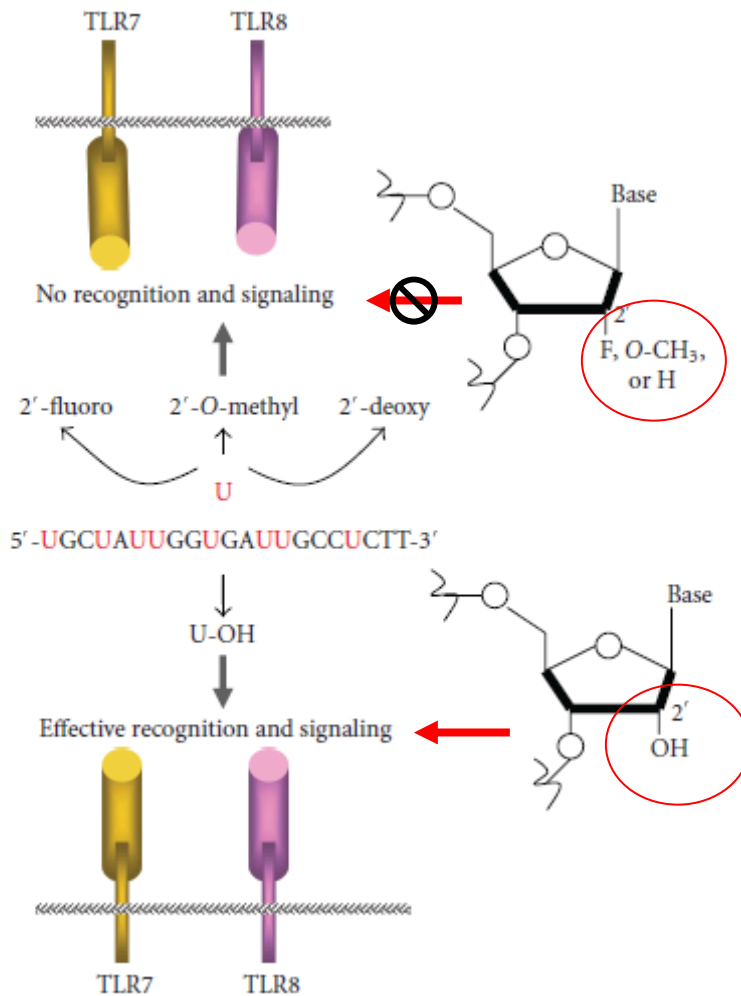


Figure 18 – Activation of TLR 7 and TLR 8 by modified RNA.

TLRs 7 and TLR 8 (yellow and pink respectively) are not activated by modified RNA with sugar modifications such as 2'F, 2'-OMe or natural DNA (-H) from (Sioud 2006). However, natural (-OH) RNA is recognized and activates TLR 7 or TLR 8.

Phosphorothioates (PS) are a common chemical modification of oligonucleotides including RNA. SiRNA molecules with PS modifications have increased stability in serum (**Figure 19C**) (Braasch et al., 2004; Winkler et al., 2010). However, PS-modified RNA increases cytotoxicity and immunogenicity by activating toll-like receptor

signaling (Winkler, Stessl et al. 2010). Boranophosphate modification also imparts increased siRNA activity and stability (**Figure 19 B**) (Shaw, Dobrikov et al. 2003; Hall, Wan et al. 2006). However, its immunological aspects have not been elucidated. Borane (BH_3) chemistry offers some unique chemical characteristics that make these compounds promising for enhancing the potential of RNA therapies (Shaw, Dobrikov et al. 2003; Hall, Wan et al. 2006). **Figure 19 A, B, C** shows the chemical structures of natural, BP (R_p isomer) and PS (S_p isomer) NTPs that are incorporated by T7 RNA polymerase and used for enzymatic synthesis of RNA. The configuration assignment for BP and PS are reversed because of the group priority around the phosphorus atom.

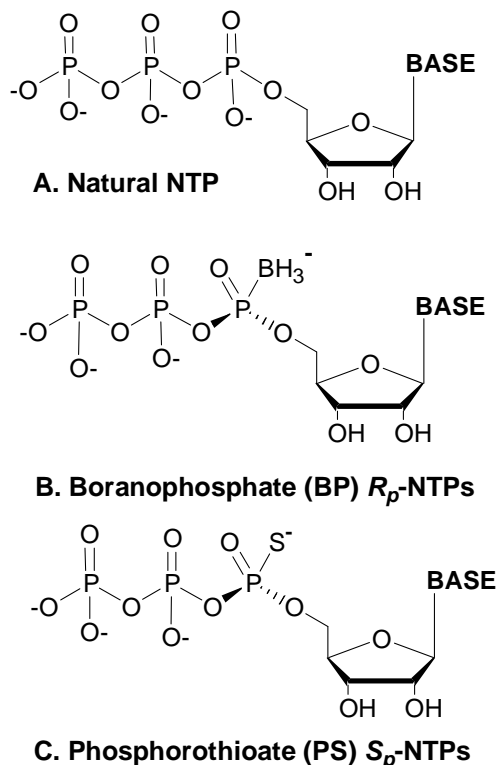


Figure 19 - Natural, BP, and PS NTPs used for synthesis of RNA40.

T7 RNA polymerase incorporates R_p -BP and S_p -PS NTPs to give RNA with S_p and R_p linkages, respectively.

We studied, in collaboration with Dr Jaewoo Lee and Professor Bruce Sullenger (Duke University Medical School, Durham, NC), a 21-mer GU-rich sequence from the U5 region of the HIV-1 RNA (ssRNA40), which is an extensively studied agonist of TLR 7 (mouse) and TLR 8 (human) (Diebold, Kaisho et al. 2004; Heil, Hemmi et al. 2004; Diebold, Massacrier et al. 2006; Diebold 2008; Gantier, Tong et al. 2008). Treatment of innate immune cells isolated from wild-type mice with ssRNA40/liposome complex resulted in activation and secretion of inflammatory cytokines TNF α , IL-6, IL-12, and IFN α , whereas ssRNA40 could not activate cells isolated from TLR 7-deficient mice (Heil, Hemmi et al. 2004).

In the current study, we compared BP-modified ssRNA40 to the natural and PS-modified ssRNA40 by quantifying the cytokine activation from mouse macrophage cells. We found that BP-modified ssRNA40 shows decreased activation of TLR 7 with increasing amounts of BP-modified nucleosides per RNA molecule. Furthermore, these fully modified BP-modified ssRNA40 did not inhibit TLR 7 activation by all PS-modified ssRNA40.

4.2 Materials and Methods

RNA synthesis

All RNAs (5'-GGC CCG UCU GUU GUG UGA CUC-3') were enzymatically synthesized using a dsDNA template (5'-TAATACGACTCACTATA GGC CCG TCT GTT GTG TGA CTC-3' and 3'-ATTATGCTGAGTGATAT CCG GGC AGA CAA CAC ACT GAG-5') (Integrated DNA technologies). This dsDNA (10 µg) was transcribed into RNA with a T7 RNA polymerase mixture (RNA polymerase, recombinant RNasin® Ribonuclease Inhibitor, and recombinant inorganic pyrophosphatase) from the Ribomax Large Scale RNA Production System and the corresponding natural and/or modified triphosphates at a 2.0 mM concentration in 100 µL of 1X transcription buffer (40 mM Tris-HCL pH 7.5, 6 mM MgCl₂, 5 mM NaCl and 10 mM spermidine). Both NTPs and NTPαSs (mixture of *R_p* and *S_p*, Trilink, San Diego, CA) were used. *R_p*-NTPαBs were chemically synthesized in our lab and HPLC-purified as described previously (He et al., 1998). The reaction mixture was incubated for 6 hours at 37° C. The DNA template was digested using RQ1

RNase-free DNase (1 U/ μ L) by incubation at 37° C for 15 min. The unincorporated NTPs were removed by G25 spin columns (GE Healthcare). The final product was ethanol precipitated, washed, re-suspended in DEPC-treated water and quantified by UV. The sample was **ethanol precipitated** as follows: The aqueous layer was transferred to a new tube and 0.1 volume (30-40 μ L) of 3M sodium acetate pH5.2 was added and as well as 5 μ L Linear acrylamide. Then, 2.5 volumes of cold ethanol (-20° C) were added. The sample was placed on powdered dry ice for 30 min (-78° C) until frozen. The sample was centrifuged at 15,000 rpm for 30 min at 4° C, the supernatant was removed with a pipette, and 500-1000 μ L cold 70% ethanol (-20° C) was added. The sample was centrifuged, the ethanol was removed, and the sample was air-dried at rt. for 15 min. The quality and size of the RNA was analyzed on a 7 M urea 10% (19:1) PAGE.

Cell lines and Cultures

Murine macrophage cells RAW264.7 (ATCC) were cultured at 37° C in 5% CO₂ incubator and grown in DMEM supplemented with 10% FCS, 100 units/mL penicillin and 100 μ g/mL streptomycin.

Preparation of RNA/liposome complex and in-vitro activation of cells

The ssRNAs were complexed to Lipofectamine 2000 (Invitrogen) or DOTAP (Roche). For RNA/Lipofectamine complex; 2.65 μ g of RNA was incubated for 15 min at RT with a solution of Opti-MEM and 2.5 μ L of Lipofectamine that was incubated for 5 min before mixing. For the RNA/DOTAP complex: HBS (HEPES buffered saline: 20 mM

HEPES, 150 mM NaCl, pH 7.4) was combined with 5 µg of RNA and an equal volume solution of 12.5 µg DOTAP in HBS and incubated for 15 min at RT.

The Raw264.7 cells were seeded onto 24-well plates at 5×10^5 cells /500 µL and stimulated with the RNA/Liposome complex at a concentration of 10 µg/mL RNA. After 24 hours, the culture supernatants were collected and stored at -80° C till used.

Cytokine ELISA

The amounts of TNF α and IL-6 in the culture supernatants were quantified using mouse TNF α ELISA set II and mouse IL-6 ELISA kits following manufactures protocol (BD Bioscience).

4.3 Results

Mouse macrophage cells were stimulated with ssRNA40 containing various amounts of natural, PS or BP nucleosides (**Table 5**). For intracellular delivery, these RNAs were complexed with liposomal transfection agents including Lipofectamine 2000 or DOTAP. We enzymatically synthesized natural, stereoregular (Rp)-PS and (Sp)-BP RNA40 to study the borano RNA-TLR7 interaction.

Table 5 –Sequence of the ssRNA40 used in the cell experiments.

Each 21-mer RNA was synthesized with either natural and/or modified NTPs; BP modified nucleosides are shown in boldface and phosphorothioates are shown underlined.

Name	NTPs	Sequence ssRNA40 (33% G, 29% C, 33% U, 5% A)
Natural	U,A,G,C	GGC CCG UCU GUU GUG UGA CUC
Thio(4S)	<u>U,A,G,C</u>	<u>GGC CCG UCU GUU GUG UGA CUC</u>
1B-U α B	U, ^b A,G,C	GGC CCG UCU GUU GUG UGA CUC
1B-U α B	^b U,A,G,C	GGC CCG UCU GUU GUG UGA CUC
1B-C α B	U,A,G, ^b C	GGC CCG UCU GUU GUG UGA CUC
1B-G α B	U,A, ^b G,C	GGC CCG UCU GUU GUG UGA CUC
2B-(C α B & U α B)	^b U,A,G, ^b C	GGC CCG UCU GUU GUG UGA CUC
2B-(C α B & G α B)	U,A, ^b G, ^b C	GGC CCG UCU GUU GUG UGA CUC
2B-(G α B & U α B)	^b U,A, ^b G,C	GGC CCG UCU GUU GUG UGA CUC
3B-G α B,U α B, C α B)	^b U,A, ^b G, ^b C	GGC CCG UCU GUU GUG UGA CUC
4B	^b U, ^b A, ^b G, ^b C	GGC CCG UCU GUU GUG UGA CUC

Guanosine (G), Adenosine (A), Cytidine (C), Uridine (U), b-boranophosphate, s-phosphorothioate

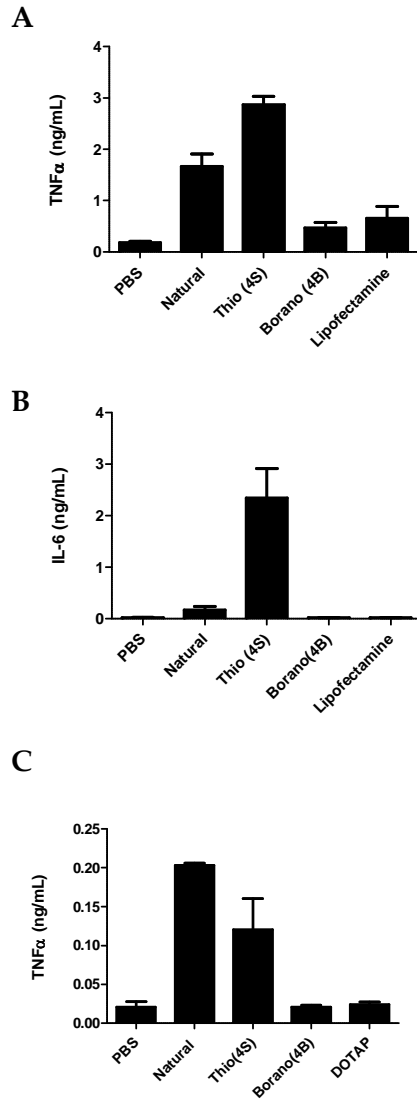


Figure 20 – A comparison of stimulation of cell- induced cytokine production by natural and PS- or BP-modified ssRNA40s.

Stimulation is dependent on modification chemistry and amount. (A) Activation of TLR7 by quantifying TNF α secretion after stimulation with either natural, fully PS (Thio(4S)), or fully BP (Borano(4B)) ssRNA40. (B) Interleukin-6 (IL-6) was also quantified after stimulation and Borano(4B) compared to Natural and Thio(4S). (C) RNA40/DOTAP complexes showed a similar trend to Lipofectamine complexed molecules where the Borano(4B) had close to background levels of TNF α production.

TNF α and IL-6 cytokine production by macrophage cells was compared after stimulation with different types of backbone modifications (**Figure 20A and 20B**). Fully-modified ssRNA40 (Thio(4S)) resulted in a two-fold greater production of TNF α compared to the natural ssRNA40 (natural) (**Figure 20A**). By comparison, the boranophosphate fully modified ssRNA40 (Borano(4B)) showed a six-fold reduction in TNF α production compared to Thio(4S) and a 3.6 fold reduction compared to natural.

When a second cytokine, IL-6, was measured, the Thio(4S) likewise had a significant production of IL-6 in contrast with Borano(4B) and natural, which had near-background levels of IL-6 (**Figure 20B**). In addition, using a different liposomal carrier DOTAP, the trend of Borano(4B) having significantly lower cytokine production

compared to Thio(4S) and natural was maintained (Figure 20C).

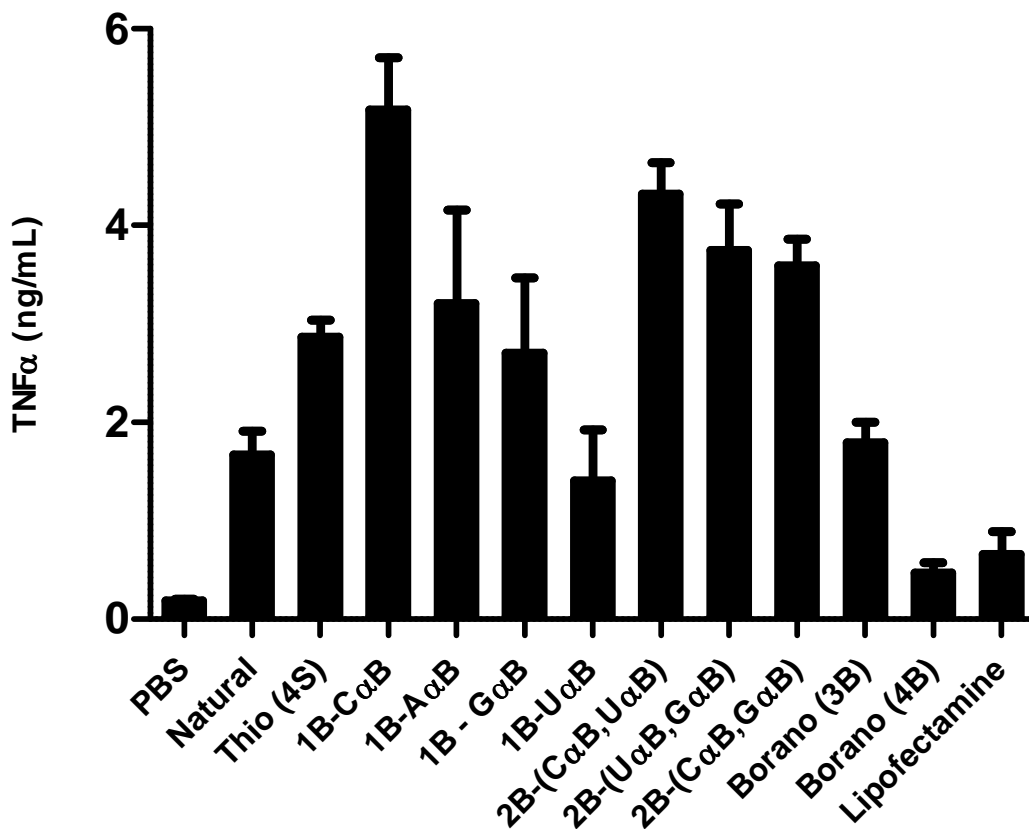


Figure 21 – TNF α production as a function of number of BP linkages in ssRNA40. Different amounts of boranophosphate linkages show a trend of decreasing production of TNF α with increasing amount of boranophosphates. Natural and thio controls show activation of TLR 7 in contrast with fully modified boranophosphate linkages (4B). However, decreasing the number of boranophosphate linkages to 3 (3B) to 2 (2B) or to 1 (1B) show an increase in TNF α production. PBS (phosphate buffered saline) show background levels TNF α for untreated cells. All have standard error from three independent experiments.

We then asked whether the cytokine production depended on the ratio of natural:borane nucleotides (and nucleobase composition). We added back different

amounts of natural nucleotides into the Borano(4B) RNA chain. RNA with three and four modified NTPs had a significant reduction in TNF α production (**Figure 21**). But unlike the reduction in TLR 7 activation seen with fully boronated ssRNA40 (**Figure 20**), oligomers with one (1B-U α B, 1B-G α B, 1-A α B or 1B-C α B) or two (2B-(U α B, G α B), 2B-(U α B, C α B), 2B-(C α B, G α B)) borano modifications yielded higher levels of TNF α production when compared to fully-modified Thio(4S) (**Figure 21**).

Furthermore, it was important to determine if boranophosphate could compete with the TLR activation due to activation Thio(4S). Therefore, Borano(4B) ssRNA40 plus Thio(4S) ssRNA40 were complexed with Lipofectamine 2000 and co-transfected into mouse cells. We observed that Borano(4B) did not block activation of TLR 7 by the Thio(4S) ssRNA40 (**Figure 22**). This suggests that the borano (4B) ssRNA40 does not bind to the receptor preventing its activation. We hypothesize that the change in chemical properties of the fully-borano ssRNA40 allows it to pass unrecognized by the receptor and does not trigger the secretion of IL-6 or TNF α cytokines.

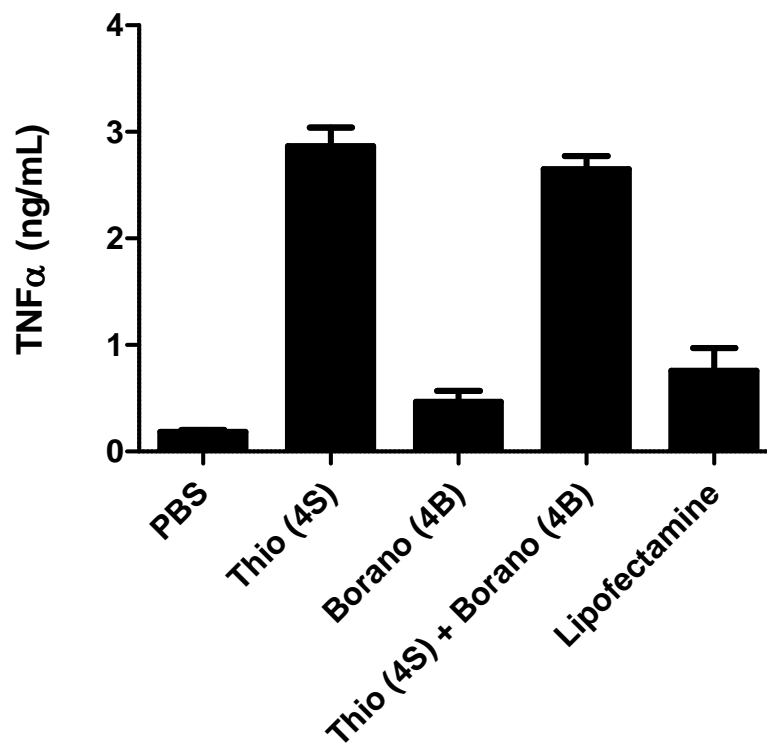


Figure 22 - Competition assay between Thio(4S) and Borano(4B).

Thio(4S) activated TLR 7, however to determine if Borano(4B) interfered with that activation, we co-transfected thio(4S) with Borano(4B). Borano(4B) does not block the activation of TLR 7/8 by Thio(4S) suggesting that the boranophosphate does not bind to the receptor or prevent the stimulation by the Thio(4S). Thus, Borano(4B) is not antagonist of TLR 7 and we hypothesize that it is not recognized by the TLR 7 receptor once in the endosomes.

This latter result is in contrast with the 2'-F or 2'-OMe modifications, which were shown to inhibit activation of TLR 7 and 8 (Allerson, Sioufi et al. 2005; Kariko, Buckstein et al. 2005; Morrissey, Lockridge et al. 2005; Robbins, Judge et al. 2007; Judge and MacLachlan 2008). Co-transfection of the unmodified RNA and the 2'-OMe RNA suppressed the activation of the TLR 7 by the unmodified RNA, suggesting that when

the 2'-OMe is bound to the TLR receptor and it prevents activation (Robbins et al., 2007; Sioud, 2006a, c; Sioud et al., 2007).

4.4 Discussion

Changing the non-bridging oxygen to a sulfur (S) atom or to a BH₃ molecule does not change the net negative charge in the phosphate linkage, but would change the chemistry of the RNA molecule and its interactions with its environment. In phosphates and phosphorothioates, there is a single atom substitution of O to S, whereas in boranophosphates there is a molecular substitution of O to BH₃. Similarly to the phosphoryl oxygen in natural RNA the sulfur has two lone-pairs of electrons available in its outer shell to coordinate to water as well as the cationic regions of proteins. In contrast, the BH₃ group does not have available lone-pair electrons; thus, replacing the non-bridging oxygen with BH₃ on the RNA reduces the coordination of water and proteins available, it also decreases its localized negative charge (Summers and Shaw 2001). Consequently, the overall surface charge of the borano modified RNA is likely less negative compared to the RNA with oxygen and sulfur substitutions. This prevents the fully modified boranophosphate backbone from interacting in the same manner as the natural or PS backbones since it is more lipophilic. We hypothesize that the decreased negative charge in the molecular surface of BP-RNA could lead to a change in the affinity and specificity of the RNA to proteins. This picture is further supported by

the co-transfection of the Thio(4S)/ Borano(4B) mixture which still activated the production of TNF α , which may suggest that the Borano(4B) does not bind to the receptor at all or in the same way (**Figure 22**).

4.5 Conclusions

Fully BP-modified ssRNA40 does not activate TLR7, and there is a trend of decreased production of TNF α with an increased number of borano modifications on the RNA strand. RNA containing one or two BP modifications still activates TLR 7 and RNA containing three or four modified NTPs result in a decrease of production of TNF α . We hypothesize that this activity difference is due to the change in surface charge density of the boranophosphate RNA molecule, its conformations, and its interaction with the receptor. Although natural, PS-, and BP RNA all carry the same net negative charge, when BH₃ is added to the backbone there is a decreased overall surface negative charge density compared to the more negative surface charge density in natural and phosphorothioate backbones. Further research should investigate the interaction of BP RNA with other TLR receptors such as TLR3, which is activated with dsRNA.

This research led us to analyze the applications of BP-RNA and design molecules for therapeutic applications such as siRNA or RNA aptamers, where immune activation by the oligonucleotide is undesirable.

Chapter 5: Investigation of boranophosphate siRNA: activity and Immunogenicity

5.1 Introduction

RNA interference (RNAi) occurs naturally in cells to control gene expression and is an evolutionary conserved cellular pathway (Fire, Xu et al. 1998). The RNAi pathway can be used to study gene expression or to specifically target genes for therapeutic applications. In siRNA therapies, an RNA of a specific sequence (19-27 nucleotides long) can be targeted to a small region of a messenger RNA (mRNA) and used to modulate gene expression of the corresponding gene (Zamore, Tuschl et al. 2000; Elbashir, Lendeckel et al. 2001). RNAi is triggered by short hairpin or long double-stranded RNAs that are processed by an enzyme complex named dicer into siRNAs (Fire, Xu et al. 1998; Elbashir, Lendeckel et al. 2001) (**Figure 23**). The antisense strand of the siRNAs is loaded into the RISC complex (RNA-induced silencing complex), which targets the mRNA of the gene with homology to the siRNA sequence **Figure 23** (Zamore, Tuschl et al. 2000; Hannon 2002). In addition, although controversial, the antisense single-stranded siRNA (AS ss siRNA) was shown to have almost as potent activity as the double-stranded siRNA (ds siRNA) (Holen, Amarzguioui et al. 2003).

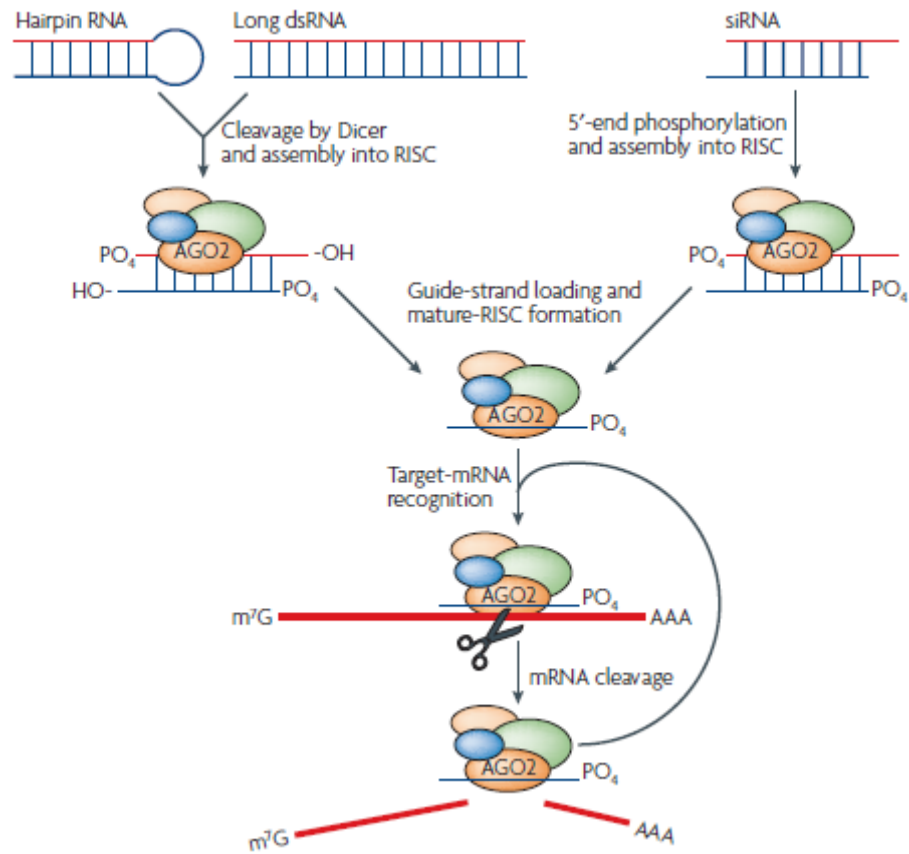


Figure 23 - RNA interference (RNAi)

RNA interference pathways for loading siRNA into the RISC complex. RNAi is initiated by a protein named dicer, which processes long and hairpin RNA duplexes into siRNA molecules. The siRNA is loaded into the RISC protein complex and targets the mRNA of the gene of interest (Rana 2007).

The RISC protein complex catalyzes the cleavage of a single phosphodiester bond on the mRNA target (Schwarz, Tomari et al. 2004). The cleavage site of the mRNA is the between the 10-11 nucleotide in the sequence of the duplex formed between the siRNA and mRNA counting from the 5'-end of the antisense strand of the siRNA (Elbashir, Martinez et al. 2001). The catalytic activity that leads to the mRNA cleavage was shown to be the Argonaut2's (ago2) catalytic domain, named p-element induced

wimpy testis or PIWI, in the RISC complex (Liu, Carmell et al. 2004; Meister, Landthaler et al. 2004). Ago2 contains a RNaseH fold that catalyses the cleavage of the mRNA (Figure 23) (Song, Smith et al. 2004). The RISC protein complex endonucleases are dependent on interactions between the mRNA/siRNA duplex and Mg^{+2} ions during cleavage (Schwarz, Tomari et al. 2004). During catalysis, at least one Mg^{+2} interacts with the non-bridging oxygen of the scissile phosphate (Figure 24). In addition, this mechanism suggests that the mRNA cleavage mechanism is similar to that of RNaseH and other RNase III families (Manoharan 2004). Further, we hypothesize that the addition of a modification on the siRNA strand that interrupts the Mg^{+2} chelation might interfere with efficient mRNA cleavage.

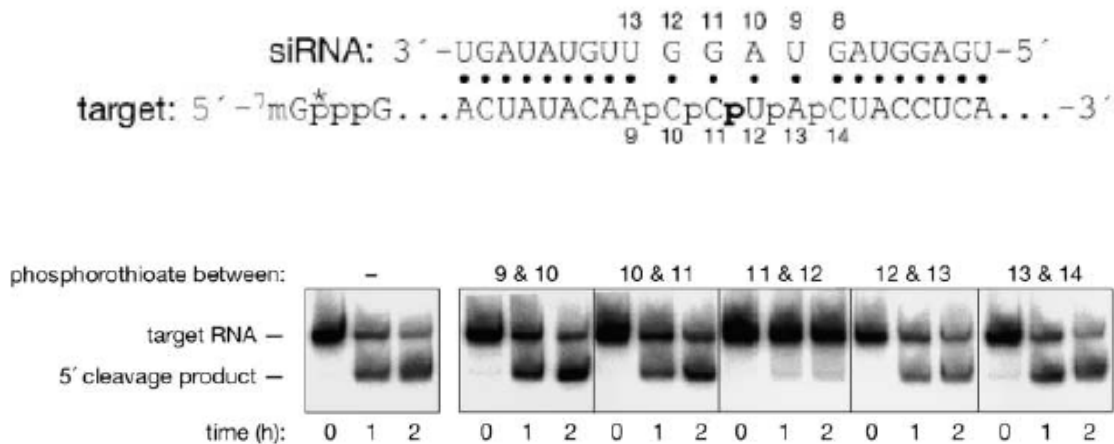


Figure 24 - Scissile phosphate is a Mg^{+} ligand (Schwarz et. al. 2004)

The anti-sense strand has to be 5'-phosphorylated before it is loaded into the RISC pocket and it is anchored within the conserved region of the ago2 termed PIWI

domain (Song, Smith et al. 2004). Chemical modifications in the siRNA have the potential to interfere with the loading process and the catalytic efficiency of the RISC complex.

This project focuses on downregulating the cell surface protein *P*-glycoprotein (*P*-gp), which is produced by the MDR1 gene, using BP-modified siRNA. The best-characterized cause of multidrug resistance (MDR) is the over-expression of *P*-gp (Karwatsky, Lincoln et al. 2003). *P*-gp is an ATP-binding cassette (ABC) protein that causes energy-dependent efflux of many unrelated hydrophobic drugs from cancer cells and is a cell-surface glycoprotein composed of 12 transmembrane helices arranged into two domains (**Figure 25**) (Karwatsky, Lincoln et al. 2003). Appended to each domain on the cytosolic side are two ATP-binding subunits which fuel the “vacuum cleaner” action of the *P*-gp, intercepting drugs and releasing them from the cells before they reach high-affinity targets (Hall, Handley et al. 2009). The MDR1 gene that encodes for the *P*-gp is considered a molecular target in multi-drug resistant cancer (Xu, McCarty et al. 2005). The rise of MDR-caused-resistance to anticancer chemotherapy drugs has long been recognized as a major obstacle in clinical anticancer treatment and emphasis has been placed on identifying chemosensitizers (Fisher, Abramov et al. 2007). A possible therapy to correct the over-expression of *P*-gp involves using gene regulators such as siRNA that would be administered in conjunction with chemotherapy drugs to decrease resistance (Fisher, Abramov et al. 2007). A number of strategies using siRNA has been shown to

downregulate P-gp1 and inhibit its drug efflux function, thereby preventing drug resistance (Bartsevich and Juliano 2000; Xu, Kang et al. 2004; Xu, McCarty et al. 2005; Fisher, Abramov et al. 2007).

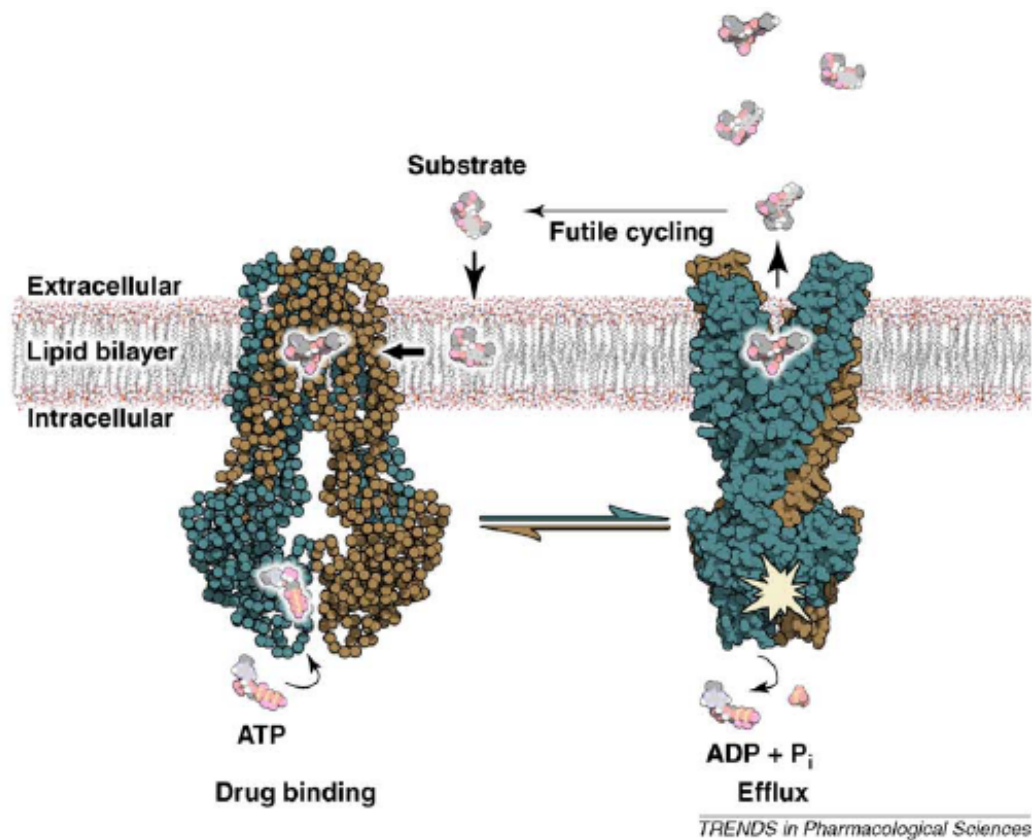


Figure 25 - P-gp structure and mechanism of substrate removal from cytosol.

P-gp is a cell-surface glycoprotein and appended to each domain on the cytosolic side are two ATP-binding subunits. ATP and the substrate bind to the protein, ATP is reduced to ADP and pyrophosphate to shuttle the substrate from the cytosol to the exterior of the cell (Figure adapted from Hall, Handley et al. 2009).

We have previously shown that BP-siRNA is active as both ss and ds molecules in HeLa cells to downregulate the enhanced green fluorescent protein (EGFP) (Hall, Wan et al. 2006). Hall et al. found that the silencing activity of ss BP-siRNA was

comparable to the ds-BP siRNA and, in some cases, more potent than the PS-modified siRNA (Hall, Wan et al. 2006). Furthermore, the activity of the BP-siRNA was long-lasting, with significant silencing observed one week after transfection (Hall, Wan et al. 2006). In the current study, in collaboration with Mike Fisher and Professor Rudy Juliano (Pharmacology Department, University of North Carolina, Chapel Hill), we screened BP-modified siRNA for activity in downregulation of *P*-gp in NIH-3T3 cells. We compared a group of BP, PS, and altriol-modified siRNAs. These were complexed with Lipofectamine 2000 before transfection and the % *P*-gp on the cell surface was quantified by immunostaining using flow cytometry. We, in collaboration with Dr Jaewoo Lee and Professor B. Sullenger (Duke University Medical School, Durham, NC), also analyzed the same siRNA molecules for their activation of the TLR 7 receptor by measuring the production of the inflammatory cytokine TNF α in Raw264.7 cells. We wanted to determine the relationship between the BP-RNA siRNA downregulation of *P*-gp and the activation of toll like receptor 7 (TLR 7).

5.2 Experimental procedures

Cell culture

Downregulation of P-glycoprotein (P-gp)

The MDR-3T3 cells were obtained from Michael Gottesman (National Cancer Institute). The 3T3 cells contain a plasmid with the human MDR1 gene (Kane, Reinhard et al. 1989).

Pro-inflammatory cytokine analysis (Tumor Necrosis Factor α , TNF α)

Murine macrophage cells RAW264.7 (ATCC) were cultured at 37° C in a 5% CO₂ incubator and grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 units/mL penicillin, and 100 μ g/mL streptomycin.

Cytokine ELISA

The amounts of TNF α in the culture supernatants were quantified using mouse TNF α ELISA set II and mouse IL-6 ELISA kits following manufactures protocol (BD Bioscience).

Modified and Natural siRNA enzymatic synthesis

Table 6 - siRNA sequences targeting the MDR1 gene mRNA.

Name	Modified NTP used	siRNA sequence 5'-3'
Shaw ORF1	none	GG UUC GAA UAG CUG UCA AUA CUU
ORF1(as)	none	UUC GAA UAG CUG UCA AUA* CdTdT
ORF1(s+as)	none	UUC GAA UAG CUG UCA AUA* CdTdT
C α B	CTP α B	GG UUC GAA UAG CUG UCA AUA CUU
A α B & G α B	ATP α B, GTP α B	GG UUC GAA UAG CUG UCA AUA CUU
2'FC	2'- F CTP	GG UUC GAA UAG CUG UCA AUA CUU
2'FC & G α B	2'- F CTP, GTP α B	GG UUC GAA UAG CUG UCA AUA CUU
U α B & G α B	UTP α B & GTP α B	GG UUC GAA UAG CUG UCA AUA CUU
3B-(A α B, U α B, G α B)	ATP α B, UTP α B, GTP α B	GG UUC GAA UAG CUG UCA AUA CUU
Borano(4B)	ATP α B, UTP α B, GTP α B,	GG UUC GAA UAG CUG UCA AUA CUU
Thio(4S)	ATP α S, UTP α S, GTP α S,	GG UUC GAA UAG CUG UCA AUA CUU
2470	altriol modified	UUC GAA UAG CUG UCA AUA* CdTdT

The name signifies the modified NTP used during transcription, as = antisense, (as+s) = antisense and sense strand, 4B = all borano NTPs, 4S = all thio modified.

The following modified nucleotides and combinations were used to synthesize the siRNA: C α B, A α B & G α B, 2'FC, 2'FC & G α B, U α B & G α B, 3B-(A α B, U α B, G α B), Borano(4B) and Thio(4S) (Table 6). These RNAs were enzymatically synthesized using

the appropriate dsDNA template and the corresponding modified/natural NTPs. The name signifies the added modified NTP used during enzymatic synthesis. The other siRNAs (Shaw ORF1, ORF1(as), ORF1 (s+as), 2470 (altriol modified) were chemically synthesized and obtained from our collaborators Mike Fisher and Professor Rudy Juliano. To anneal the dsDNA template, each DNA template strand (Integrated DNA Technologies) was added in equimolar amounts (6 nmol) in 100 μ L of annealing buffer (10 mM Tris-HCL pH 7.5, 10 mM MgCl₂). The mixture was heated to 95° C for 5 min and allowed to cool to RT temperature for 2 h. The dsDNA template (10 μ g), was incubated with a 10 μ L T7 RNA Polymerase mixture (RNA polymerase, recombinant RNasin® Ribonuclease Inhibitor, and recombinant inorganic pyrophosphatase) from the Ribomax® Large Scale RNA Production System along with the corresponding natural and/or modified ribonucleoside triphosphates at a 2.0 mM concentration in a 1.5 mL micro centrifuge tube. The final volume consisting mostly of 1X reaction buffer (80 mM HEPES-KOH pH 7.5, 24 mM MgCl₂, 2 mM spermidine, 40 mM DTT) was 100 μ L, the reaction mixture was incubated at 37° C in a water bath set at that temperature to allow the polymerase to incorporate the NTPs. Natural NTPs and 2'-F modified NTPs were obtained from Epecentre Inc. *R_p*-NTP α Bs were chemically synthesized in our lab and HPLC-purified as described previously (He and Shaw 2001). The reaction mixture was incubated for 6 hours at 37° C. The DNA template was digested using 10 μ L RQ1 RNase-free DNase (1 U/ μ L) by incubation at 37° C for 15 min. The unincorporated NTPs

were removed by G25 spin columns (GE Healthcare). The final product was ethanol precipitated, re-suspended in DEPC treated water and quantified by UV. The sample was **ethanol precipitated** as follows: The aqueous layer was transferred to a new micro centrifuge tube (1.5 mL) and 0.1 volume (30-40 μ L) of 3M sodium acetate pH 5.2 was added and as well as 5 μ L Linear acrylamide. Then, 2.5 volumes of cold ethanol (-20 ° C) were added. The sample was placed on powdered dry ice for 30 min (-78° C) until frozen. The sample was centrifuged at 15,000 rpm for 30 min at 4° C, the supernatant was removed with a pipette, and 500-1000 μ L cold 70% ethanol (-20° C) was added to the pellet. The sample was centrifuged, the ethanol was removed, and the sample was air-dried at rt. for 15 min. The quality and size of the RNA was analyzed on a 7 M urea 10% (19:1) PAGE gel. The RNA was stored at -20° C in 1X Dharmacon siRNA buffer if need within a few days or as lyophilized powder for storage during long periods of time.

Preparation of siRNA/liposome complex for siRNA downregulation of P-gp.

The ds siRNA was prepared by heating the sense and antisense strand at 70° C for 2 min and cooling at RT for 5 min in 1X siRNA buffer (Dharmacon).

Either ss or ds siRNA were complexed with Lipofectamine 2000 (Invitrogen). For RNA/Lipofectamine complex; the siRNA was diluted in Opti-MEM and then incubated for 30 min at RT with a solution of Opti-MEM and lipofectamine that had been

previously incubated for 5 min before mixing. The final ratio of Lipofectamine: RNA was 1 to 1 in a total volume of 50 μ L.

The cells were transfected as described by Fisher et. al (2007) (Fisher, Abramov et al. 2007). The NIH-3T3 cells were seeded onto 24-well plates at 2×10^4 cells / 500 μ L in 10% FBS/DMEM-H and transfected with the RNA/liposome complex at a final concentration of 50 nM siRNA per well incubated at 37 ° C, 5% CO₂ for 4 h. The media was removed and replaced with 2% FBS/DMEM-H and incubated for an additional 68-72 h (Xu, Kang et al. 2004).

Preparation of RNA/liposome complex for TLR 7 *in vitro* activation experiments

The ssRNAs were complexed to Lipofectamine 2000 (Invitrogen). For RNA/Lipofectamine complex; 2.65 μ g of RNA was incubated for 15 min at RT with a solution of Opti-MEM (volume to make 50 μ L) and 2.5 μ L of Lipofectamine also in Opti-MEM (volume 50 μ L) that had been incubated for 5 min before mixing (total volume 100 μ L). The Raw264.7 cells were seeded onto 24-well plates at 5×10^5 cells /500 μ L and stimulated with the RNA/liposome complex at a concentration of 10 μ g/mL RNA. After 24 hours, the culture supernatants were collected and stored at -80° C till used.

Immunostaining of *P-gp*

After treating the NIH-3T3-MDR1 cells with natural or modified siRNA, the cells were washed with PBS, trypsinized and resuspended in PBS with 10% serum (Fisher,

Abramov et al. 2007). Each sample was transferred to a new tube and the cell clumps broken down by gently pipetting. Tubes were spun for 5 min and the supernatant was aspirated avoiding the cell pellet. 20 μ L FITC-labeled *P*-gp mouse anti-human antibody in a storage buffer solution containing bovine serum albumin (BSA) and <0.09% sodium azide (BD Biosciences) was diluted in phosphate buffered saline (PBS) before it was added to each sample and incubated at 4° C for 30 min. The antibody was washed with 100 μ L of PBS and supernatant was removed. The cells were resuspended in PBS and analyzed on a Becton Dickinson flow cytometer using Ciero software (Cytomation, Fort Collins, CO) (Fisher, Abramov et al. 2007). This experiment was done with assistance of Mike Fisher at Professor Juliano's Lab.

5.3 Results

5.3.1 *P*-gp downregulation (MDR1 gene) by boranophosphate modified siRNA

To determine the effect that BP modifications have on siRNA activity, we synthesized modified siRNA oligonucleotides with various numbers of modifications and transfected NIH-3T3 cells (**Chapter 5, Materials and Methods, Table 6**). The modifications included 2'F sugar modifications, 2'F with NTP α B or only NTP α B. The sample name indicates the modified NTPs used. The siRNA was complexed with Lipofectamine as ds siRNA or ss siRNAs. Three controls were used: the **ORF 1** control was chemically synthesized and contains dTdT at the 3'-end to stabilize against nucleases and it is either single-stranded (**ORF1 as**) or double-stranded (**ORF1 as+s**)

siRNA molecules. **Shaw ORF** control is a chemically synthesized RNA that contains UU at the 3'-end since RNA polymerase cannot add dTdT when enzymatically synthesizing RNA.

The controls **Shaw ORF**, enzymatically synthesized control with 3'-UU, had a high percentage of *P*-gp downregulation as a single-stranded or double-stranded molecule. On the other hand the chemically synthesized **ORF 1**, which was chemically synthesized with 3'-dTdT had activity as double-stranded siRNA (ds siRNA) but had no activity as single-stranded siRNA (ss siRNA). In contrast, enzymatically synthesized natural siRNA had activity as ss siRNA and ds siRNA. Enzymatically synthesized siRNAs have 3'-UU and also a 5'-phosphate when compared to the **ORF 1 (Figure 26)**. Further the BP siRNA that had been transcribed with one or two NTP α Bs or a mixture of 2'F and NTP α Bs, had activity against p-gp. No significant difference in activity as either single- or double- stranded siRNAs was seen between the BP-RNA and the 2'F&BP RNA. However, the activity of the single-stranded BP-siRNA decreased as the number of BP modifications was increased to three or four (**Figure 26 3B and 4B**). The activity of the **3B** and **4B** was lost when these were subsequently transfected as ss siRNAs, but activity returned when the BP-siRNA was annealed to the sense-strand to make the ds BP-siRNA. Fully modified PS-siRNA **thio(4S)**, had the same effect as fully modified **4B** BP-siRNA; the ss siRNA lost activity but when it was complexed to the

sense-strand to make the ds siRNA, the molecule showed higher activity as measured by the increased % of p-gp on the cell surface (Figure 26).

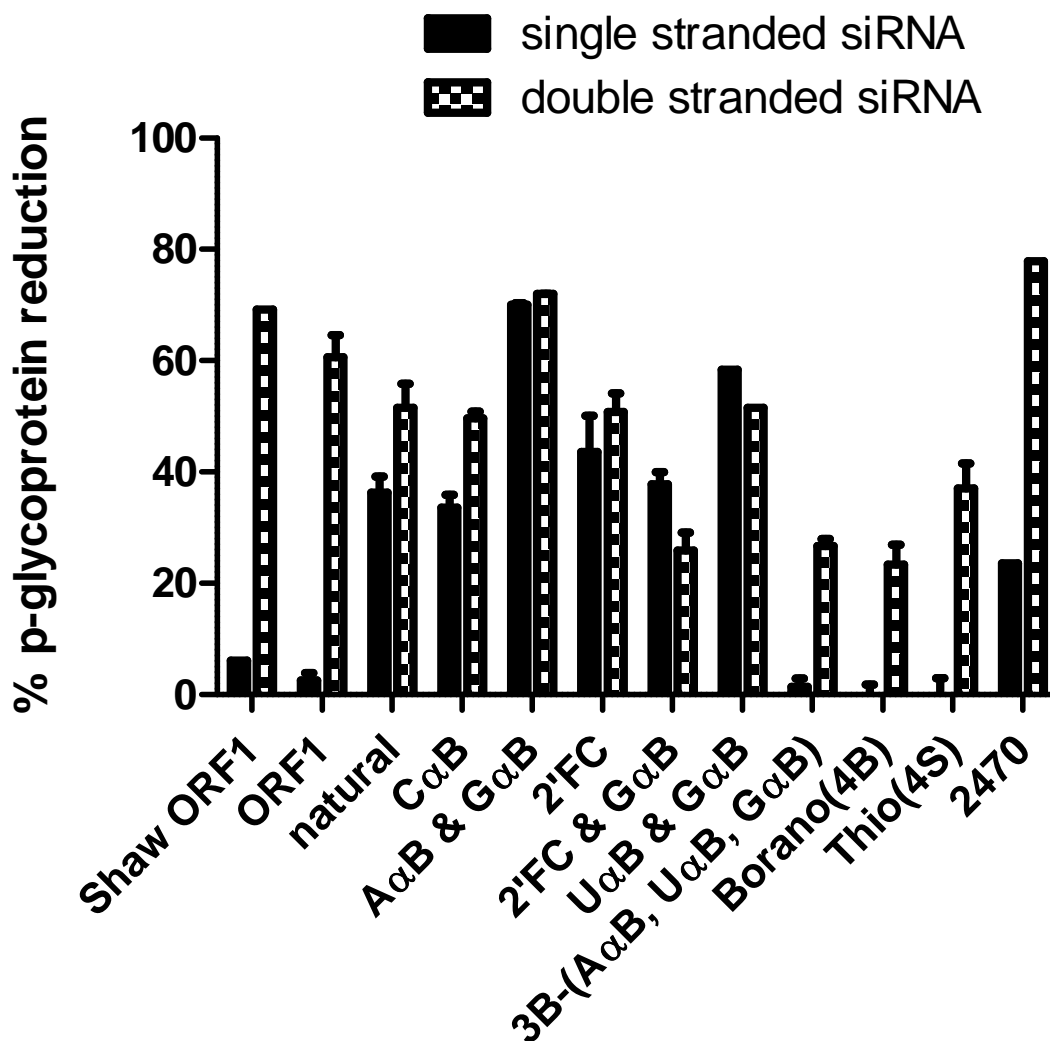


Figure 26 - MDR1 downregulation by modified siRNA as either a single-stranded molecules (black) or double-stranded (black/white pattern).

Natural not modified NTPs used and enzymatically synthesized. The sample name denotes the modified NTP used during transcription for the other samples. Three controls were used: the ORF 1 control was chemically synthesized and contains dTdT at the 3'-end and either single-stranded (ORF1 as) or double-stranded (ORF1 as+s) siRNA molecules. Shaw ORF1 control is a chemically synthesized RNA that contains

UU at the 3'-end. Standard error was calculated using 3 separate experiments and shown as an error bar.

Cytokine TNF α production by BP-modified MDR1 single-stranded siRNA

We wanted to contrast the MDR1 siRNA activity to downregulate *P*-gp to the activation of TLR7 by measuring the production of TNF α produced by this sequence. The MDR1 siRNA was the same sequence tested in the previous *p*-gp experiments and was transcribed with natural and modified NTPs with: all natural NTPs, three NTP modifications (**3B**), four modifications (**4B**), and full **PS** modifications. In **Chapter 4**, we showed that fully or 90% modified BP ssRNA40 did not activate TLR 7 we wanted to analyze the immunogenicity of the MDR1 siRNA sequence. We stimulated Raw246.7 cells with MDR1 siRNAs that had previously been tested in the siRNA assay with **natural, thio (4S)**, and borano (**3B** and **4B**) complexed to a Lipofectamine carrier.

For single-stranded MDR1 siRNA, we found that there was a loss of siRNA downregulation when three or four borano modifications were added to the RNA transcripts (**Figure 27**). In addition, we confirmed that for the MDR1 siRNA sequence, the **Borano(4B)** did not activate TNF α production (**Figure 27 a**). However, as previously shown, it was also not active as a ss siRNA in the MDR1 assay (**Figure 27 b**). In contrast, **Thio(4S)** activated the production of TNF α , but similarly to the BP RNA it was not active as a ss siRNA molecule in downregulating *P*-gp.

The **Borano(4B)** gained some of its siRNA activity when annealed to the sense siRNA strand. This was also seen with **Borano(3B)** and **Thio(4S)**, suggesting that the siRNA activity may be linked to an immunogenic reaction from the sense-strand (**Figure 26**) or to the change in structure of the single-stranded siRNA when modified with BP or PS.

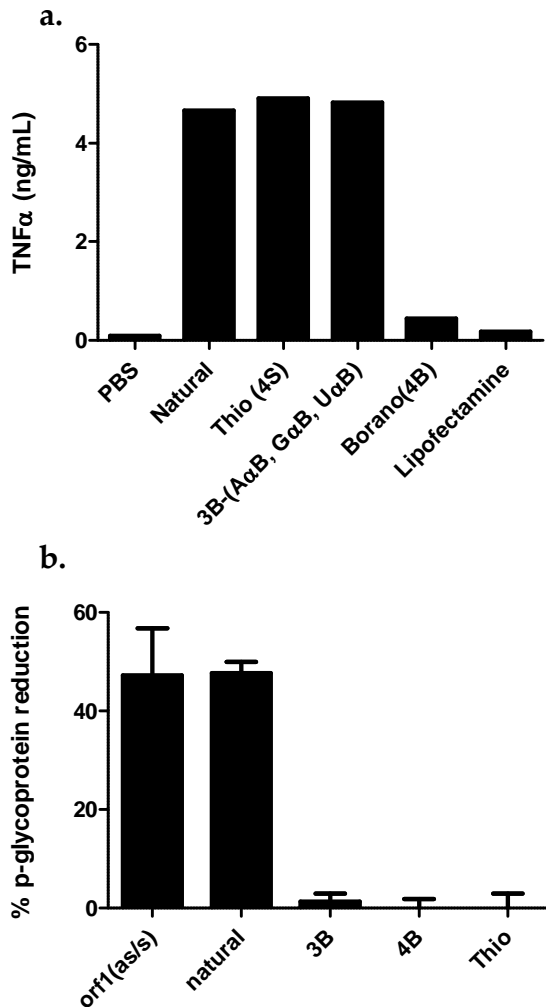


Figure 27 – Comparison of *P*-gp percent downregulation by modified single stranded MDR1 siRNA and its immunogenicity.

(a) TNF α production after stimulation with natural, 4S, 3B, 4B RNA complexed to Lipofectamine. Fully modified BP-RNA, Borano(4B), does not stimulate the production of TNF α (ng/mL) in Raw264.7 cells as compared to Thio(4S) and natural RNA which activated the production of TNF α . (b) In NIH-3T3 cells, natural RNA activated *P*-gp downregulation in contrast to the complete loss of MDR1 downregulation activity of 3B and 4B modified siRNA. Taken together the results suggest that there is a fine balance between immunogenicity and siRNA activity.

5.4 Discussion

RNAi is an evolutionarily conserved gene silencing process that can be exploited in cancer therapy to correct aberrant genes. However, the actual downregulation effects of siRNA must be distinguished from non-specific off-target effects such as the activation of TLR receptors. It has been reported that G- and U-rich ss siRNA stimulate dendritic cells, which can lead to an immune response (Heil, Hemmi et al. 2004; Diebold, Massacrier et al. 2006; Sioud 2007; Gantier, Tong et al. 2008; Robbins, Judge et al. 2009). In addition, it has been shown that there is an interferon response associated with siRNA synthesized by T7 RNA polymerase, as well as chemically synthesized natural siRNA (Hornung, Guenther-Biller et al. 2005; Sioud 2007; Robbins, Judge et al. 2009).

The off-target effects of siRNA are not well understood and are dependent on both the siRNA (e.g., length, sequence, and type of modification) and the cell type. Chemical modifications of the RNA backbone or at the 2' position of the sugar can change the immune response in a cell-specific and modification-specific manner leading to a lack of immune response in the case of 2'-OMe and 2'F (Robbins, Judge et al. 2007; Sioud 2007). In **Chapter 4** we tested a known TLR 7 activating sequence, BP- ssRNA40, in mouse cells for activation of TLR 7. This is not an siRNA molecule, but rather a sequence derived from the HIV RNA that is named ssRNA40 and is known to activate TLR 7. Using this sequence, we found that fully modified ssRNA40 did not activate TLR 7 but yet there was some stimulation with one or two nucleotides modified. In the

current study, we took this research further and wanted to test a specific MDR1 targeting siRNA for downregulation of the *P*-gp protein and contrast that with the activation of TLR 7.

We found that increasing the number of BP-modifications on the antisense strand of the siRNA reduced the siRNA activity of the molecule in the MDR1 model and inversely correlated to the activation of TLR 7. This could be due to a number of factors including (a) incorporating electron-deficient boron can tweak the chemical properties of the molecule, changing its interactions with the RISC complex, mRNA or TLR 7 molecules (b) the BH_3 not coordinating with Mg^{2+} ions in the RISC complex, or (c) the disruption of A-form RNA.

The lack of siRNA activity and TLR activation of the fully modified siRNA may be also due to the chemical properties of the BH_3 group compared to the single oxygen atom (O). Changing the non-bridging oxygen to a sulfur (S) atom as in the phosphorothioate backbone modification (PS) or to a BH_3 molecule (BP) does not change the net negative charge in the phosphate linkage, but changes the chemistry of the RNA molecule and its interactions with its environment. In addition, normal (PO) and phosphorothioate (PS) each have two lone pairs of electrons for coordinating and bonding. In contrast, the boranophosphate (BP) does not have lone pairs of electrons available and the negative charge is distributed towards the phosphorous atom, making this atom less positive compared to natural or PS. We hypothesize that the decreased

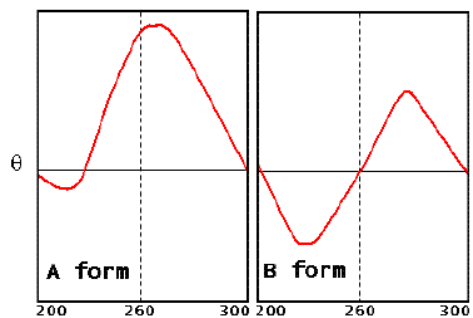
negative charge in the molecular surface of BP-RNA could lead to a change in the affinity and specificity of the modified RNA to the mRNA and also proteins such as Ago2 in the RISC complex or TLR 7 receptors. The surface charge that results from the non-bonding electrons on an oxygen or sulfur is absent in the case of BH₃ substitution and the negative charge is distributed towards the phosphorous atom. Similarly to the phosphoryl oxygen in natural RNA, often denoted as the non-bridging oxygen, the sulfur has available electrons in its outer shell to coordinate to water as well as the cationic regions of proteins. In contrast, the BH₃ group does not have available hydrogen bonding electrons and hence replacing the non-bridging oxygen with BH₃ on the RNA decreases its localized negative charge (Summers and Shaw 2001). Consequently, the overall surface charge of the BP-RNA is less negative compared to oxygen and sulfur substitutions. This prevents the fully modified BP backbone from interacting in the same manner as the natural or PS backbones.

The activity of the RISC complex has been shown to be Mg²⁺ dependent and one of the non-bridging oxygen in the mRNA target directly interacts with this ion (Schwarz, Tomari et al. 2004). Nucleotides 10-11 in the siRNA interact with this scissile phosphate (11-12) and cleavage occurs as seen on top of **Figure 24**, which was reproduced from Schwarz et. al (Schwarz, Tomari et al. 2004). This suggests an RNaseH mechanism of action. In previous studies the BH₃ was shown to reduce RNaseH activity

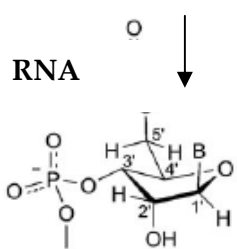
during when a BP-DNA was annealed to natural RNA (Wang, Dobrikov et al. 2003). We hypothesize that BP-siRNA in the RISC complex will reduce the efficiency of the catalytic cleavage of the mRNA by the ago2 protein.

In the current study, the fully modified, single-stranded BP-siRNA was not active in downregulating *P-gp* and also, it did not activate TLR 7. We hypothesize that this may be due to the change in the single-stranded RNA structure from the natural A-form to a more B-form structure. In general, it is known that the RNA or DNA form two types of structures: an A-form helix which has a shallow trough at approximately 210 nm and a large peak at approximately 262-264 nm and a B form helix has a trough at 220 nm and a peak at 270 nm. The BP-modified single-stranded RNA oligonucleotide spectra, obtained by Laura Moussa, was compared with the CD spectrum of the unmodified RNA transcripts of the same sequence, which had a maximum at 267 nm and a minimum at 208, which is indicative of an A-form-like RNA structure (**Figure 28 B**). The modified single-stranded RNA showed a shift in the maximum from 267 nm to 273 nm, indicative of a more B-form-like helix, although the minimum did not shift. Both the minimum and the maximum absorbances in BP-RNA were reduced in intensity compared to the normal RNA.

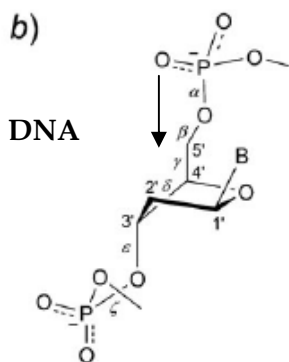
A.



a)



b)



B.

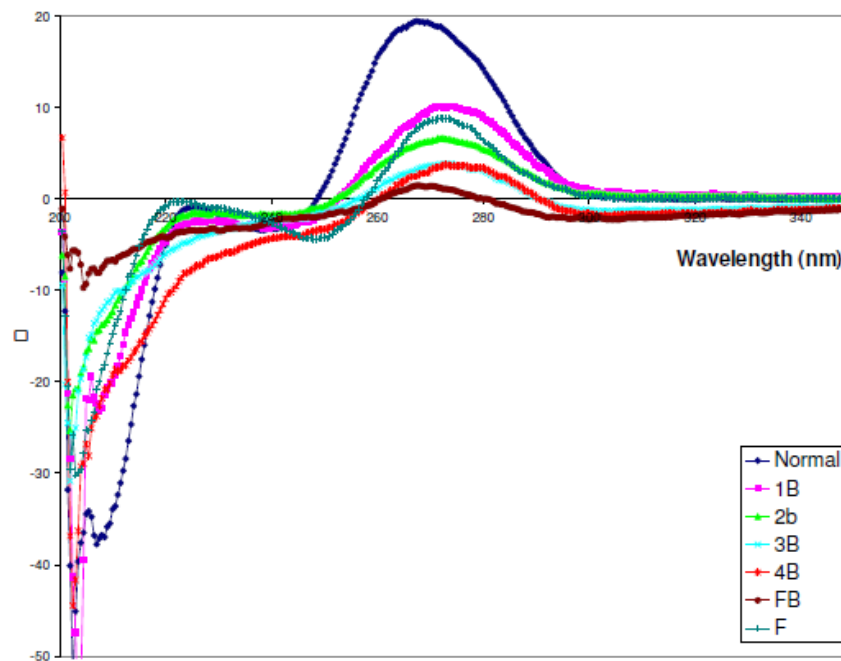


Figure 28 – Natural and modified RNA CD spectra.

(A) General A-form and B-form oligonucleotide CD spectra with the A form helix with a shallow trough at approximately 210 nm and a large peak at approximately 262-264 nm and a B-form helix has a trough at 220 nm and a peak at 270 nm with approximately the same magnitude. (B) Natural and BP-RNA CD spectra of 5'- CCA GAG ACA ACU GAU CUC UUU-3' transcripts shows the extensive effect on the peak height at 260 nm suggesting a change in oligonucleotide structure when BP modified single stranded siRNA (Laura Moussa Duke Dissertation 2009). The modifications are as follows: 1B = G α B, 2b = (C α B, G α B), 3B = (C α B, G α B, U α B), 4B = all bases BP, FB = (2'F C, 2'F U, G α B), F = (2'F C, 2'F U).

We hypothesize that this may be due to a reduction in base stacking within the molecule when the backbone was BP modified. This could lead to the reduction of efficiency of loading of the single-stranded antisense siRNA into the RISC complex. Once an RNA is loaded into the RISC complex, it seeks its complementary mRNA and needs to form an A-type helix for a more efficient catalytic effect (Chiu and Rana 2002). The CD spectra in **Figure 29** shows the effect on double-stranded RNA, and there is a less pronounced shift of RNA-like A-form to a more DNA-like B form helix with increasing number of boranophosphate modifications. We hypothesize that the reduction of siRNA activity as single-stranded molecules are 'rescued' by the annealing of the sense-strand to form the double stranded siRNA. This could explain why the activity is restored when the same fully modified siRNA (4B) molecule is annealed to the natural sense strand. It follows that BP-modified single-stranded siRNA might not be loaded efficiently into the RISC complex so almost no molecules are loaded and thus there is no downregulating activity. For the double-stranded siRNAs, the sense strand

assists in the loading into the RISC complex and Argonaute 2 (Ago2) degrades the target mRNA

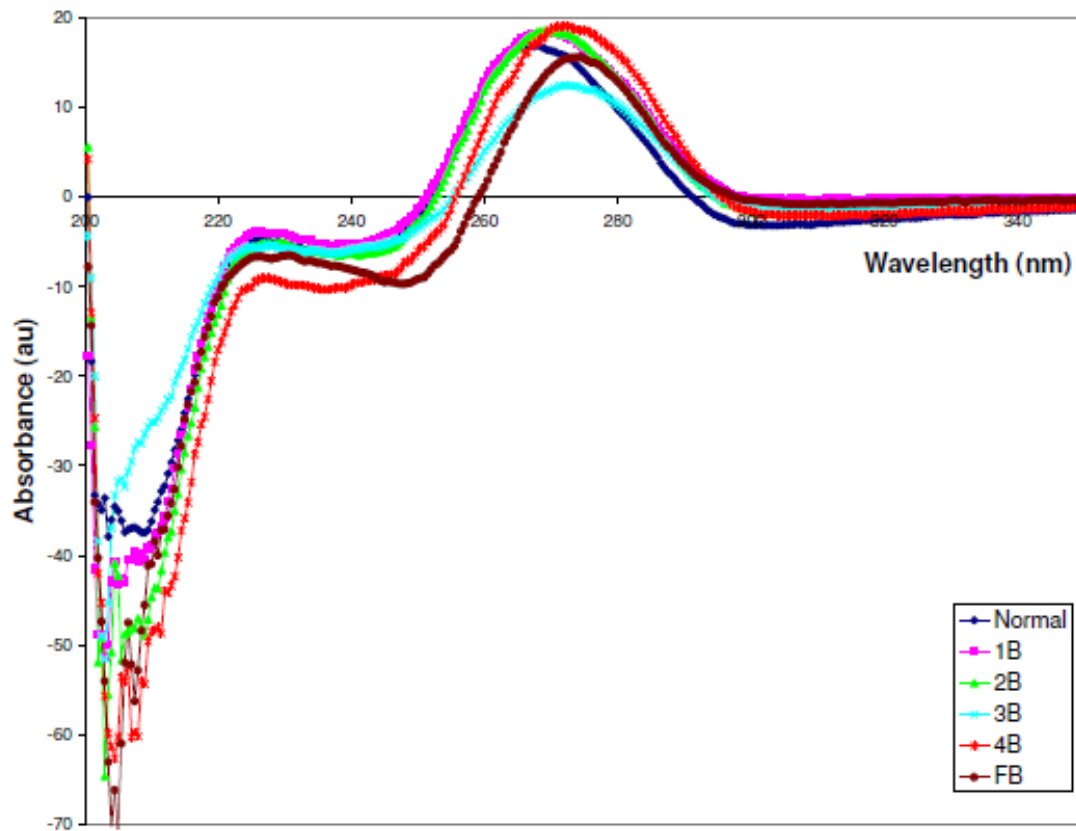


Figure 29 - Heteroduplex dsRNA CD spectra, natural and modified RNA.

Heteroduplex dsRNA with one strand modified as shown in the figure legend of the following sequence 5'- CCA GAG ACA ACU GAU CUC UUU-3' and annealed to corresponding natural RNA to form a duplex. There is less of a change in structure when the BP-RNA is annealed to a natural RNA. The modifications are as follows: 1B = G α B, 2b = (C α B, G α B), 3B = (C α B, G α B, U α B), 4B = all bases BP, FB = (2'F C, 2'F U, G α B), F = (2'F C, 2'F U). (Laura Moussa, Duke Dissertation 2009)

The next step for this project is to analyze for the degradation at the mRNA level of MDR1 using real-time PCR (Soutschek, Akinc et al. 2004). This method generates first-strand cDNA synthesized using a gene-specific primer of the gene of interest and followed by PCR. If there is knockdown by siRNA, the mRNA levels would be significantly lower. The RT-PCR method of quantifying mRNA was done previously by Fisher et al. (2007) for the altrial-modified RNA (Fisher, Abramov et al. 2007). This needs to be confirmed for the BP siRNA to make sure that the downregulation is not due to an off-target effect. It is especially important to distinguish the innate immune affects from the loading efficiency of double-stranded siRNA molecules. We hypothesized earlier that there is a significant change in single-stranded RNA structure and this would prevent the loading of the modified BP-siRNA into the RISC. However when this was annealed to its sense strand, the structure was similar to the normal duplex, and the activity was observed in tandem. The observation of the reduction of mRNA with the corresponding % p-gp reduction would allow us to deduce the siRNA results are only due to RNAi activity.

Chapter 6: Boranophosphate RNA Aptamers and SELEX

6.1 Introduction

6.1.1 Aptamers

Until recently, it was thought that RNA had only three roles in cells; mRNA, ribosomal RNA, and transfer RNA. Following the discovery of genomic technologies such as sequencing and PCR, new roles for small RNAs have been discovered. One of these is selection and subsequent use of RNA aptamers to control protein activity. An aptamer is a short oligonucleotide sequence (RNA or DNA) that has been selected by an *in-vitro* evolution process to bind a protein target with high affinity and specificity (Ellington and Szostak 1990; Tuerk and Gold 1990; Gold 1995). Aptamers can function as a receptor agonists and as targeting delivery agents when coupled to other oligonucleotides, such siRNA (Ellington and Szostak 1990; Tuerk and Gold 1990; Gold 1995; McNamara, Andrechek et al. 2006; McNamara, Kolonias et al. 2008). Aptamers can be used for clinical purposes (as macromolecular drugs). The number of therapeutic targets for selection and use of aptamers is large and diverse and includes soluble proteins, transcription factors, cell-surface receptors, and other intracellular proteins (Barbas and White 2009). The most extensively characterized inhibitory aptamer is the RNA aptamer that targets the vascular endothelial growth factor (VEGF) (Burmeister, Lewis et al. 2005; Fraunfelder 2005; Ng and Adamis 2005; Ng and Adamis 2006; Ng, Shima et al. 2006; Shukla, Namperumalsamy et al. 2007; Barakat and Kaiser 2009). The

VEGF aptamer, named pegaptanib, was approved by the FDA in December 2004 for the treatment of wet age-related macular degeneration (AMD) and is currently being tested in patients (Ng, Shima et al. 2006). This is a good example of the versatility of aptamers. This aptamer was selected from a 2'-F pyrimidine-modified RNA library, which was synthesized by incorporating 2'F UTP and 2'F CTP using a mutant T7 RNA polymerase enzyme. The aptamer was stabilized against 3' endonucleases by the addition of a 3'-3' deoxythymidine cap (dTdT) (Ruckman, Green et al. 1998). After the selection aptamer was further modified, with all but two purine nucleosides modified with 2'-OMe in order to further increase the stability of the molecule (Green, Jellinek et al. 1995). In addition, in order to decrease the clearance time, the aptamer was conjugated with a 40-KDa PEG linker that resulted in an aptamer named NX1838, which has a half-life of 9 hours *in vivo* porcine model, compared to unmodified RNA which is eliminated in minutes (Tucker, Chen et al. 1999).

There are several advantages of aptamers over antibodies as therapeutic agents (Gold 1995; Gold, Polisky et al. 1995; Gold, Singer et al. 1997). Some advantages include: A) less immunogenic reactions are seen with aptamers compared to antibodies even when administered in 1000-fold greater doses than the therapeutic dose (Gold, Singer et al. 1997); B) aptamers can be synthesized and selected under a variety of conditions *in vitro*, do not require a biological system for production and thus are more versatile as therapeutics; C) an antidote can be developed to reverse the effects of the aptamer

simply by taking advantage of base pairing between short sequences in the aptamer and the complementary sequence of the antidote. Rusconi et al. hypothesized those oligonucleotides complementary to RNA aptamers could base pair to the aptamer, interrupt its activity, thus function as antidotes reversing the effect of the aptamer against the target protein (Rusconi et al., 2002). Further, the Sullenger laboratory has demonstrated this antidote effect by using an antidote to inhibit of factor IXa and VWF aptamers; they showed that the complementary oligonucleotide bound to the aptamer. The binding of the antidote causes a conformational change that rendered it inactive and thus reversed its inhibitory activity (Nimjee, Rusconi et al. 2005; Oney, Lam et al. 2009);

D) aptamers are smaller in size compared to proteins; truncated aptamers are 8 to 14 KDa, i.e. about 25 to 40 nucleotides long whereas antibody proteins are greater than 20KDa. Their smaller size thus allows for access to a wide range of epitopes on the target that are not accessible to antibodies (Alexis, Basto et al. 2008; Levy-Nissenbaum, Radovic-Moreno et al. 2008); and E) the addition of chemical modifications to the RNA aptamers that can be amplified and re-selected is the most significant difference between aptamers and antibodies. Modifications can be added to the aptamer molecule to give it properties such as enzymatic stability and chemical functionality, and yet retaining the basic chemical properties that allow the RNA to fold based on the nucleobase complementarity.

6.1.2 Systematic Evolution of Ligands by EXponential enrichment (SELEX)

Aptamer molecules are selected against a variety of protein targets or small chemicals via a procedure termed Systematic Evolution of Ligands by EXponential enrichment (SELEX) (Figure 30) (Gold 1995; Gold 1995; Gold, Polisky et al. 1995; Gold, Singer et al. 1997; Singer, Shtatland et al. 1997).

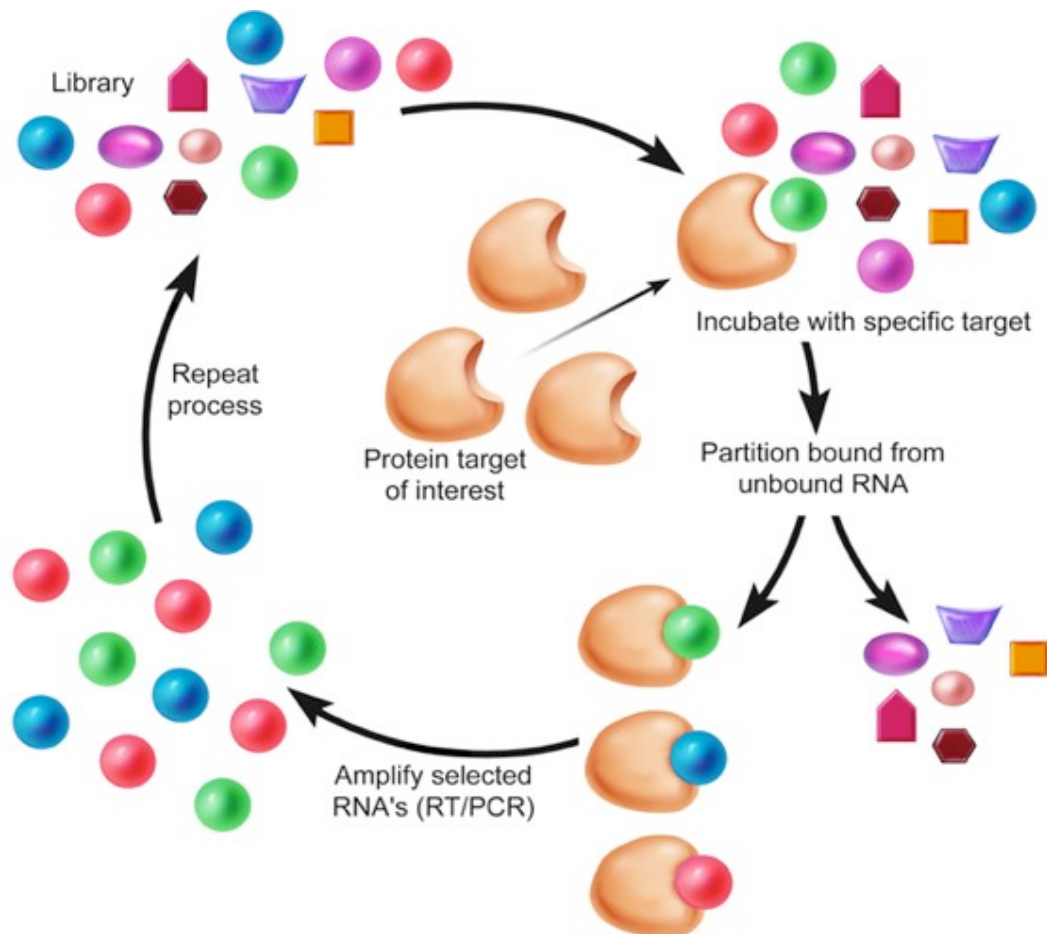


Figure 30 - The SELEX method.

A random RNA library, containing 10^{15} different sequences, was incubated with the target protein and the bound RNA partitioned. The bound RNA was then reversed

transcribed and amplified before the process was repeated. (SELEX figure adapted (Nimjee, Rusconi et al. 2005)

The SELEX procedure screens a diverse library of RNA or DNA molecules *in vitro* against a protein target (Gold 1995). The library is enzymatically synthesized by a RNA or DNA polymerase and four NTPs (typically ATP, CTP, GTP, or their deoxy analogs and UTP for RNA and TTP for DNA) from a dsDNA template. The template contains a random sequence region (20-60 nt) flanked by two constant regions. The random region of the aptamers contributes to the complexity of the library by allowing for 10^{10} - 10^{15} different sequences because of the diversity of the sequences. Each sequence can form complex 2-dimensional conformations based on pairing of the nucleobases to give rise to double stranded scaffolds which give rise to multiple 3-dimensional structures. These complex structures allow the aptamer to bind to the target with high specificity and activity. The DNA template contains a promoter sequence for enzymatic synthesis and two constant regions that contain PCR primer-binding sites for amplification as well as restriction sites for cloning. The promoter sequence is specific to the RNA polymerase being used and is required for the initiation of transcription by T7 RNA/DNA polymerase. The template is transcribed to create a library containing many copies of 10^{14} - 10^{15} different sequences. The library is incubated with the target protein or a molecule of interest. Then, a nitrocellulose filter is used to isolate the small percentage (0.1%) of the RNA molecules that bind to the protein with high affinity after many rounds of partition and amplification. Since oligonucleotides have very low affinity to

the nitrocellulose filter compared to the protein's high affinity each round of selection only isolates the bound RNA molecules. Thus, only RNA aptamers that bind to the protein are retained in the nitrocellulose membrane (Woodbury and Von Hippel P. H. 1983). The target-bound aptamers are separated, reverse-transcribed to cDNA, and then amplified by PCR before starting the next round. The stringency, and hence the selectivity and affinity, of the aptamer selection can be modulated by changing the salt concentration of the binding and wash buffers. In addition, the surfactant can be changed from bovine serum albumin (BSA) to CHAPS depending on the background binding observed.

The enrichment of the RNA library is determined by filter binding assay after each round. Usually after 8 to 15 rounds there is significant enrichment, the partitioned RNA molecules are cloned and sequenced. This repetitive process enriches the library with sequences that bind to the protein target with some affinity. Once the saturation of enrichment is measured, the RNA is reverse-transcribed, cloned, and sequenced. In a family of clones, there are usually a few sequence motifs (6-12 nucleotides long) that are necessary for target binding. The selected aptamer clones can be truncated to the necessary motifs to give a smaller and more specific aptamer. The individual sequences can be tested for specificity and affinity to the desired target.

6.1.3 Thrombin Target Focus of this Project

The thrombin protein is a key protein in the blood clotting pathway and it is a good selection target because it has multiple sites where negatively charged oligonucleotides can bind (Bock, Griffin et al. 1992). Thrombin is a standard target to investigate the value of new aptamer chemistries. In addition to the catalytic site, thrombin has an anion-binding exosite 1, which binds fibrinogen, and an exosite 2 that binds heparin. DNA and RNA aptamers bind to different sites on the thrombin protein, where DNA binds to exosite 1 and RNA binds exosite 2 (Nimjee, Oney et al. 2009). Both exosites are positively charged patches on the protein that allow for electrostatic interactions with the negatively charged aptamers (Nimjee, Oney et al. 2009).

In this study here, we propose that the unique chemistry of borane and borane in combination with 2'-F (doubly modified) RNA aptamers will allow the RNA to fold into shapes that may have high affinity for epitopes found in the target protein. Based on previous studies, it was expected that the borane aptamers would have a diversity of shapes leading to different protein binding motifs compared to natural and only 2'F RNA aptamers by virtue of different the different charge distribution and lipophilicity of the BH₃ group (Lato, Ozerova et al. 2002).

6.1.4 Thrombin and coagulation

Thrombin is a serine protease that is involved in platelet activation and aggregation, endothelial cell activation and coagulation (Coughlin 2000; Celikel,

McClintock et al. 2003). The excess or aberrant thrombin generation leads to thrombosis. This can lead to stroke, deep vein thrombosis and peripheral vascular disease which are leading causes of mortality in America (Cavendish, Fugit et al. 2004). In the cell-based model of coagulation, thrombin generation occurs on the surface of platelets during the propagation phase (Monroe, Hoffman et al. 2002). The Xa/Va (prothrombinase) complex plays a vital role in activating pro-thrombin to thrombin on the platelet surface, leading to stable clot formation **Figure 31** (Monroe, Hoffman et al. 2002; Allen, Hoffman et al. 2006; Roberts, Hoffman et al. 2006; Hoffman and Monroe 2007).

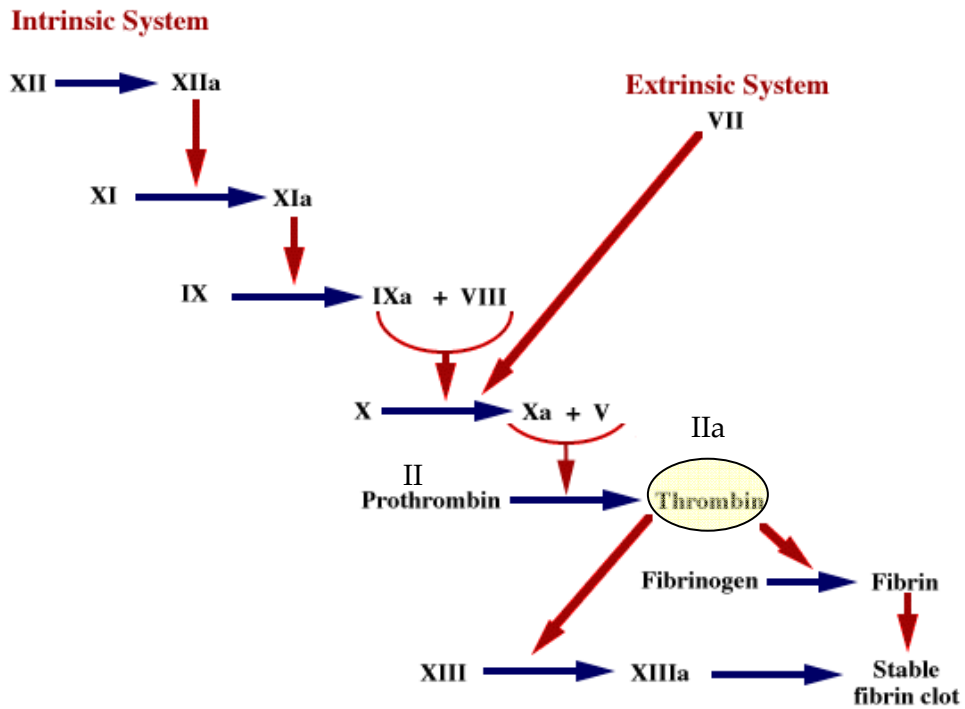


Figure 31 – Blood coagulation signaling cascade.
 Coagulation cascade leads to the formation of blood clots. Thrombin catalyzes the formation of fibrinogen to fibrin which leads to the formation of blood clots. (Figure adapted from (Monroe, Hoffman et al. 2002))

The process of coagulation proceeds in distinct but overlapping steps: 1) initiation, 2) amplification, and 3) propagation (Monroe, Hoffman et al. 2002). These steps take place in two cells types: one is the tissue-factor (TF)-bearing cells and platelets.

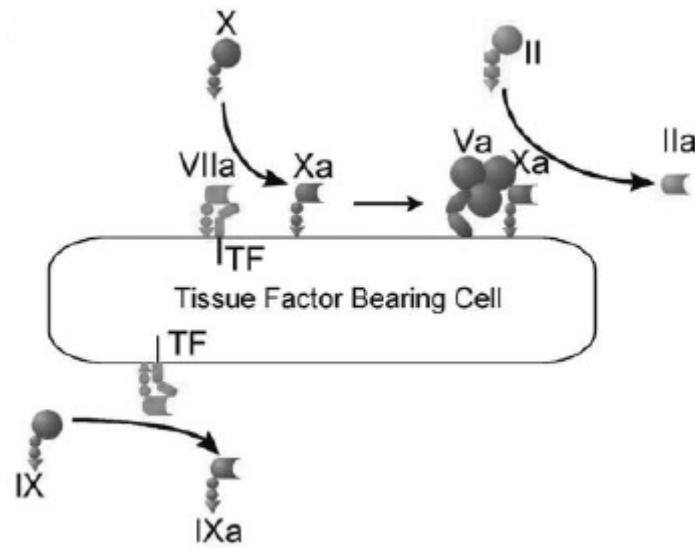


Figure 32 - Extrinsic pathway on TF-bearing cells initiation phase.

This small amount of thrombin activates platelets and leads to the propagation phase, in which a large amount of thrombin is produced. (Figure adapted from (Monroe, Hoffman et al. 2002))

TF-bearing cells generate a small amount of thrombin and activate platelets for the propagation phases, in which a large production of thrombin occurs (**Figure 32**).

Although platelet adhesion occurs at the site of injury, increasing the amount of thrombin can increase the coagulation activity compared to adhesion alone (Baglia and Walsh 1998; Monroe, Hoffman et al. 2002). Thus, the propagation phase leads to a large production of thrombin to clot fibrinogen and this leads to coagulation of blood (**Figure 33**).

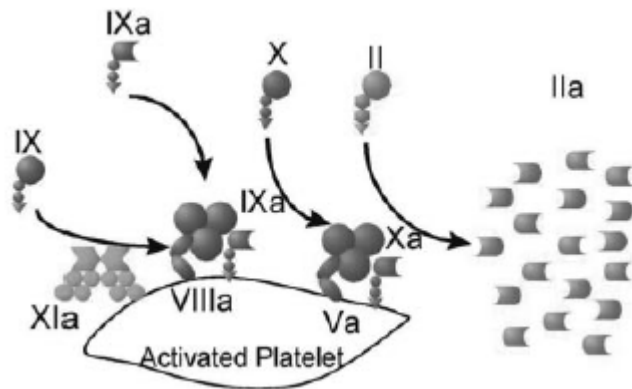


Figure 33 - Activated platelet factor Xa formed on the platelet surface leads to a burst of thrombin generation (Monroe, Hoffman et al. 2002).

Thrombin plays a key role in coagulation during the activation and propagation phases. The production of thrombin during the amplification phase leads to what is known as hemophilia. In hemophilia, platelet adhesion occurs normally and there is a small amount of thrombin produced on TF-bearing cells but there is no activation by FIXa/FVIIa. This prevents the burst of thrombin from happening, which prevents normal homeostasis (Monroe, Hoffman et al. 2002). It has been shown that targeting thrombin with aptamers, either RNA or DNA, increases the clotting time *in vitro* (Bock, Griffin et al. 1992; White, Rusconi et al. 2001) and *in vivo* (Griffin, Tidmarsh et al. 1993). In addition, targeting thrombin to reduce coagulation with aptamers was shown to have high efficacy and a lack of adverse effects in comparison with heparin control in canine models during cardiopulmonary bypass (DeAnda, Coutre et al. 1994). Therefore, thrombin is an attractive target for anticoagulation therapy.

6.1.5 Modified RNA Aptamers

The incorporation of chemically modified NTPs into the RNA aptamer molecules is advantageous as it adds nuclease resistance and gives the molecule additional chemical functionalities for increased binding and selectivity to the target (Shaw, Dobrikov et al. 2003; Hall, Wan et al. 2006; Shaw 2007). In order for the aptamers to be nuclease-resistant, modifications to the sugar (2'-F or 2'-OMe) and/or the phosphate backbone's non-bridging oxygen (-S) are added. A 2'-F modification instead of 2'-OH on the sugar has proven to be a successful modification for aptamer libraries (White, Roy et al. 2008). The incorporation of chemically modified NTPs is limited by the ability of RNA polymerase to recognize and incorporate the modified NTPs into the elongating transcript. In fact only 2'F, 2'NH₂, PS, and BH₃ are accepted by the wild type T7 RNA polymerase with varying efficiencies, where in our experiments, 2'F was incorporated better than BH₃ which was better than PS.

We have used enzymatically synthesized RNA libraries modified with BP-linkages on the phosphate backbone, and in combination with 2'F sugar modifications, we proposed to select *in vitro* a family of modified aptamers that selectively bind the thrombin with high affinity. The 2'FC&UαB RNA is a novel combination of both a sugar and backbone modifications that has not been used previously for SELEX. Our lab has shown that BP-modified RNA has a significantly greater half-life compared to natural RNA in the presence of bovine pancreatic RNases (Hall, Wan et al. 2006). The

unique polarity in P-B bonds should allow the borane-aptamers to bind a new diverse array of ligand sites in the thrombin molecule. In P-B linkages some of the negative charge is distributed away from the boron towards the phosphorus because of the decreased electronegativity of the boron (B) compared to oxygen (O), thereby changing the polarity of the bond (Sergueeva, Sergueev et al. 2001). In addition, the borane-group does not have lone pair electrons like oxygen, so borano-RNA molecules will interact differently with metal ions and proteins relative to oxygen. Importantly, NTP α Bs are compatible with T7 RNA polymerase during the enzymatic synthesis of the modified library. They are also compatible with reverse transcriptase used during the enrichment of the library step and PCR. It was shown by our lab in collaboration with Lato et al. (2002) that borane modification is compatible with every step of the SELEX method against ATP-bound to a column (Lato, Ozerova et al. 2002). Boron analogues and macromolecules are considered to have low toxicity in rodents (Hall, Burnham et al. 1993). To understand the role of boranophosphate modification on RNA aptamer specificity, known aptamers to ATP were retrofitted with boranophosphate backbone modifications (Lato, Ozerova et al. 2002). One example was retrofitting aptamers that recognized coenzyme A (CoA) (Lato, Ozerova et al. 2002; Saran, Frank et al. 2003). This aptamer lost its activity when GTP was modified but only partially lost its activity when UTP was modified (Lato, Ozerova et al. 2002). This suggests that boranophosphate backbone has some influence over the structure of the RNA and thus its affinity (Lato,

Ozerova et al. 2002). *In vitro* selection of GTP α B and UTP α B libraries was done against C8-linked-ATP agarose in binding buffer L (50 mM Bis-Tris pH 6.4, 200 mM NaCl, 10 mM MgCl₂) by Lato et. al. and obtained a collection of ζ -fold aptamers and non- ζ -fold aptamers (Lato, Ozerova et al. 2002). The ζ -fold had been extensively studied after selection with natural RNA against the ATP molecule (Sassanfar and Szostak 1993; Burgstaller, Kochoyan et al. 1995; Dieckmann, Suzuki et al. 1996; Burke and Gold 1997). When the selection was done with boranophosphate RNA against ATP, in addition to the ζ -fold aptamers, some non- ζ -fold aptamers were obtained. These non- ζ -fold aptamers required the borano modification to maintain their activity, suggesting that the -BH₃ was directly involved in binding at specific positions (Lato, Ozerova et al. 2002).

We wanted to evaluate the SELEX method using a BP-modified RNA aptamer library. We selected against a well characterized protein, thrombin, and compared two RNA libraries: 1) containing boranophosphate U α B and 2) library 2'-FC & U α B modified. The 2'FC&U α B RNA is a novel combination of both a sugar and backbone modifications that has not been used in SELEX before. In this study, we focused on using BP-modified U α B and mixed 2'F&U α B RNA aptamer libraries in each step of the SELEX process to optimize conditions for this modification. This would allow us to determine if there was borano effect when selecting for aptamers targeted to thrombin. The steps that involve modified RNA selection are: transcription, incubation with the protein target, reverse transcription, and amplification. The enzyme compatibility with

the BP-RNA and combined 2'FC & U α B RNA is essential at every step of the SELEX process. Previous selections using BP modifications were done against a small molecule target ATP, bound to a column. We wanted to take this a step further and select against a protein using a nitrocellulose filter. We were unable to enrich the BP-RNA and combined 2'FC & U α B RNA libraries and obtain an aptamer to thrombin. We did however, hypothesize that it was the non-specific binding of the BP-RNA to the bovine serum albumin (BSA) in the binding buffers that prevented the enrichment of the aptamer library.

6.2 Materials and methods

DNA template – Klenow extension

The DNA library template (denoted Sel2 Library) was 5'-GGGAGGACGATGCGG-N40-CAGACGACTCGCTGAGGATCC-3', where "N40" denotes the random region 40-nucleotides in length (Oligos etc Inc.). This region gives the library its complexity and allows the formation of 10^{15} different possible sequences. The DNA template contains a T7 RNA polymerase promoter region; two flanking constant regions contain restriction sites for cloning as well as two PCR primer binding sites. The total length of the DNA template is 107 nucleotides long. The ssDNA template was annealed to the primer and elongated with the exonuclease-minus Klenow fragment of the DNA polymerase (Promega) and dNTPs (A, C, T, and G) (Promega). The DNA template strand (1 nmol) was mixed with the DNA primer (2 nmol) at a 1:2 ratio in annealing buffer (10 mM Tris-

HCl pH 7.5, 10 mM MgCl₂) in a total volume of 90 μL. This reaction mixture was heated to 95 ° C for 2 min and allowed to cool to rt. The Klenow reaction was assembled on ice with 0.5 mM dNTPs, 1X Klenow buffer (50 mM Tris-HCl (pH 7.2 at 25° C), 10 mM MgSO₄, 0.1 mM DTT, and 30 units of Klenow enzyme (Promega) to give a final volume of 500 uL. One unit of enzyme is defined as the amount of enzyme that incorporates 10 nmol of total deoxyribonucleotides into TCA-insoluble material in 30 minutes at 37° C (Protocol, Promega). The dsDNA (2 μL approximately 1 μg/μL) was run in a 3% agarose gel to analyze for full-length product.

Enzymatic synthesis of RNA libraries

All RNAs libraries were enzymatically synthesized using a dsDNA template (as discussed above using the Klenow enzyme).

This dsDNA template (10 μg) was incubated with a T7 RNA Polymerase mixture (RNA polymerase, recombinant RNasin® Ribonuclease Inhibitor, and recombinant inorganic pyrophosphatase) from the Ribomax Large Scale RNA Production System and the corresponding natural and/or modified triphosphates at a 2.0 mM concentration in 100 μL 1X buffer (80mM HEPES-KOH pH 7.5, 24 mM MgCl₂ , 2 mM spermidine, 40 mM DTT). The NTPs used in the transcription reaction were; the natural NTPs (Promega), a mixture of *Rp* and *Sp* NTPαSs (TriLink) were used. *Rp* NTPαBs were chemically synthesized by Marcus Cheek and HPLC-purified as described in **Chapter 3**. The reaction mixture was incubated for 6 hours at 37° C. The DNA template was digested

using RQ1 RNase-free DNase (1 U/ μ L) by incubation at 37° C for 15 min. The unincorporated NTPs were removed by G25 spin column (GE Healthcare) by loading the 100 μ L reaction mixture in the middle of the Sephadex column. The final product was ethanol precipitated, re-suspended in DEPC treated water (10-25 μ L) and quantified by UV (RNA ratio 260/280 between 1.90-2.0). The sample was **ethanol precipitated** as follows: The aqueous layer was transferred to a new tube and 0.1 volume (30-40 μ L) of 3M sodium acetate pH 5.2 was added and as well as 5 μ L Linear acrylamide. Then, 2.5 volumes of cold ethanol (-20° C) were added. The sample was placed on powdered dry ice for 30 min (-78° C) until frozen. The sample was centrifuged at 15,000 rpm for 30 min at 4° C, the supernatant was removed with a pipette, and 500-1000 μ L cold 70% ethanol (-20° C) was added. The sample was centrifuged, the ethanol was removed, and the sample was air-dried at rt. for 15 min. The quality of the RNA was analyzed on a 7 M urea 10% (19:1) PAGE gel (as described in **Chapter 2**).

Dephosphorylation of RNA

Shrimp Alkaline phosphatase (SAP) (Promega) was used to remove the 5'-phosphate of ssRNA before labeling of RNA with [³²P] ATP and T4 polynucleotide kinase. SAP (1 U/ μ L) was received in storage buffer containing 25 mM Tris-HCl, pH 7.6 at 4° C, 1 mM MgCl₂, 0.1 mM ZnCl₂, and 50% glycerol. The RNA library (100 pmol) was incubated with 20 units of SAP in 100 μ L of 1X buffer (50 mM Tris-HCl, pH 9.0 at 37° C and 10 mM MgCl₂). After incubating at 37° C for 15 minutes the SAP enzyme was heat

inactivated at 65° C for 15 minutes. Then 300 µL of TE buffer [10 mM Tris-HCl pH8.0, 0.1 mM EDTA] was added to increase the total volume before extraction. The reaction mixture was mixed with 400 µL phenol: chloroform: isoamyl alcohol (25:24:1) (Ambion). The sample was vortexed for 1 min, spun at 15,000 rpm for 5 min at 4° C, and the supernatant was transferred to a new tube. The aqueous layer was mixed with 400 µL chloroform: isoamyl alcohol (24:1) (2X), vortexed for 1 min, and then spun at 15,000 rpm for 5 min. The aqueous layer was transferred to a new tube and 0.1 volumes (30-40 µL) of 3 M sodium acetate (pH 5.2) and 5 µL linear acrylamide were added. Then, 2.5 volumes of cold ethanol (-20° C) were added. The sample was placed on powdered dry ice for 30 min (-78° C) till frozen. The sample was centrifuged at 15,000 rpm for 30 min at 4° C. Then, the supernatant was removed with a pipette and 500-1000 µL cold 70% ethanol (-20° C) was added. The sample was centrifuged, the ethanol was removed and the sample was air-dried at RT for 15 min. The pellet was re-suspended in TE buffer (10 mM Tris HCl pH 8.0, 0.1 mM EDTA).

5'-end labeling of RNA

RNA with a 5'-phosphate was dephosphorylated with SAP as shown above, prior to labeling with T4-polynucleotide Kinase (PNK) (Promega) and [γ -³²P]ATP. T4 PNK (5-10 U/µL) purified from *E. coli* cells expressing a recombinant clone was supplied by Promega in storage buffer containing 20 mM Tris-HCl pH 7.5, 25 mM KCl, 2 mM DTT, 0.1 mM EDTA, 0.1 µM ATP, and 50% (v/v) glycerol (Promega). One unit was

defined as the amount of PNK required to catalyze the transfer of 1 nanomole of phosphate from [γ - ^{32}P] ATP to the 5'-OH end of a polynucleotide in 30 minutes at 37° C in 40 mM Tris-HCl pH 7.5, 10 mM DTT, 0.1 mM [γ - ^{32}P] ATP, and 0.5 $\mu\text{g}/\mu\text{L}$ 5'-OH polynucleotide 'end' concentration. A 3 pmol aliquot of dephosphorylated RNA library was incubated with 2 μL of 3,000 Ci/mmol [γ - ^{32}P] ATP and 10 units of PNK in 20 μL 1X PNK buffer (50 mM imidazole-HCl pH 6.6, 10 mM MgCl_2 , 5 mM DTT, 0.1 mM spermidine, and 0.1 mM EDTA). After 30 min incubation time the reaction mixture was run through a G25 column to remove excess hot ATP (GE Healthcare). The 5'-end ^{32}P labeled RNA was stored at -20° C in the reaction buffer.

Binding Curves to Measure the Enrichment of Each Round

Affinity binding curves were determined after each round of selection using a dot-blot apparatus (Bio-Rad) and two membranes the top one is the nitrocellulose membrane (Protran) that binds protein with high affinity and only the RNA that is bound to the protein, both membranes are (h) 13 cm X (L) 14 cm (Woodbury and Von Hippel P. H. 1983). The bottom is a nylon membrane (Whatmann) that collects the non-bound RNA order to quantify the total RNA library per well (Woodbury and Von Hippel P. H. 1983). All binding studies were performed in different salt concentration buffers as shown. Physiological conditions were mimicked by binding buffer F (20 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM CaCl_2 , and 0.1% BSA). Lower salt concentrations

such as binding buffer E (20 mM HEPES, pH 7.4, 50 mM NaCl, 2 mM CaCl₂, and 0.1% BSA) and binding buffer EF (1:1 mixture of E and F) were used to decrease the stringency of the selection by allowing more binding of the protein to the RNA. Human purified α -thrombin was purchased from Haematologic Technologies Inc. (Essex Junction, VT) and used in the double-filter nitrocellulose filter binding assays. RNA was dephosphorylated using bacterial alkaline phosphatase and radioactively end-labeled at the 5' end with T4 PNK and [γ -³²P] ATP as described in the preceding paragraph. Direct binding was performed by incubating ³²P-RNA with thrombin in the desired buffer.

To determine the binding affinity of RNA to different concentrations of thrombin between each round of selection, a serial dilution of thrombin was made (5 μ M to 0.078 μ M in 8 tubes). The protein was incubated with 2000 cpm/ μ L of labeled RNA in binding buffer for 5 min at 37° C. The nylon and nitrocellulose membranes that had been pre-soaked in wash buffer were assembled in the dot-blot apparatus (Bio-Rad) with the nylon at the bottom and the nitrocellulose membrane on the top. Each of the 8 samples was transferred to a well. Vacuum was applied and each well was washed with 100 μ L of wash buffer. The apparatus was disassembled and the membranes, top and bottom were separated and wrapped separately in plastic wrap. To quantify the nitrocellulose and nylon membrane-bound RNA that had been end-labeled with ³²P, the filter was exposed to a low-energy autoradiography screen inside a light-blocking cassette for four hours. The screen was then imaged on the Typhoon using the storage phosphorescence

setting. This instrument used the Typhoon Scanner Control Software, V 4.0 (Amersham) and intensities of the dots or bands were quantified using ImageQuant 5.2 software. The fraction of the nucleic acid-protein complex that bound to the nitrocellulose membrane was quantified and the non-specific binding of the radiolabeled nucleic acid (nylon membrane) was subtracted out of the binding such that only specific binding remained. This was shown as the corrected fraction bound (**corrected FB**) and was used to select the thrombin concentration for the binding reactions. The **corrected FB** = [nitro-(nylon*lowest FB #8)]/total RNA, where the nitro = counts per min on the nitrocellulose, nylon = counts per min on the nylon membrane; the fraction #8 has no protein and this corrects for any background binding of the RNA to the filter, and the total RNA = (nitro + nylon).

Binding reactions and purification on bound RNA

Using the concentration determined in the binding curves, thrombin was incubated with the RNA at 37° C in either buffer E, F or a combination of E/F. The buffers were made using the concentrations of chemicals shown **Figure 34**, where the total volume for the 10X binding buffers was 10 mL and the 1X binding buffer was 1 L. All buffers were filtered through a 0.2 µm filter using a vacuum flask. The wash buffers were stored at rt. and the binding buffers were stored at 4° C. The concentration of thrombin was started at 20% fraction bound as the starting point for the SELEX procedure and reduced as the rounds progressed to increase stringency of the selection.

1X Wash buffer E (Filter sterilize, store RT)

20 mM Hepes pH 7.4
50 mM NaCl
2 mM CaCl₂
water

10X Binding Buffer E (Filter sterilize, store 4°C)

200 mM Hepes pH 7.4
500 mM NaCl
20 mM CaCl₂
BSA (0.1%)
Water

1X Wash buffer F (Filter sterilize, store RT)

20 mM Hepes pH 7.4
150 mM NaCl
2.0 mM CaCl₂
water

10X Binding Buffer F (Filter sterilize, store 4°C)

200 mM Hepes pH 7.4
1.5 mM NaCl
20 mM CaCl₂
BSA (0.1%)
Water

1X Wash buffer E/F (Filter sterilize, store RT)

Mixture 1:1 of E and F

10X Binding Buffer E/F (Filter sterilize, store 4°C)

Mixture 1:1 of E and F

Figure 34 - SELEX binding and wash buffers E, F and E/F used in during selection.

Before the binding reaction, a pre-clear step, to remove any RNA that binds to the nitrocellulose filter, was done using a 13 mm nitrocellulose disk (Whatmann) in a total volume of 100 μ L of binding buffer. The reaction was incubated at 37° C for 30 mins and the supernatant loaded into a rust colored Centrex® tube. The flowthrough volume was adjusted to 100 μ L which was used in the binding reaction and partition with thrombin.

The fraction bound value (obtained from the binding curves), that is used to calculate the protein concentration for each round of selection, starts at 0.2% fraction bound library and can change to modulate the stringency of the selection. In addition, the ratio between protein and RNA can also be varied to increase the stringency of the selection. The starting ratio was 1: 10 protein target to RNA and then the ratio was increase in subsequent rounds to the increase stringency of the selection.

The protein and RNA were incubated in binding buffer for 15 min and transferred to a 25-mm nitrocellulose disk (Whatmann). The disk was fastened in one of the 11 available wells of the manifold (Millipore) and the manifold was attached to a vacuum (Millipore). After the sample in the centrifuge tube was transferred to the membrane disk, the vacuum was applied and the filter was washed with 5 mL of wash buffer two times. The disk was cut into 8 pie pieces and transferred to an Eppendorf tube and incubated with phenol: chloroform: isoamyl alcohol (25:24:1) pH 6 for 30 min. The sample was vortexed and DEPC-treated water was added before centrifuging the sample. The extracted supernatant, containing the RNA, was removed and an additional extraction was performed using chloroform: isoamyl alcohol (24:1). The extracted RNA was ethanol precipitated by transfer the aq layer to a new tube and adding 0.1 volume (30-40 μ l) of 3M sodium acetate pH 5.2 and 2 μ l Linear acrylamide (Ambion) and 2.5 vol of cold ethanol. After vortexing the tube was placed in powdered dry ice for 30 min (-78° C) till frozen. Then the sample was centrifuged at top speed for 20 min at 4° C (15,000 rpm). The supernatant was removed with a pipette and pellet was carefully washed with 1 mL cold 80% ethanol (-20° C). The sample was centrifuged again for 10 min at top speed and the ethanol aspirated (slant tube, pellet facing up and aspirated from beneath using a pipette) and re-suspended in DEPC-treated water (10-20 μ L).

Reverse transcription (RT) of isolated RNA from each round and PCR

The isolated RNA was incubated with a DNA 3'- primer (5'-GGGGGGATCCAGTACTATCGACCTCTGGGTTATG-3') and heated for 5 min at 65° C. Once the mixture was at room temperature, 0.2 mM dNTP mix and 10 units of AMV RT (Promega) were added to 10 units of AMV RT in 50 µL 1X buffer (50 mM Tris-HCl pH 8.3, 50 mM KCl, 10 mM NaCl₂, 0.5 mM spermidine and 10 mM DTT). This reaction mixture was incubated at 37° C for 30 min. The AMV RT was deactivated by heating to 95° C for 5 min. This cDNA reaction was used to set up 10 PCR reactions with 5 µL of RT in each PCR tube. Each PCR tube contained as follows: 20 µL of 5X GoTaq buffer proprietary buffer (Promega) the RT reaction was added 5'-primer (5'-GGGGGAATTCTAATACGACTCACTATAGGGAGAGAGGAAGAGGGATGGG-3') and 3'- primer (5'-GGGGGGATCCAGTACTATCGACCTCTGGGTTATG-3'). The DNA primers (1 uM of each), 1.5 mM MgCl₂, 0.2 mM dNTP mix, and GoTaq flexi polymerase (Promega) were combined in a 100 µL total reaction volume. Using a Thermo-cycler® machine the PCR cycle conditions were: 94° C /5' --> (94° C /30" 55° C /30" 72° C /30") x 20 cycles --> 1 min 72° C --> 4° C. An aliquot was run on a 3% agarose gel and the DNA product size was 107 bp as measured using DNA markers (Fermentas). The PCR reactions were concentrated using a A10 filter (Facol) and centrifuged at 5,000 rpm, then washed twice with 2 mL TE buffer (10 mM Tris pH 7.0, 0.1 mM EDTA). The PCR product was used to make the enriched RNA for the next round of selection.

6.3 Results

Enzymatic synthesis of RNA libraries containing borano-modifications

Starting with a ssDNA template that contains a random sequence region a 3'-primer is annealed (**Figure 35 Step 1**) and using a Klenow Fragment (exo⁻) enzyme that 'fills' in to make the dsDNA template to be used in enzymatic synthesis of the library (**Figure 35 Step 2**). The klenow Fragment (exo⁻) is a DNA polymerase I with an N-terminal truncation which retains its polymerase activity but has no 3'-5' exonuclease activity (Derbyshire 1988) . The dsDNA template obtained by Klenow fill in (**Figure 35 Step 2**), was incubated with T7 RNA polymerase and either modified (BH₃ or 2'F) or natural NTPs (**Figure 35 Step 3**) to obtain the desired RNA aptamer libraries. For the natural and UαB libraries, the corresponding NTPs were transcribed with the wild type T7 polymerase to obtain full product RNA. For the enzymatic synthesis of 2'-FC & 2'-FU and the UαB & 2'FC modified RNA libraries; a mutant T7 enzyme was used (Epicentre).

Step 1 – Annealing Template (blue) to primer (red)

ssDNA

5' -GGGGGGATCCAGTACTATCGACCTCTGGGTTATG-3'
3' -ATCGACCTCTGGGTTATG (N40) CCCATCCCTCTTCCTC-3'

Step 2 – Klenow Extension to make dsDNA

Exo⁻ Klenow Fragment (DNA polymerase)
dNTPs (T, A, C, G)



dsDNA

5' -GGGGGGATCCAGTACTATCGACCTCTGGGTTATG (N40) GGGTAGGGAGAAGGAG-3'
3' -CCCCCTAGGTCATGAATCGACCTCTGGGTTATG (N40) CCCATCCCTCTTCCTC-3'

Step 3 – Enzymatic synthesis of RNA

T7 RNA polymerase
rNTPs (U, A, C, G) or replacing a natural rNTP with:
rNTP α B (^bU, ^bA, ^bC, ^bG)



RNA
5' -GGGUUAUG (N40) GGGUAGGGAGAAGGAG-3'
(RNA library – 10¹⁴ different sequences)

Figure 35 – RNA library synthesis.

The first step is to anneal the template to primer (Step 1). This allows the Klenow enzyme to “fill in” and make the dsDNA template (Step 2). The dsDNA is then used to synthesize RNA using T7 RNA polymerase, and it is at this step that NTP α Bs are used.

The transcripts were analyzed using a 10% (19:1) PAGE gel, in which molecules migrate depending on their size to change ratio. A longer DNA or RNA would take more time migrating through the gel and a shorter DNA or RNA would migrate faster. We found that the full-length transcripts were obtained by natural and modified NTPs.

Figure 36 shows full-length transcripts for natural (**Figure 36 (2) lane A**), 2'-F-modified pyrimidines (**Figure 36 (2) lane B**), U α B (**Figure 36 (3) lane C**) and UTP α B & 2'FC RNA (**Figure 36 (3) lane D**).

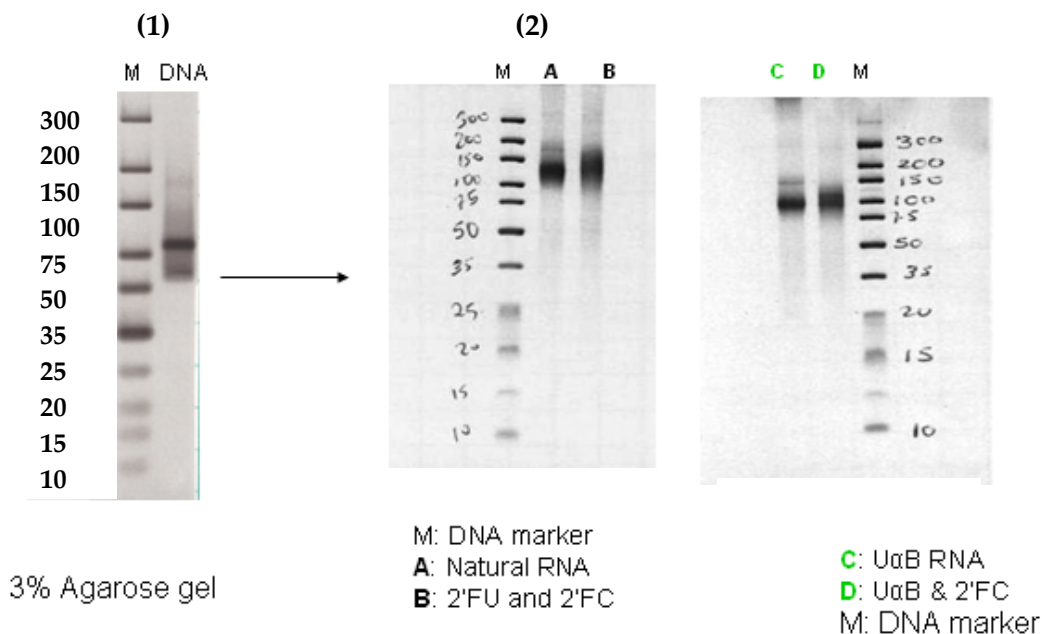


Figure 36 – Natural and modified RNA libraries analyzed by 10% PAGE/7 M Urea. Boranophosphate RNA libraries synthesized from dsDNA (1) dsDNA migrates between the 75-100 mer DNA marker bands. This dsDNA template was used with T7 RNA polymerase and a mixture of natural or modified NTPs. (2) Transcription using natural NTPs RNA (Lane A) and sugar modified pyrimidines (Lane B) results in full length RNA product since it migrated between DNA markers 100-150. (3) Transcription with borano UTP α B replacing natural UTP (C) and UTP α B and sugar modified 2'-F CTP (D) also show full length products and the migration length between markers 100-150 as expected.

The appearance of bands between the 100 mer and 150 mer DNA marker bands suggests the incorporation of the desired modified NTPs into the transcript because it is a mixture of four NTPs (U, A, G, C) either modified or natural per transcription

reactions. Thus, if a modified NTP was not incorporated, there would not be a band corresponding to full-length products. Since RNA folds into numerous ways depending on its sequence, it is expected that there will be broadening of the bands as seen in the PAGE gel below.

The yields obtained for modified libraries using a nanodrop UV spectrometer were somewhat less when compared to natural RNA transcription reactions with wt T7 RNA polymerase, where natural RNA was obtained at an average of 58.5 μL per 100 μL reactions. The U α B RNA was significantly lower, and it was 21.0 μg per 100 μL reaction. In addition, 2'FC&2'FU transcription reactions using the mutant T7 RNA polymerase gave an average of 55.6 μg per 100 μL reaction compared to 15.5 μg per 100 μL reaction with 2'FC&U α B. The reduction in yields with BP-modified RNA raises the concern that there might be a reduction of enrichment after every selection round under the conditions used.

Binding affinities of modified RNA during SELEX

The binding of an RNA molecule to a target protein can be modeled as a “one ligand, one binding site”. The binding of a ligand (RNA) to a single binding site is defined by the concentration of the binding site (B_{max}) and the concentration of its unbound ligand when the binding site is 50% full (K_d). The RNA aptamers that bind with high affinity have a low K_d and high B_{max} . After each round of selection we are

looking for high affinity binding of the enriched RNA libraries using a filter binding assay.

A UTP α B RNA library was synthesized (general scheme shown in **Figure 35**) and incubated with thrombin in buffer **E**. The different salt concentrations in the buffers are shown in **Figure 34**. Buffer **E** has a low salt concentration and allows for less stringent binding events. We can compare the starting library at round 0 in buffer **E** (purple) to buffer **F** (red) and also compare subsequent rounds of selection (**Figure 37**). The library binds with higher affinity and higher B_{\max} to thrombin at lower stringency. In contrast, in the physiological salt concentrations in buffer **F**, the affinity is significantly reduced.

UTP α B RNA SELEX

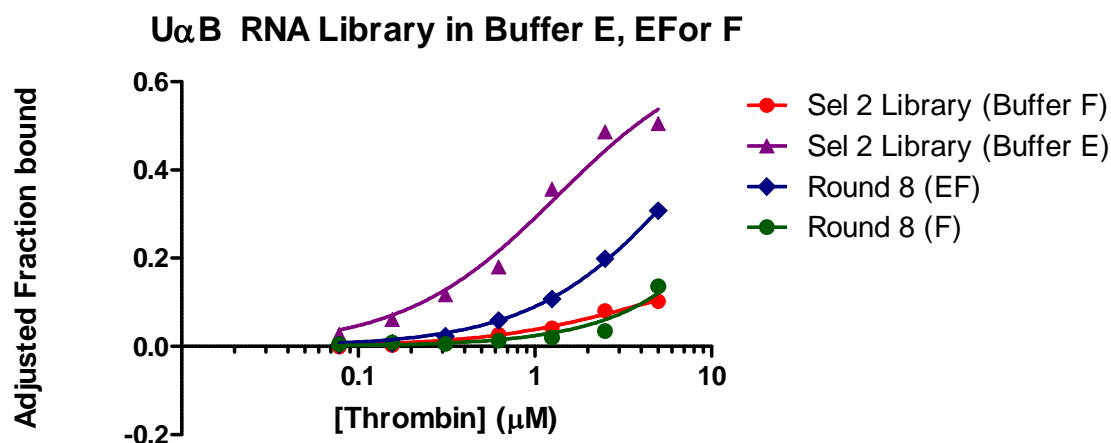


Figure 37 - UTP α B RNA SELEX.

The progress of the selection against thrombin was followed using a nitrocellulose filter binding assay. Red circles (●) represent the starting RNA library (Sel2) in buffer F. Purple triangles (Δ) represent Sel2 in low salt buffer E. Blue diamonds (◇) are round 8 in buffer E/F and green circles (●) are round 8 in buffer F. The x-axis shows the human thrombin concentration (μ M) and the y-axis shows the fraction of RNA bound to the protein adjusted for non-specific filter binding of the RNA. (Prism graphing software used)

The selection was started in buffer E and the salt concentration was changed to E/F (1:1 v/v), which gives an intermediate salt concentration. **Figure 38** shows all eight rounds of selection in different buffers. At round 5, there was an increase in affinity in buffer E so the selection was switched to buffer EF (**Figure 38**). After round 8, the bound RNA assayed in buffer EF and buffer F. No enrichment was seen compared to the starting library round 8 E/F (blue) and round 8 F (green) **Figure 37**. In addition, **Table 7**

shows the protein: RNA ratios used to decrease astringency and drive the enrichment of the RNA pool.

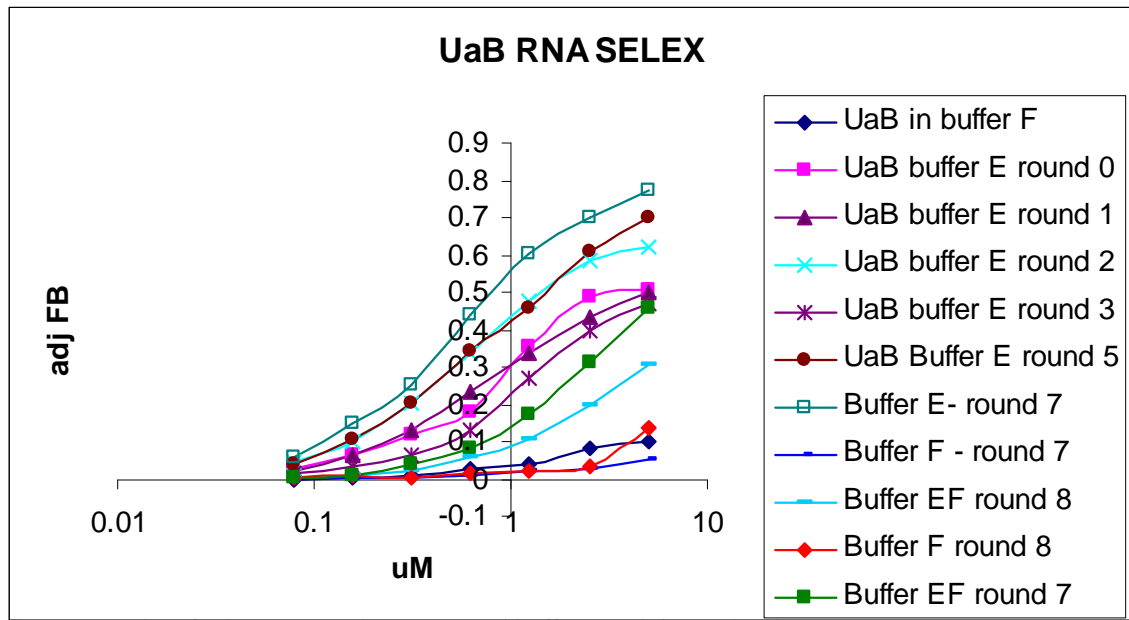


Figure 38 - Detailed SELEX UTP α B RNA rounds 0 to 8.

SELEX UTP α B RNA rounds 0 to 8 in the noted buffer show lack of enrichment after 8 rounds (Buffer F round 8, red triangle Δ). At round 2, (light blue x) there was a slight increase in affinity compared to round 0 (pink squares, \square). However, it was lost at round 3 (dark purple star *). At round 7 still in buffer E (dark green open square \square), there was another increase in affinity and the buffer was switched to E/F for round 8 (light blue -) where we saw a decrease in affinity. (Excel software used).

Table 7 – U α B RNA and thrombin conditions used at each round of selection. %FB is the adjusted fraction bound percentage that corresponds to the [thrombin] concentration obtained from binding curves. The [thrombin] μ M was used during binding reaction between the RNA and thrombin protein. In addition, the RNA: protein ratios were varied between some of the rounds of selection to increase the stringency.

Buffer	Round #	[Thrombin]	% FB used	RNA:Protein
E	0	0.94 μ M	20%	1 to 5
E	1	0.63 μ M	20%	1 to 10
E	2	0.31 μ M	20%	1 to 10
E	3	0.9 μ M	20%	1 to 8
E	4	0.63 μ M	13%	1 to 8
E	5	0.31 μ M	20%	1 to 10
E	6	0.31 μ M	20%	1 to 10
EF	8	0.31 μ M	10%	1 to 10

When the RNA: protein ratio was increased to 1: 8 and the %FB used was 13%, there was a loss of affinity in round 5 (**Table 7, Figure 38**).

UTP α B & 2'-F SELEX

A UTP α B and 2'-FC library was synthesized and selected against thrombin to examine the effect of the BP modification on the enrichment of the library pool.

Previous selections against thrombin were accomplished with two 2'-F -modified pyrimidines (U and C); we wanted to directly compare the effect of substituting 2'F U for UTP α B. This was a way to determine if there was a 'borano effect' during selection.

The starting library was tested in buffer E (purple) and F (red) and there was an expected rise in affinity in the less stringent buffer E. The selection in buffer E progressed faster for the 2'FC & UTP α B RNA compared to the UTP α B RNA selection,

and by round 3 there was a rise in affinity. The selection buffer was then changed to E/F for another round. Round 4 RNA was tested in buffer E and F and no increase in affinity was observed (Figure 39). There was also a decrease in affinity in buffer E/F at this round.

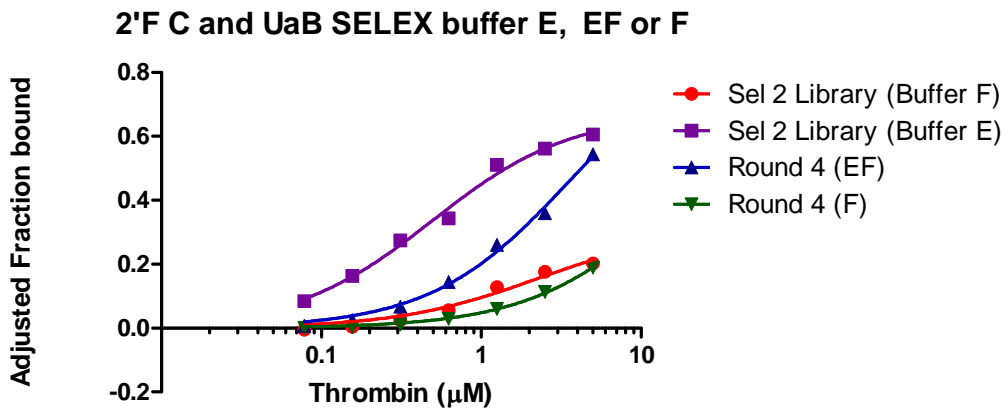


Figure 39 - 2'-F & U α B RNA SELEX, showing lack of enrichment after 4 rounds of selection using different selection buffers.

The progress of the selection against thrombin was followed using a nitrocellulose filter binding assay. Red circles (●) represent the starting RNA library (Sel2) in buffer F. Purple squares (◻) represent Sel2 in low salt buffer E. Blue triangles (Δ) are round 4 in buffer E/F and green inverted triangles are round 4 in buffer F. The x-axis shows the human thrombin concentration (μM) and the y-axis shows the fraction of RNA bound to the protein adjusted for non-specific filter binding of the RNA.

The UTP α B&2'FC RNA selection was enriching faster and the RNA: protein ratios were changed from 10 to 12. This was very conservative change. In addition, the %FB was decreased from 27% to 15%, which was faster than the changes used in the UTP α B selection (Table 8).

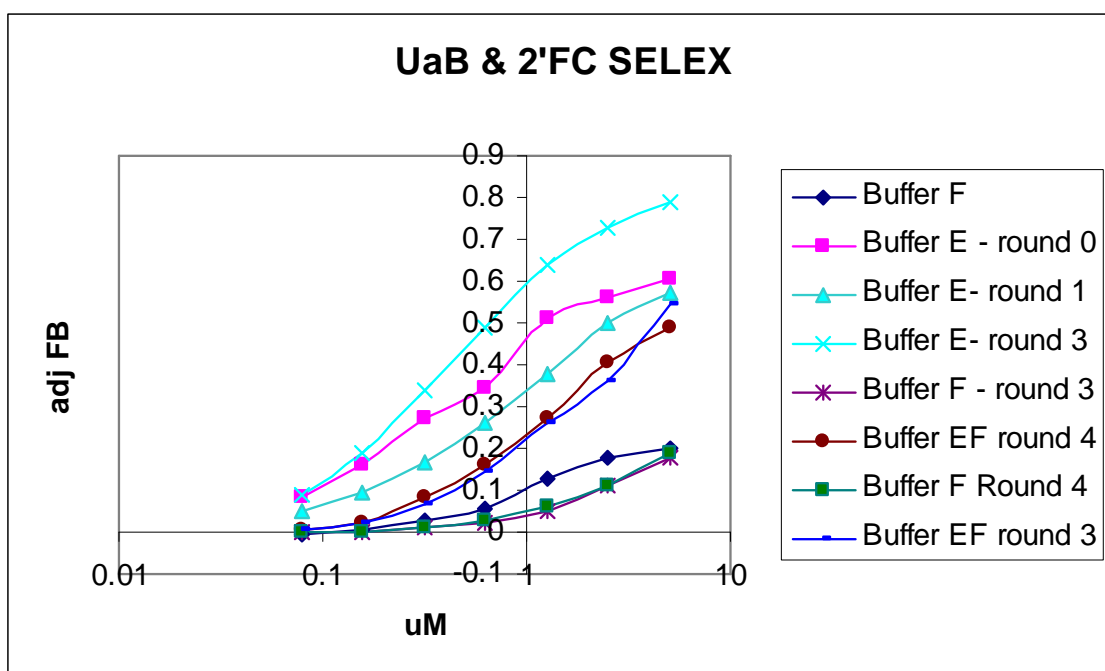


Figure 40 - Detailed SELEX (2'F-C and U α B), rounds 0 to 4. SELEX (2'F-C and U α B) Library showing four rounds of selection starting in buffer E (pink squares \square). Since no significant binding by the library was seen in buffer F (blue diamonds \diamond) the selection buffer was changed to less stringent buffer E (pink squares \square). After three rounds in buffer E there was slight increase in affinity (buffer E, round 3, light blue \times). After, the slight increase we changed to buffer E/F, which is a 1:1 mixture of E and F for round 4. The RNA isolated in round 4 was assayed in F, E/F and E but there was a loss of affinity. After, four rounds of selection there was no increase in affinity in buffer F round 4 (green square \square).

Table 8 – RNA and protein ratios used at each round

Buffer	Round #	[Thrombin]	% FB used	RNA:Protein
E	1	0.31 uM	27%	1 to 10
E	2	0.31 uM	27%	1 to 10
E	3	0.31 uM	16.70%	1 to 10
EF	4	0.625 uM	15%	1 to 12

Other modified Sel2 RNA libraries were made with different modified NTPs and there was a remarkable difference in affinity in buffer F. **Figure 41** shows that GαB RNA bound with higher B_{max} to thrombin compared to other boranophosphate modifications and its affinity profile is similar to 2'FC&2'FU modified RNA.

RNA Library to Thrombin Modification Comparison

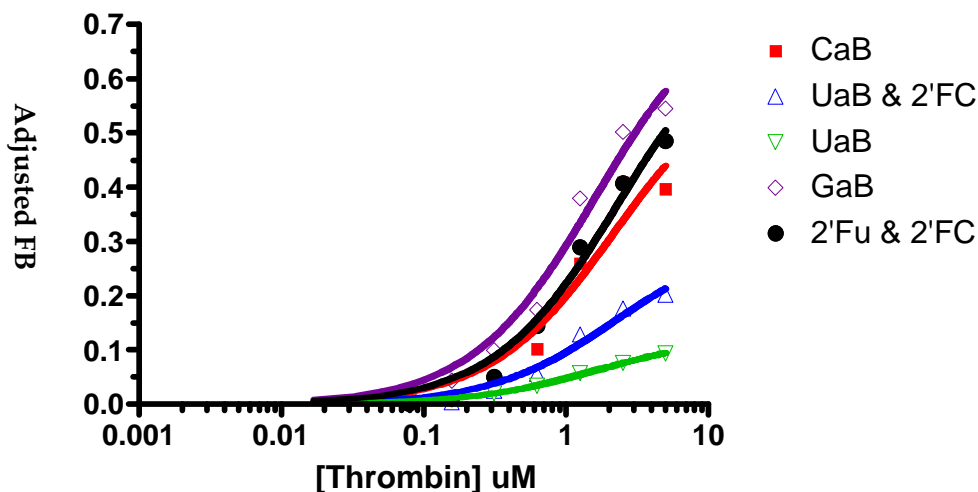


Figure 41 -Library modifications have an effect on library affinity to thrombin. Changing the nature and amount of modifications in the RNA library modulated the affinity of the thrombin. GαB modified (purple diamond ◊) had the highest affinity to thrombin. Followed by the 2'FU & 2'FC modified RNA (black circles ●). UαB

(green upside down triangle) had the lowest affinity and combining U α B & 2'FC (blue triangle Δ) increased the affinity slightly.

In addition, UTP α B RNA bound similarly to 2'FC&UTP α B in buffer F, but during the selection we saw a difference in the enrichment of the pools as the selection process continues (**Figure 38** and **Figure 40** respectively). This suggests a significant contribution of the borano modification to the affinity to the target.

Next, we asked whether starting the selection with GTP α B in buffer F would allow for the selection process to get enriched more readily with fewer rounds of selection, as compared to starting in buffer E and then increasing the ionic strength of the buffer to E/F. The starting G α B RNA library bound to thrombin with high affinity in buffer ; however, after the pre-clear step using nitrocellulose membrane, the affinity decreased. This suggests that we removed a high amount of non-specific binding RNAs that bound to the nitrocellulose filter or the BSA. After one round of selection, the affinity seen in the starting library was lost (orange triangle (Δ) **Figure 42**).

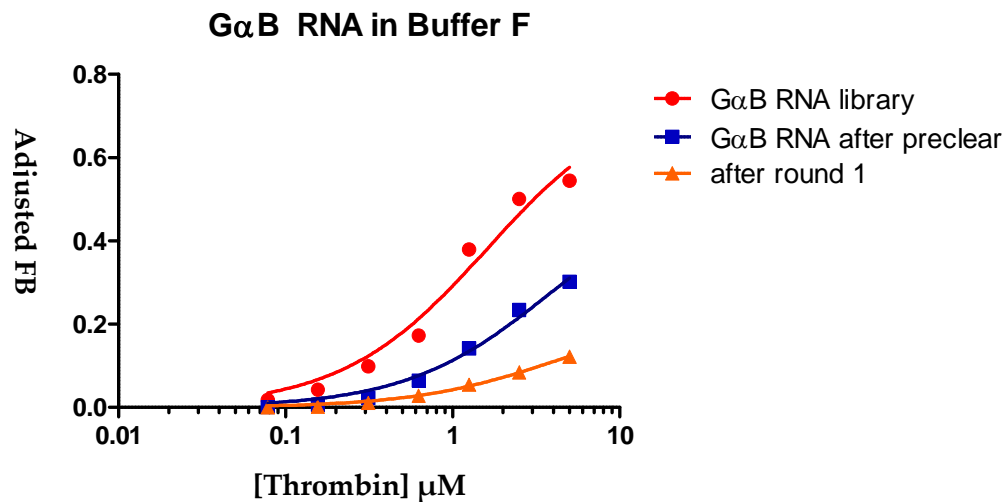


Figure 42 - G α B RNA library round 0 to 1.

G α B RNA library lost affinity after round 1. Using a nitrocellulose filter binding assay the Red circles (●) represent the starting G α B RNA library in buffer F. Blue squares (□) represent Sel2 in buffer F. Orange triangles (△) are round 1 in buffer F where there is loss of affinity after round 1 of binding with thrombin. The x-axis shows the human thrombin concentration (μM) and the y-axis shows the fraction of RNA bound to the protein adjusted for non-specific filter binding of the RNA (corrected FB = [nitro-(nylon*lowest FB #8)]/total RNA).

Using BP- RNA as Templates for Reverse Transcription to cDNA using AMV RT

We wanted to analyze the cDNA products obtained by reverse transcription when using BP-RNA and 2'FU& U α B RNA as a templates for AMV RT compared to the natural RNA and 2'FU&2'FC RNA. Reverse transcription of several types of modified RNA (G α B, 2'FC & U α B, 2'F U & 2'F C)with AMV RT produced some over-extended DNA products and lowered yields compared to natural (**Figure 43**). G α B RNA (**Lane 1**), 2'FC&U α B RNA (**Lane 2**), natural (**Lane 3**) and 2'FC&2'FU (**Lane 4**) in **Figure 43** show the cDNA product obtained from AMV RT enzyme. The enzyme was able to efficiently

reverse transcribe the natural RNA (**Figure 43, Lane 3**) but was less efficient with the modified RNA (**Figure 43, Lane 1, Lane 2, lane 4**). The 2'FC&U α B RNA is a novel combination of both a sugar and backbone modifications. Modifications of the RNA, such as 2'F or BPs, change the overall structure, chemical properties and the bulkiness of the RNA and this may reduce the efficiency of the enzymatic reaction. Nevertheless, it has been shown by numerous selections that using 2'F (C/U) modifications can be useful for selecting aptamers and AMV RT is the enzyme of choice (White, Rusconi et al. 2001; Ng and Adamis 2006).

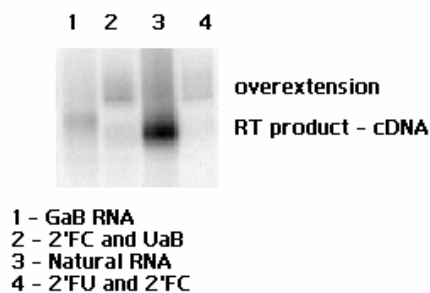


Figure 43- Using modified templates with AMV RT to obtain cDNA. GTP α B RNA (1), 2'FC&UTP α B (2), natural (3) and 2'FU&2'FC (4) were incubated with AMV RT and the RNA to obtain a 83 mer cDNA. The cDNA was end labeled with 32 P and T4 kinase. The product was analyzed using 10% (19:1) PAGE/ 7 M urea and over-extension product bands of 3-5 nucleotides were observed with 2 and 4 but with natural or U α B RNA. The yields were significantly lower with the modified RNA templates.

The entire cDNA product, including overextended products obtained from the reverse transcription with AMV RT, was used in the PCR reaction to yield full-length

DNA products after every round (**Figure 44**). The amplified products were then used to synthesize the RNA for the next round. This step leads to the enrichment of the RNA pool because only the bound RNA, that was bound to thrombin and was thus retained in the nitrocellulose filter, was reverse transcribed and amplified with PCR.

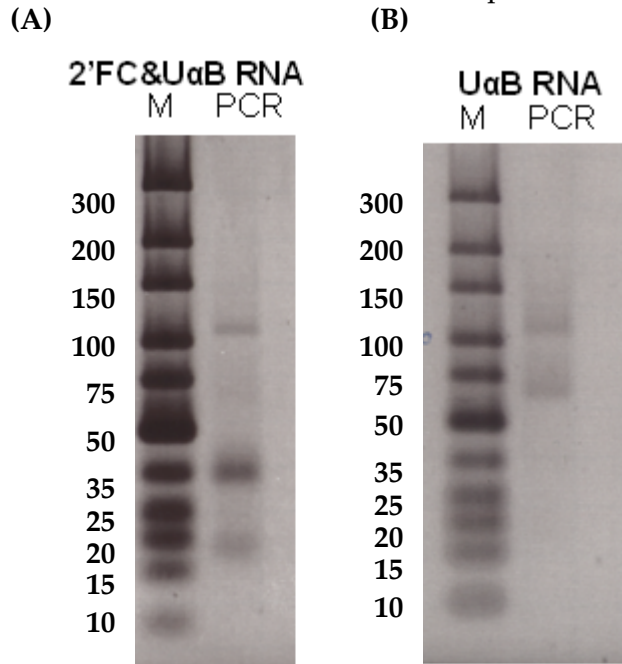


Figure 44 - PCR amplified DNA analysis using a 10% (19:1) PAGE/ 7 M urea.

The cDNA obtained from the AMV RT reaction was amplified by PCR with Taq DNA polymerase. (A) We analyzed the PCR product after the 2'FC&UαB RNA was reverse transcribed and amplified with PCR. A full length product was observed coinciding with the 100 mer DNA marker. In addition, the primers were seen to migrate by markers 35 and 20. (B) The PCR product obtained after the UαB RNA was reverse transcribed and amplified. A PCR product band was seen at 100 mer migration level in addition to a 60 mer. 10 μg of the PCR reaction was used as DNA templates for the synthesis of the RNA for the next round of selection.

As seen in **Figure 44**, the amplification of the cDNA gives 100 mer products that match the length of the template. The 5' end primer includes the promoter region for the transcription reaction with the T7 RNA polymerase. The UαB RNA has a 30% lower

yield of PCR products compared to the 2'FC&U α B RNA. This might suggest a less efficient AMV RT reaction leading to less enrichment of the RNA pool.

6.5 Discussion

Boranophosphate RNA aptamers libraries were studied for their use in the SELEX method. Thrombin was used as the target protein because it is a well-studied target for aptamer selection. There was a reduced enrichment after 8 rounds of selection observed with UTP α B RNA and after 4 rounds of selection with 2'FC& UTP α B RNA selections. Although the 2'FC& UTP α B RNA pool was enriched faster than the UTP α B RNA, both failed to increase to the normally acceptable level of enrichment using the selection conditions for the 2'F (C/U) RNA that had previously worked for thrombin protein (White, Rusconi et al. 2001). It should be noted however that 13-15 rounds of selection have been done in previous selections with thrombin and other targets. We choose here not to continue the selection but consider changing the selection buffers in future experiments. We hypothesize that the low level of enrichment under the standard conditions used for the 2'F (C/u), could be due to the properties of the NTP α Bs or the overall boranophosphate RNA chemical properties that prevented the enrichment of the pool during selection.

Another type of phosphorous modified NTPs , the phosphorothioate NTP α S; have been shown to be less efficient substrates and competitive inhibitors for phosphatases (Eckstein and Sternbach 1967). In addition, previous work in our lab has

shown that thymidine 5'-boranophosphate (5'-TMP α B) was dephosphorylated less readily compared to the natural molecule (Kociecki J., dissertation 2006). This modified monoester was hypothesized not to coordinate Zn⁺² like the natural monoester because of the lack of non-bridging oxygens that interact with Zn⁺² and the guanadinium group of Arg-166 (Kociecki J., dissertation 2006). We hypothesize that the end-labeling reaction of the modified RNA aptamers for the binding assay might not show accurate binding curves if the modified RNA is labeled less efficiently. This is consistent with our results for the 5'-end modified RNA such as GTP α B RNA libraries where we saw an apparent loss of affinity after round 1 (**Figure 42**).

Another consideration is the fidelity or kinetics of the T7 polymerase during synthesis of the RNA aptamers at each round of selections. If the RNA yield is lower during enzymatic synthesis after each selection round, this could lead to a loss of high affinity aptamers. Although previous work in the lab showed that NTP α Bs are incorporated into mRNA and translated into the desired protein as measured by efficient translation into the luciferase protein (Wan Jing, Dissertation), we were unable to duplicate these results. This might shed light on the decrease of enrichment if there are too many mutations in the RNA. Although at first mutations increase the number of different sequences, in further rounds this will pose a problem.

The reverse transcription step after the isolation of the bound RNA after each round of incubation with the protein depends on the activity and fidelity of the AMV RT

enzyme and the reaction yield may be affected by the properties of the BP-modified oligonucleotides. Reverse transcription with AMV RT needs to be further optimized to increase its yields. Future experiments should also include sequencing of the cDNA and quantify the fidelity of the RT by measuring the deletions, insertions, substitutions of BP compared to; 2'F modifications, mixed 2'FC&UαB and natural.

The 3D arrangement and chemistry of the target amino acids in thrombin with RNA aptamers could be important for example: the ion pair formation between the phosphate backbone in the RNA and the thrombin protein as described by Long et. al may disrupted by the BP modification by preventing ion pairing and changing the backbone structure (Long, Long et al. 2008). The known RNA aptamer to thrombin toggle-25t forms a stem-loop that forms a complex tertiary structure and binds thrombin through a series of adenine-arginine contacts at exosite 2 (Long, Long et al. 2008). There is extensive π - π stacking within the RNA structure, and seven out of the eleven single-stranded bases are paired with either the protein's arginine residues or other bases (**Figure 45**) (Long, Long et al. 2008).

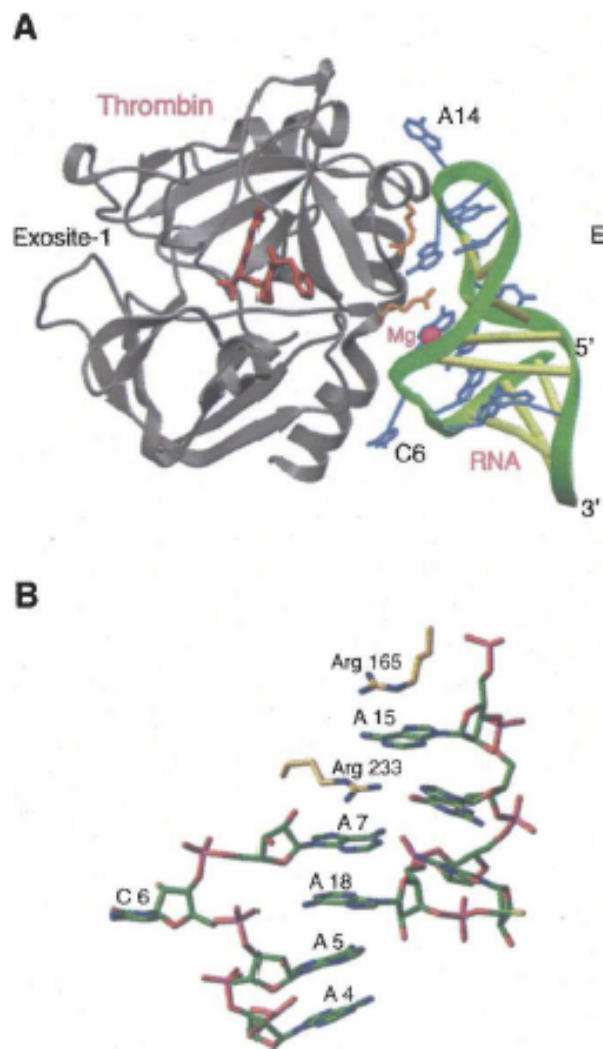


Figure 45 – The crystal structure of the RNA aptamer (2’F C & 2’F U) bound to thrombin.

(A) Toggle-25t aptamer bound to thrombin at exosite 2. (B) There are extensive interactions between RNA bases and protein interface forming an A-Arg zipper.

One hypothesis is that there may be fewer ion-pairing and weaker non-specific electrostatic interactions between the boranophosphate modified RNA with the protein’s exosite 2. The reduced negative charge density of the borano RNA may allow the Sel2

library to bind non-specifically to the thrombin protein, like the oligosaccharide heparin. Heparin binds non-specifically to the exosite 2 without complex direct hydrogen bonding interactions of the exosite 2/RNA aptamer (Long, Long et al. 2008). This lack of complexity makes heparin bind with lower affinity, i.e., K_d of 6 μM (Olson, Halvorson et al. 1991) compared to the toggle-25t with a K_d of 0.5 nM (White, Rusconi et al. 2001).

Another possibility is that the BP-RNA is not binding exosite 2 at all and may be binding another site with less specificity. We hypothesize that targeting the positively-charged exosite 1 or 2 will lead to a lack of enrichment in the pool as selection progresses. This may be due to the lack of appropriate sites, binding specificity or affinity of the modified RNA aptamer to thrombin. Also, many more rounds of selection may be necessary for a successful selection.

Non-specific binding was not due to the binding of the RNA to the filter since the background was less than 2% consistently. We hypothesize that the non-specific binding of borane analogs to BSA might have been a problem. Thus, one alternative method of decreasing “background levels” is to perform a negative selection using BSA during the early rounds of selection. If that does not work, then the removal of BSA and replacement with CHAPS might be acceptable (Blake, Sullenger et al. 2009). A CHAPS surfactant molecule will not bind the oligonucleotides as readily as the 66,776 Da proteins BSA (**Figure 46**).

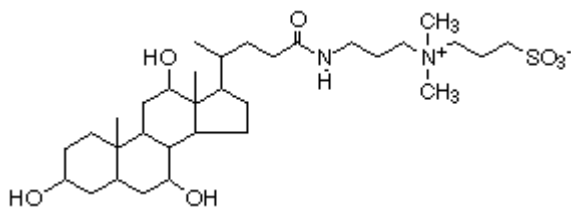


Figure 46 – CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]propanesulfonic acid) chemical structure of surfactant for selection buffers.

In conclusion, we were not able to successfully obtain BP-RNA aptamers to thrombin after several rounds of selection. Although it was shown that SELEX could be done against the small target ATP (Lato, Ozerova et al. 2002), this does not necessarily translate to selecting against proteins. There are more experiments that need to be done to optimize the SELEX process to complement the BH_3 chemical properties. The failure could be due to the incompatibility of the chemical properties of the boranophosphate with the standard selection conditions or the non-specific binding to the BSA in the buffers. A protein with smaller patches of positive charged sites might be a better target for the BP-RNA aptamers to form more specific interactions.

Chapter 8: Future directions

Chemical modifications added to RNA molecules modulate properties of the RNA *in-vivo* and *in-vitro*. An advantage of NTP α Bs is that they are good substrates for RNA and DNA polymerases, which allows the enzymatic synthesis of RNA oligonucleotides of varying lengths. In addition, this modification imparts different structure and activity properties to the BP-RNA. Therefore, a wide range of oligonucleotide therapeutic applications are possible. We explored the ability of the innate immune system to recognize BP-RNA and activate the production of cytokines. We found that fully modified BP-RNA was not an activator of TLR 7. This finding has implications for the applications of RNA in therapeutic settings, since innate immune activation is not desired, when administering oligonucleotides to modulate protein or gene activity. Further research would determine if other endosomal TLR receptors are modulated by BP-RNA or BP-DNA. In addition, this would need to be analyzed by *in-vivo* models such as a mouse or rat.

This follows that the gene regulation activity of siRNA needs to be analyzed as possible applications of BP-RNA. We screened the BP-RNA for its ability to downregulate p-glycoprotein as a siRNA in RNAi. We conclude that there is a fine line between loss of innate immune response and the gene downregulation of BP-siRNA molecules. We hypothesize that BP disrupts the structure and charge distribution so

extensively that it does not bind with the same affinity to proteins or other oligonucleotides. Thus, there is a loss of downregulation activity by the siRNA in tandem with a loss of immunogenicity. Fully modified siRNA was not active in the MDR 1 model, which is hard to downregulate, yet it has not been tested in a less challenging model such as the downregulation of EGFP genes. We suspect that BP-siRNA activity is target dependent and a less stringent target than MDR1 would be better downregulated. In addition, the RT-PCR (real-time PCR) experiments to analyze the reduction of the target at the mRNA level needs to be done. We cannot distinguish innate immune activation and siRNA downregulation. Future studies would also have to include crystal structures of the BP-RNA and affinity assays to catalytic components like the ago2, in the RISC complex.

For the application of BP-RNA in aptamer selections, the SELEX process needs to be further studied with the NTP α B. The SELEX process depends on the activity of many enzymes such as T7 RNA polymerase, PNK, T4 kinase, and AMV RT. If the efficiency of these enzymes is low then the modified RNA pool will not be enriched. In this study we saw a lack of enrichment during SELEX indicating that the selection conditions need to be further optimized with the BP modification. We need to start by changing the buffer's BSA component to CHAPS to reduce the non-specific binding, but keeping the salt conditions used in these experiments (buffers E and/or F). To translate the BP-RNA lack of immune activity, a selection with fully BP-modified RNA libraries might be

attempted with a protein target that has a varied charge surface allowing for more complex interactions. Further, the known thrombin aptamer, toggle-25, should be retrofitted with BP modifications systemically to test our hypothesis on the soundness of the thrombin as a BP-RNA aptamer target.

Appendix A: Gemcitabine triphosphate (dFdCTP) polymerization with mutant Y639F T7 RNA polymerase

Introduction

Pancreatic Cancer and Gemcitabine Therapy

Pancreatic cancer (PC) is a difficult cancer to diagnose; it is very aggressive, only 6% of patients survive 5 years after a diagnosis (Cancer Facts and Figures 2011, p19). The pancreas is a gland organ that produces several important hormones including insulin, glucagon, and somatostatin. Standard therapy for patients with metastatic pancreatic cancer is administration of gemcitabine (2', 2'-difluoro 2'-deoxycytidine monohydrochloride), sometimes termed dFdC, **Figure 47** (Burris, Moore et al. 1997; Mini, Nobili et al. 2006; Sultana, Ghaneh et al. 2008) in combination with other chemotherapy agents (Sultana, Tudur Smith et al. 2008). Gemcitabine is a cytidine (C) analogue that is phosphorylated into its triphosphate form inside the cell (**Figure 47 C**, dFdCTP); the triphosphate targets cells in the S-phase of the cell cycle and then gets incorporated into DNA, which leads to apoptotic cell death (Huang, Chubb et al. 1991; Ruiz van Haperen, Veerman et al. 1993).

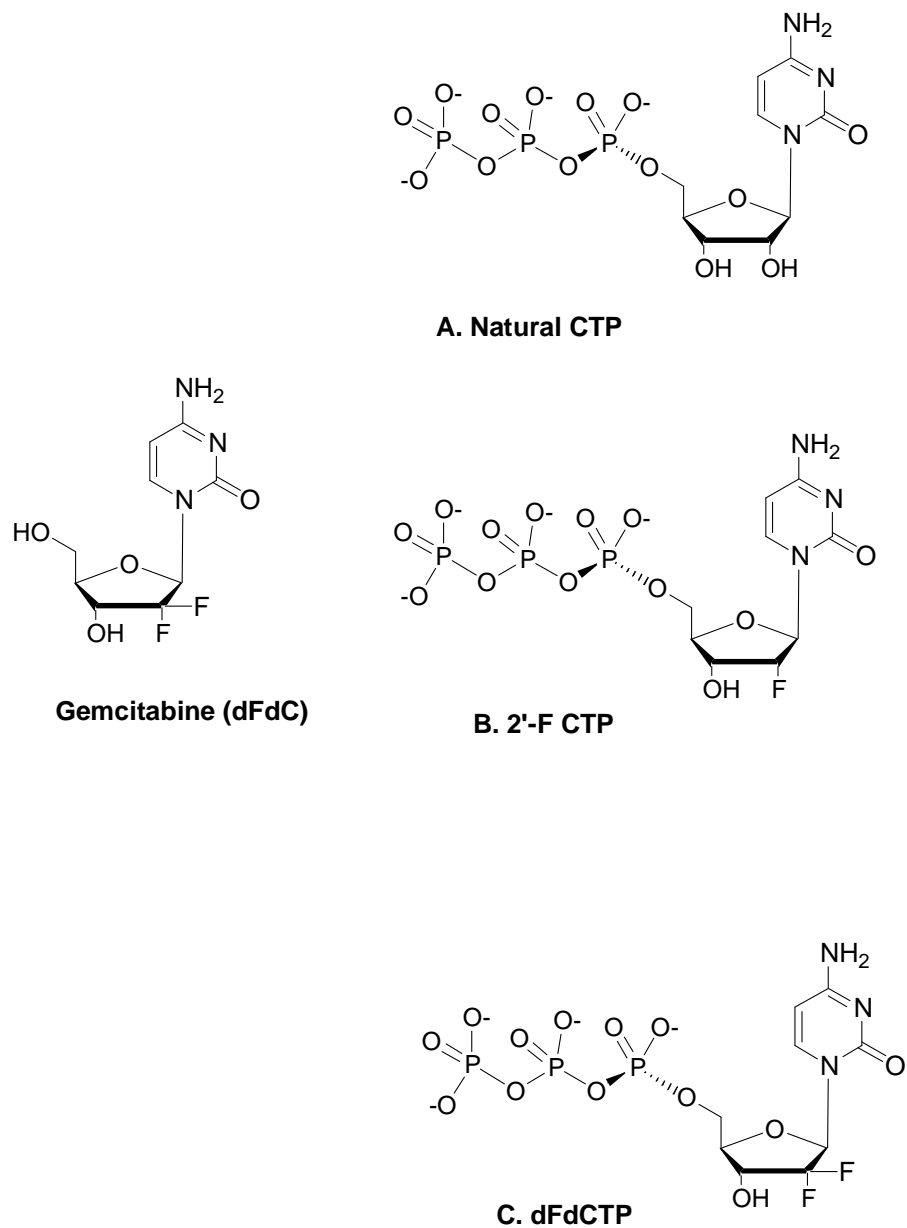


Figure 47 – Gemcitabine (dFdC) and (A) Natural CTP, (B) 2'F CTPs and (3) dFdCTP

The efficiency of gemcitabine depends on its delivery to cancer cells and its efficient endogenous enzymatic phosphorylation to its triphosphate form (dFdCTP). Using a targeted delivery molecule like an aptamer may facilitate specific delivery of

gemcitabine into cancer cells. In addition, polymerized gemcitabine molecule attached to an aptamer would allow for more molecules per cell to be delivered at one time and may lead to apoptosis of the pancreatic cancer cells more rapidly and efficiently.

We analyzed the ability of the mutant Y639F T7 RNA polymerase, which incorporates 2'F modified NTPs into RNA, to incorporate the triphosphate form of the gemcitabine (dFdC) which is termed dFdCTP. The dFdCTP was synthesized by Marcus Cheek (Duke University) and the gem-RNA wing was enzymatically synthesized by the author of this dissertation (Mariam Sharaf).

The gem-RNA wing was annealed to an epidermal growth factor (EGFR) aptamer to allow for aptamer mediated delivery of the polymerized gemcitabine (gem-RNA). The activity of this EGFR aptamer-gem-RNA was examined in pancreatic cancer *pancreas-Paca2* cells in collaboration with Dr Partha Ray and Dr Rebekah White (Duke Medical School).

Epidermal Growth Factor Receptor (EGFR) and the EGFR Targeting Aptamer

The epidermal growth factor receptor (EGFR), a 170-kd transmembrane glycoprotein (Carpenter and Cohen 1990), is a member of the type 1 receptor tyrosine kinase (TK) family (Jorissen, Walker et al. 2003). This receptor is a cell surface protein that binds to epidermal growth factor (EGF) (Jorissen, Walker et al. 2003). Binding of the protein to a ligand induces receptor dimerization and tyrosine auto-phosphorylation (**Figure 48**). The dimerization leads to the activation of a signaling cascade that leads to

cell aberrant cell growth, metastasis and angiogenesis. Mutations in this gene are associated with many types of cancers (Krohn, Wiegand et al.; Harari 2004). The EGFR receptor was shown to undergo endocytotic internalization (Sorkin 2004; Qian, Peng et al. 2008; Lee, Kim et al. 2009) and we hypothesize that this aptamer would be a useful carrier of the gem-RNA specifically to cancer cells.

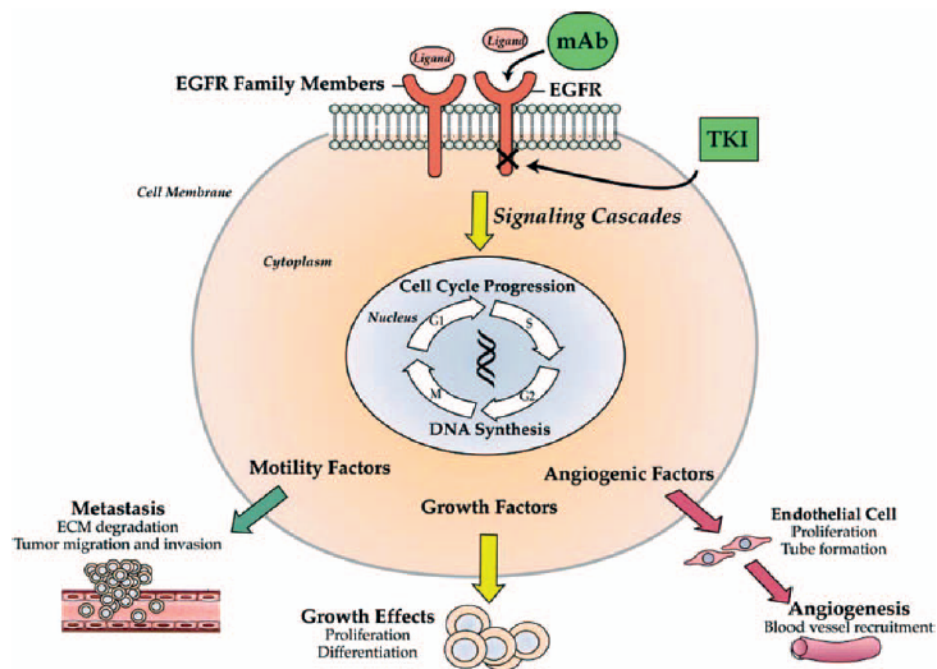


Figure 48 – Epidermal Growth Factor Receptors (EGFR) signaling pathway. EGFR pathway highlighting potential downstream effects of aberrant activity of EGFR receptors such as metastasis, cell proliferation and angiogenesis. EGFR aptamer binds with high affinity and specificity to the EGFR receptor, interrupting the activation of the signaling cascades.

The EGFR aptamer was obtained by SELEX techniques using the purified extracellular domain of human EGFR (Li, Larson et al. 2009). The aptamer was shown to bind EGFR expressing cells and was internalized (Li, Larson et al. 2009). Previous work

has shown that aptamers can deliver cytotoxic drugs specifically to tumor cells (Huang, Shangguan et al. 2009). This aptamer-drug molecules can be used to enhance the efficacy of the drugs and deliver drug specifically to cancer cells (Huang, Shangguan et al. 2009). It follows that we proposed and successfully used an EGFR aptamer to deliver the Gem-RNA wing into pancreatic cancer cells. The experiments in this Chapter were designed to confirm that the synthesis of dFdCTP was possible as wells as the synthesis of the gem-RNA using the mutant T7 RNA polymerase, finally that the gem-RNA anneals to the EGFR aptamer leading to apoptosis and cell death.

T7 RNA polymerase to polymerize the phosphorylated gemcitabine wing (gem-RNA)

T7 RNA polymerase, which was obtained from the T7 bacteriophage, catalyzes initiation and synthesis of RNA in the 5'→ 3' direction using a DNA template (Chamberlin and Ring 1972; Melton, Krieg et al. 1984). A T7 RNA polymerase expressed in *E. coli* is used *in-vitro* with a double-stranded DNA (dsDNA) template and a mixture of four NTPs(U, G, C, A) to synthesize RNA (Chamberlin and Ring 1972; Chamberlin 1974). The enzyme requires a double-stranded promoter region to initiate the synthesis (Chamberlin 1974; Milligan, Groebe et al. 1987). Synthetic DNA made on a synthesizer was shown to be a template for T7 RNA polymerase just as readily as plasmid DNA (Milligan, Groebe et al. 1987). The transcription by T7 RNA polymerase can be broken down into the following steps: initiation, elongation, and termination (Chamberlin and Ring 1972; Chamberlin 1974; Milligan, Groebe et al. 1987). The change between initiation

and elongation relies on crucial interactions between the template, triphosphates, and the enzyme. This step determines if the polymerase will continue transcription or starts the transcription again (Milligan, Groebe et al. 1987). During synthesis, specially for short RNA transcripts, shorter RNA than the desired length product are observed because the polymerase cycles between the promoter region to +1 to +8 and releases short RNAs from the initial transcription complex (ITC) (Mukherjee, Brieba et al. 2002). If the polymerase keeps going past nucleotide +9, this moves the polymerase from the promoter region to the rest of the DNA template and forms a stable elongation complex (von Hippel and Berg 1989; Mukherjee, Brieba et al. 2002). This elongation step gives the full-length product and often a longer transcript. Longer than desired length RNAs are seen because the T7 RNA polymerase may add one to three non-templated nucleotides at the end of the transcript before termination giving a range of sized RNA (Milligan, Groebe et al. 1987).

It was shown that incorporation efficiency of gemcitabine into DNA was about twice that of the incorporation into RNA *in vitro* (Ruiz van Haperen, Veerman et al. 1993). In addition, dFdC inhibits both DNA and RNA polymerase. There is a certain amount of dFdC expected in the NTP mixture from the natural degradation products of dFdCTP. Low incorporation efficiency by the polymerase due to inhibitory of properties of dFdC leads to low yields when making a gemcitabine RNA (gem-RNA). To increase the incorporation of dFdCTP and thus increase the yield of the gem-RNA, we used the

mutant polymerase that has been shown to incorporate 2'-F NTPs. The Y639F mutant T7 RNA polymerase was found to incorporate 2'-sugar modified NTPs like 2'F and 2'-NH₂ but not bulky substituent's like 2'-OMe (Padilla and Sousa 1999). This enzyme had been mutated at the tyrosine 639 and changed to phenylalanine which made the enzyme unable to discriminate between hydrogen and non-hydrogen bonding 2'- ribose substitutions on NTPs (Padilla and Sousa 2002; Sousa and Mukherjee 2003). We used this enzyme to incorporate the dFdCTP into RNA. We hypothesized that the two 2'F groups in the 2' position on the sugar of the dFdCTP are small atoms and should not increase the bulk significantly.

This polymerized gem-RNA wing was delivered into cells using an EGFR (Epidermal Growth Factor Receptor) aptamer and its activity in mia-Paca2 cells was assayed.

Materials and methods

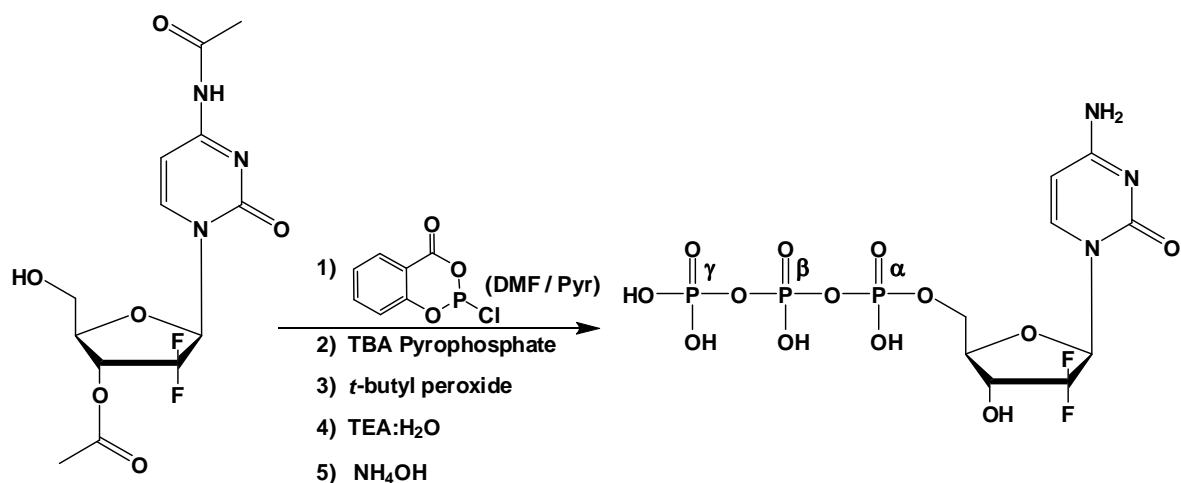
The wing sequence that attaches to the EGFR aptamer to deliver gem-RNA into cells was enzymatically synthesized with the mutant T7 RNA polymerase (Y639F) with either a 3 or 5 nucleotide links between each dFdCTP incorporation.

Synthesis of Gemcitabine Triphosphate

(Synthesis by Marcus Cheek, Chemistry Department Duke University)

Gemcitabine triphosphate was successfully synthesized using a modified one pot phosphorochloridite procedure originally developed by Ludwig and Eckstein(Ludwig

and Eckstein 2002). The Gemcitabine triphosphate was purified through ion exchange chromatography and characterized (see below) using ion-trap MS (positive/negative ion mode), ^{31}P -NMR, and ^1H -NMR. Final purification was carried out on reverse phase HPLC. For future applications, it should be straightforward to synthesize the α -*P*-borano modified Gemcitabine triphosphate by the method developed by the Shaw lab(He, Hasan et al. 1998).



Scheme 1 - Synthesis of Gemcitabine triphosphate using the corresponding nucleoside.

DNA template design and preparation

All DNA the templates were obtained from IDT (Integrated DNA technologies). The orange colored **C**'s indicate where the modified NTP would be incorporated.

5-linker short-RNA (30 mer)

5'-AAT TTA ATA CGA CTC ACT ATA GGG AGA GATGATGATGAT C GA TGA C
GATGA-3'

We designed the DNA template using the T7 promoter region (5'-AAT TTA ATA CGA CTC ACT ATA-3'), followed by spaced (either 3/4 or 5 nucleotides in between the gemcitabine). The final DNA template for synthesis and testing on cells:

5 NTP-linker between each dFdCTP (C) (52 mer):

5'-AAT TTA ATA CGA CTC ACT ATA GGG AGA GAT GAT GAT C GATGA C
GATGA C CTGGT C ATGG C GGG C ATTTAATTT -3'

3/4 NTP-linker between each dFdCTP (C) (44 mer):

5'-AAT TTA ATA CGA CTC ACT ATA GGGAGAGATGAT C GAT C
GAT C CTGGT C ATGG C GGG C ATTTAA TTT -3'

The DNA templates either a 5 NMP-linker between each dFdCTP (C) or 3/4 NMP-linker between each dFdCTP (C) (6 nmol) shown above were annealed to their complementary DNA strand at an equimolar ratio in 90 μ L of annealing buffer (10 mM Tris-HCL pH 7.5, 10 mM MgCl₂). These were heated to 95° C for 5 min and allowed to cool to rt. for 2 hours.

Gem-RNA synthesis

The gem-RNA was transcribed using 5 μ g of the dsDNA templates shown above and 2 μ L of the Durascribe T7 enzyme mix (Epicentre®) containing the mutant T7

enzyme, with either 5 mM or 2 mM NTP mix (ATP, UTP, GTP, either CTP or dFdCTP), in a 1X transcription buffer (40 mM Tris-HCL pH7.5, 6 mM MgCl₂, 5 mM NaCl and 10 mM spermidine) The reaction was incubated overnight at 37 ° C in a water bath. The DNA template was digested using RQ1 RNase-free DNase (1 U/μL) by incubation at 37° C for 15 min. The unincorporated NTPs were removed by G25 spin columns (GE Healthcare). The reaction mixture was mixed with 400 μL phenol: chloroform: isoamyl alcohol (25:24:1). The sample was vortexed 1 min, spun at 15,000 rpm for 5 min at 4° C, and the supernatant transferred to a new tube. The aqueous layer was mixed with 400 μL chloroform: isoamyl alcohol (24:1) (2X), vortexed 1 min, and spun at 15,000 rpms for 5 min in a centrifuge. The aqueous layer was transferred again to a new tube and 0.1 volume of 3M sodium acetate pH 5.2 was added and as well as 2 μL Linear acrylamide. Then, 2.5 volumes of cold ethanol (-20 ° C) was added and the sample was placed on powdered dry ice for 30 min (-78° C) until frozen. The sample was centrifuged at 15,000 rpm for 30 min at 4° C, the supernatant was removed with a pipette, and 500-1000 μL of cold 70% ethanol (-20° C) was used to wash the pellet. The sample was centrifuged again and the ethanol was removed, and the sample was air-dried at rt. for 15 min. The quality and size of the RNA was analyzed on a 7 M urea 10% (19:1) PAGE gel.

CellTiter-Glo® Luminescent Cell Viability Assay

(This assay was performed by Partha Ray at the Duke Medical School)

Briefly, pancreatic cancer Mia-Paca2 cells (3000 cells/well) were seeded on a 96 well plate, the day before the experiment. The cells were treated with EGFR aptamer-gemcitabine wing (400 nM). Gemcitabine (1000 nM) and EGFR aptamer-2'F wing (400 nM) were used as controls. 48 hrs after the application, CellTiter-Glo assay was performed to analyze cell viability. Each application was done in triplicate set.

Results and discussion

Gemcitabine (dFdC) has been shown to inhibit both DNA and RNA polymerases. We hypothesized that the phosphorylation of this nucleoside to its triphosphate form (dFdCTP), will allow it to be incorporated into an RNA strand using T7 RNA polymerase. To synthesize the gem-RNA wing, we used the Y639F T7 RNA mutant, which is able to incorporate 2'F CTP or 2'F UTP into RNA transcripts (Ruiz van Haperen, Veerman et al. 1993). We compared the incorporation of dFdCTP instead of 2'F CTP into RNA at low (2 mM) and high (5 mM) NTP concentrations.

The first sequence used to test our hypothesis was a 5-linker short-gem-RNA which did not include the EGRF binding sequence. We found that the yield was higher for the 2'FC compared to dFdCTP at either low (2 mM) or high (5 mM) NTP concentrations. At 5 mM NTP concentration the 2'FC transcription yield was 3 times higher than the dFdCTP yields, suggesting some method of enzyme inhibition at high dFdCTP concentration. However, when the NTP concentration was lowered to 2 mM the yield for the dFdCTP increased significantly in contrast with the 2'FC RNA yield,

which was the same. The length of the transcript was analyzed on a PAGE gel (**Figure 49**). Multiple bands between 20-35 DNA marker (**M, Figure 49**), were observed for both 2'FC (**Lane 1, Figure 49**) and dFdCTP (**Lane 2, Figure 49**). This is due to the known non-templated addition of NTPs by T7 RNA polymerase.

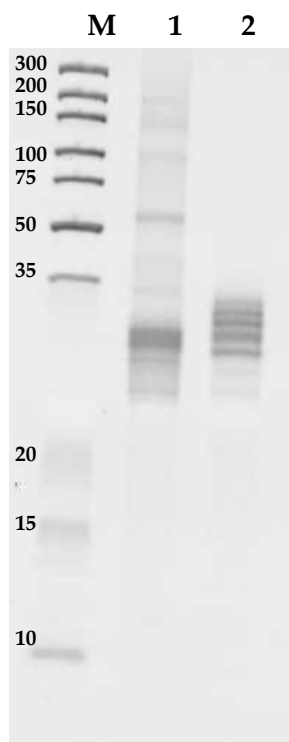


Figure 49 - Gemcitabine RNA (short-gem-RNA)

Gemcitabine RNA (short-gem-RNA) without the sequence to bind to the aptamer. Lane1. 5-linker short-gem-RNA. Lane 2. 5-linker short-2'-F RNA. Full length products were seen in lane 1 and 2, the bands migrated at the expected rate compared to the DNA markers (Between 20-35). Multiple bands were observed corresponding to non-templated addition of a few nucleotides by T7 RNA polymerase.

In addition, we tested a sequence with a reduced the number of NTPs interspaced between each dFdCTP and also added a sequence complementary to the EGFR aptamer (5'-CTG GTC ATG GCG GGC ATT TAA TTT-3') and found that reducing it from 5 to 3/4 nucleotides between each gemcitabine did not make a difference in the yield of the transcript. The length of the RNA between 5 and 3/4 nucleotide linker was 52 and 44 nucleotides in length respectively as seen during the analysis of the RNA with PAGE. In **Figure 50**, **lane 1** shows the 44-mer 3/4-linker gem-RNA and **lane 2** the control RNA a 44-mer 3/4-linker 2'-F RNA, both show full length products and migrate with the 50 mer DNA marker. There is a difference in migration between DNA and RNA but the approximate length of the RNA is seen and compared to the other sequences on the gel. **Lane 3** 52-mer 5-linker gem-RNA and **Lane 4** 52-mer 5-linker 2'-F RNA, migrate between 50 and 75 DNA marker bands. All lanes show abortive products at 35 mer DNA marker bands.

For therapeutic applications the size of the molecule is important, so the shorter one was used to determine if the gem-RNA caused apoptosis in cell based assays.

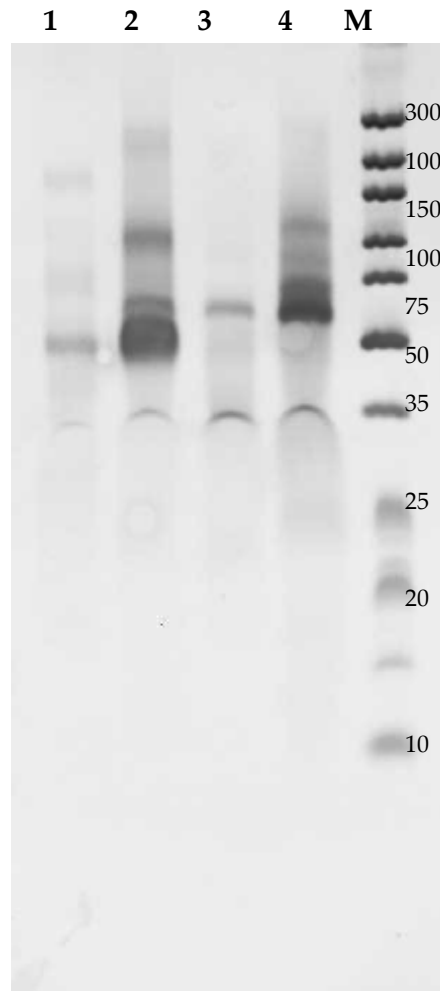


Figure 50 - Analyzing gem-RNA 5 or 3/4-nucleotide linker wings on PAGE and comparing to the control 2'-F RNA.

Lane 1. 44-mer 3-linker gem-RNA and Lane 2 44-mer 3/4-linker 2'-F RNA show full length products and migrate with the 50 mer DNA marker. Lane 3 52-mer 5-linker gem-RNA and Lane 4 52-mer 5-linker 2'-F RNA, migrate between 50 and 75 DNA marker bands. All lanes show abortive products at 35 mer DNA marker bands and extended products at 100-150 marker bands.

Mia-Paca2 cells were transfected with EGFR aptamer-gemcitabine wing (3/4-gem-RNA) (400 nM). As controls, Gemcitabine (1000 nM) and an EGFR aptamer-2'F wing (400 nM) were used. A colorimetric assay was performed to analyze cell viability (**Figure 51**). The untreated cells showed the same level of apoptosis as the 2'F winged

EGFR aptamer. There was an increase in apoptosis seen with the gem-RNA winged aptamer shown by the decrease in cell number (Figure 51).

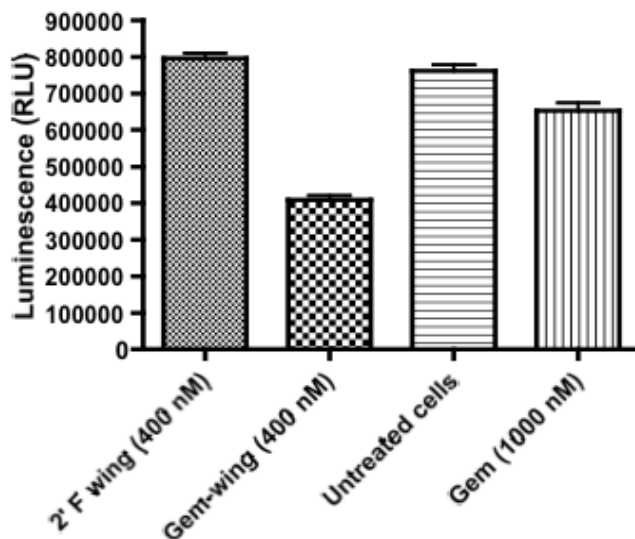


Figure 51 – Gem-RNA activity in mia-pac2 cells.

The cells were treated with EGFR aptamer-gemcitabine wing (EGFR-3/4-gem-RNA) (400 nM). Gemcitabine (1000 nM) and EGFR aptamer-2'F wing (400 nM) were used as controls. There was a significant increase in apoptosis with the 3/4-gem-RNA wing compared to the 2'FC wing control.

We propose that the delivery of gemcitabine in a polymerized form by the EGFR aptamer is advantageous, because the gemcitabine is delivered concentrated in a targeted manner to the cell surface of the receptor. It is expected that the gem-wing with the EGFR aptamer would be deposited near or at the cell surface and internalized via the EGFR receptor. We propose that the wing would be degraded into the monophosphate

molecule and phosphorylated with the cellular kinases into the active triphosphate form. There by, increasing the load concentration of the gemcitabine drug in the cancer cell increasing its efficacy. This study was conducted as a first step to the targeted delivery of cytotoxic drugs. Future studies will include in-vivo experiments, as well as imaging experiments to follow the gem-RNA wings degradation and phosphorylation to the potent compound.

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Biography

The author was born on May 26th, 1980 in Russia to an Afghani father, Nematullah Sharaf and Ecuadorian mother, Rocio Palma. She lived in Afghanistan for one year and moved to Ecuador and lived there for 10 years. In 1992 she moved to Sydney, Australia where she graduated in 1997 from Sydney High school located in Maroubra Beach. The adventure continued when the family moved to the United States where Mariam attended the University of North Carolina in Chapel Hill and graduated with a BS in chemistry in 2002.

After working in Boston in two small biotechnology companies, she returned to North Carolina to start the PhD program at Duke University. While at Duke she received numerous fellowships and awards. Fellowships received included the Kathleen Zeilik Fellowship from the Duke University Chemistry Department (Summer 2009), the Carl Storm Underrepresented Minority Fellowship (July 2009), National Institute of Health minority fellowship from the grant titled "Pharmacodynamics of Genes and Oligonucleotides" (Aug '05-May '06), and the Burroughs Wellcome Fellowship from the Duke University Chemistry Department (Jan '07-Aug '07).

Mariam Sharaf presented her research as posters and gave presentations at several domestic and international scientific conferences:

1. RNAi 2010 Gene Regulation by Small RNAs, Oxford University, Oxford UK.
(Mar '10) Presentation: Boranophosphate-modified RNA as potential anti-cancer therapeutic: Aptamers and RNA interference
2. Gordon Conference on Nucleosides, Nucleotides and Oligonucleotides, Newport RI (July '09). Poster: Utility of Boranophosphate RNA in Targeting Disease
3. Oligonucleotide Therapeutics Society, Boston MA (Oct '08). Poster presentation: Boranophosphate Modified RNA Aptamers for Use in SELEX
4. Symposium on RNA biology VII: RNA, Tool and Target, Chapel Hill NC (Oct '07). Poster: Enzymatic Synthesis of Boranophosphate RNA Aptamers for Use in SELEX

Publications

Sharaf, M., Lee, J., Cheek M, Shaw, Boranophosphate RNA does not induce the production of inflammatory cytokines TNF α or IL-6. (Manuscript in process for submission to NAR)

Sharaf, M., Fisher, M., Cheek, M., Shaw, B.R., Juliano, R., "Inhibition of MDR1 expression with boranophosphate-modified siRNAs a structure activity study" (Manuscript in progress for submission to Oligonucleotides)

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