STRUCTURAL AND CHEMICAL STUDIES OF RNA AS ANTI-CANCER TARGET AND SIGNALING MOLECULE

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RAJOSHI CHAUDHURI

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STRUCTURAL AND CHEMICAL STUDIES OF RNA AS ANTI-CANCER TARGET AND SIGNALING MOLECULE

Rajoshi Chaudhuri, Candidate for the Doctor of Philosophy degree

University of Missouri-Kansas City, 2011

ABSTRACT

Ribonucleic acids have been found to have a major role in causation and propagation of diseases. Targeting such therapeutically important RNA can be a method of treatment of various diseases. Two such therapeutically important RNA discussed in this dissertation are telomerase and extracellular microRNA.

Telomerase is a ribonucleoprotein whose role has been implicated in cancer. Telomerase catalyses telomere extension by using a portion of its RNA as a template. During this process a RNA/DNA heteroduplex is formed. We have attempted to inhibit telomerase by targeting this heteroduplex using nucleic acid binders such as intercalators. In this dissertation, we sought to understand the mechanism of telomerase inhibition by intercalators, using a structural approach. The affinity of binding of ethidium bromide to the RNA/DNA heteroduplex was examined by photocrosslinking ethidium azide to the telomerase RNA and using a primer extension assay. Ethidium azide had a greater affinity for the RNA/DNA heteroduplex compared to the telomerase RNA alone.

Designing specific inhibitors using ethidium bromide as the scaffold proved to be challenge due to the off target binding to other nucleic acids. Thus, the next part of the dissertation dealt with identifying weak inhibitors of telomerase and then increasing the

affinity for the target enzyme by developing interactions with the protein portion of the enzyme. Naproxen was used as a scaffold to introduce functionalities which would interact with the inner surfaces of the protein portion of the enzyme. Peptide libraries using naproxen as the scaffold having varying lengths and motifs were designed. The synthesized derivatives of naproxen were tested for their ability to inhibit telomerase in order to obtain a molecule which was a better inhibitor of telomerase than naproxen alone.

The final portion of the dissertation deals with identifying and targeting extracellular microRNA released from cancerous cells. MicroRNA are small (22-25 nucleotides) RNA duplex which regulate gene expression by cleaving the target mRNA or by blocking protein formation. We hypothesized that microRNA released in the extracellular fluid behave as signaling molecules. Extracellular microRNA were observed in the media of culturing carcinoma cells. Thus identifying and targeting these extracellular species can be another strategy in cancer therapy.

APPROVAL PAGE

The faculty listed below, appointed by the Dean of School of Graduate Studies have examined the dissertation titled “Structural and chemical studies of RNA as anti-cancer target and signaling molecule” presented by Rajoshi Chaudhuri, candidate for the Doctor of Philosophy degree, and certify that in their opinion it is worthy of acceptance.

Supervisory Committee

Simon H.Friedman, PhD., Committee Chair

Department of Pharmaceutical Sciences

William Gutheil, Ph.D.

Department of Pharmaceutical Sciences

Chi Lee, Ph.D.

Department of Pharmaceutical Sciences

Zhongua Peng, Ph.D.

Department of Chemistry

David Van Horn, Ph.D.

Department of Chemistry

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With love,

To my mother and father

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

In recent years, ribonucleic acids or RNA have been attractive therapeutic targets. [1,2] RNA, DNA and proteins are the three macromolecules which are essential for life. All genetic information is stored in DNA in the nuclei of cells. This information is copied into messenger RNA (mRNA) by a process called transcription. In the cytoplasm proteins are synthesized using this information and the mRNA as a template. This is considered the central dogma of genetics. [3] However the role of RNA has been found be more diverse than just being a mode of transferring information between DNA and proteins. RNA has been found to have other important roles. These include:

1. Regulatory:

Several non-coding RNA have been found to be involved in regulating gene expression. One such type of non-coding RNA are microRNAs. These are small RNA (21-25 nucleotides in length) which block gene expression by RNA by binding to a complementary sequence on its target mRNA and either cleaving it or by preventing ribosomes from binding.[4] siRNAs are another example of non-coding RNA which behave in a manner similar to microRNAs but are generally not endogenous in origin.[5 6]

2. Catalytic

RNA can behave as enzymes in catalyzing chemical reactions. An example of this is observed in splicing during mRNA transcription. Splicing is the removal of introns from exons and it occurs by the RNA portion of the intron undergoing self cleavage.[7] This type of

RNA was named ribozymes (ribonucleic acid enzyme) and some common types include hammerhead ribozymes and hairpin ribozymes.[8]

3. DNA synthesis

RNA can act as template to synthesize DNA using an enzyme called reverse transcriptase. This is most commonly observed in retroviruses which contain RNA as the genetic material.[9] Telomerase is another ribonucleoprotein which synthesizes the ends of chromosomes or telomeres, using its inherent RNA as a template. [10]

4. Genetic material:

RNA has been found to be the genetic material in many types of viruses such as Influenza virus and hepatitis C virus.[11] The RNA in this case is generally single stranded but double stranded RNA is also observed in some types of viruses. This type of double stranded RNA from virus can cause RNA interference in host cells and can sometimes trigger an interferon response.[12]

These examples indicate that due to the important roles played by RNA; any deregulation in its state can result in diseases. One such example is the presence of telomerase in most forms of cancer. This is due to the mechanistic property of telomerase to extend telomeres which increases the longevity of the cancer cells. This makes it a therapeutically important RNA and targeting it is an attractive anti-cancer therapy. We have pioneered a novel method of targeting this ribonucleoprotein by targeting the RNA/DNA heteroduplex with small molecules.[13] Using a structural approach, I have sought to understand the mechanism of inhibition of telomerase by these small molecules and design specific inhibitors.

Similarly microRNAs which play a major role in gene regulation have been found to be causative reason of many diseases. We have attempted to understand if microRNAs which have been observed to exist extracellularly behave as signaling molecules and are responsible for the proliferation of cancerous cells.

Importance of inhibiting telomerase:

Telomerase has been found to be highly expressed in cancer cells.[14] The mechanistic reason for this occurrence is the role played by telomerase in maintaining the length of telomeres. Telomeres are specialized ends of chromosomes responsible for maintaining the chromosomal integrity. These are shortened during each round of DNA replication. When the telomeres reach a critical length, the cells undergo senescence and cell death. While this is the normal occurrence in somatic cells, regenerative cells and cancer cells exhibit uncontrolled proliferation due to the maintenance of the telomeric length by telomerase.[15] Since telomerase is highly expressed in cancerous cells but is absent in normal somatic cells, it can be considered as a highly specific cancer target. Additionally 80 % of cancer types have been observed to express telomerase making it a potential universal target for cancer therapy[16].[17]

Telomeres and Hayflick limit:

Chromosomes are organized structures of DNA present in the nuclei of cells. They consist of highly coiled DNA associated with proteins. Since chromosomal DNA contains several genes and other regulatory elements, any damage to the DNA can be hazardous. Thus repair mechanisms exist within a cell to repair any breaks in the DNA, such as non-homologous end joining and base excision repair. Ends of chromosomes differ from double

stand breaks in the DNA by the presence of telomeres. Telomeres ("telos" – end; "mer" – part) are G – rich tracts located at the terminal region of chromosomes.[18,19] The telomere ends are protected by telomere binding proteins (ex. POT1, TEN1).[20,21]The presence of telomeres imparts chromosomes integrity and protects the chromosome termini from degradation or end-joining.[22-26]

However, telomeres are shortened during DNA replication due to the inherent nature of the process. DNA replication proceeds in a semi-conservative manner.[27] (Figure 1) This means that each replicated DNA consists of one original parent strand and one newly synthesized strand. DNA synthesis is performed by a family of enzymes called DNA polymerase. Deoxyribonucleotides consists of two strands, the sense strands having a 5'-3' directionality and the antisense strand with a 3'-5' directionality. DNA polymerase can synthesize the DNA strand only from the 5'-3' direction. Hence during replication the two strands of the parent DNA are unwound resulting in a replication fork. Short RNA primers act as template for the DNA synthesis. The complement of the parent strand having a 3'- 5' directionality is synthesized continuously and is termed as the leading strand. The complement of the parent strand with 5' -3' directionality is synthesized in short pieces called Okazaki fragments. The RNA primer is excised and the fragments are then joined by DNA polymerase. The destruction of the RNA primer thus results in a 3' overhang and a shortening of the telomeres. (Figure 2)

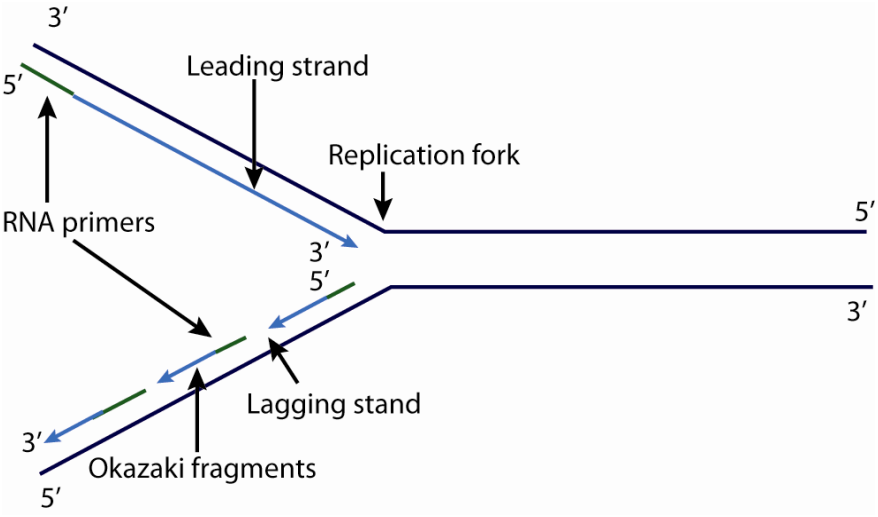


Figure 1. Semi-conservative method of DNA replication

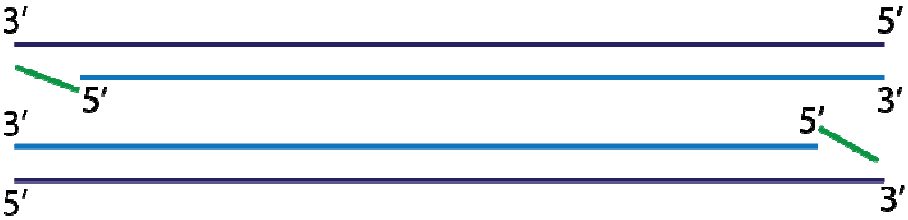


Figure 2. The end-replication problem during DNA replication

In 1965, Hayflick discovered that cells divide a definite number of times (called the Hayflick limit) before undergoing senescence and cell death. It was then discovered that there was a correlation between the number of cell divisions and the length of the telomere.[28] Telomere erosion occurs during DNA replication as described earlier. Once the telomeric length reach a critical limit the chromosome ends are recognized as double stranded DNA breaks. Thus at the Hayflick limit, the critically short telomeres cause a permanent arrest of

growth which is called replicative senescence, mortality stage 1 or M1. Cells which avoid this stage by the inactivation of checkpoint genes like p53 undergo further shortening of telomeres and then reach a second proliferative block, called mortality stage 2 or M2. This stage is generally characterized by massive cell death.[29] Cells which avoid this condition are able to do so due to the activation of an enzyme telomerase. Shortening of telomeres is not observed in the cells with activated telomerase and hence these cells attain cellular immortality.

Telomerase role in cancer cells:

As opposed to normal somatic cells, cancer cells display uninhibited proliferation. In these cells the telomeric length is not shortened and hence the cells do not undergo senescence and death. It was found that there was a correlation between the maintenance of the telomere length and an over expression of the enzyme telomerase in the cells. Telomerase was first discovered in *Tetrahymena thermophilia* in 1985 by Blackburn et al.[30] It is a ribonucleoprotein which extends the telomeres which have been shortened during DNA replication and thereby maintains the telomeric length over the critical limit. Bodnar et al. transfected two human cell lines (retinal pigment epithelial cells and foreskin fibroblasts) which do not express telomerase with vectors coding for the telomerase catalytic subunit. They observed that these cells elongated telomeres and exhibited longer viability compared to the telomerase negative controls.[31]

Telomerase structure and function:

Telomerase is essentially a ribonucleoprotein whose catalytic function depends on two components: 1. the reverse transcriptase protein (TERT) and 2. the telomerase RNA (TR

or TERC). Ligner et al. first identified the TERT region from *Euplotes aediculatus* as protein p123.[32] Although telomerase has several structural similarities to other known reverse transcriptase (RT), it has certain unique features such as possessing an intrinsic RNA and the capacity to add multiple repeats of telomeres through extension and translocation.

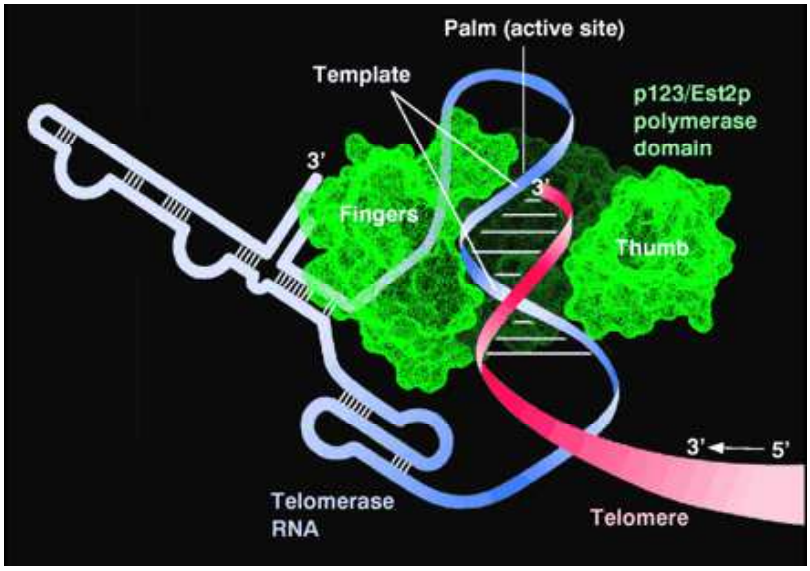


Figure 3. Schematic representation of telomerase[32]

Sequence comparison between telomerase TERT region and prototypical RTs revealed that the universally conserved RT motifs were present in the TERT.[33 34]Additionally telomerase has an approximately 400 amino acid N terminal extension and an approximately 150 -200 amino acid C-terminal extension. Thus the telomerase structure can be divided into four domains:

1. N –terminal domain which contains the moderately conserved GQ block
2. RNA binding domain containing conserved motifs CP, QFP and T
3. Reverse transcriptase domain containing the conserved RT motifs and

4. The C-terminal extension

One difference that telomerase has from other RTs is a presence of a “linker” region between the N-terminal motif and the rest of the protein. This region is very diverse and can range from 20 amino acids to 500 amino acids. This suggests a large conformation flexibility between the N terminal region and the rest of the protein.

N-terminal region:

In some organisms like *S. cerevisiae*, the N-terminal domain of telomerase is not required for activity in-vitro and the residues exhibit weak nucleic acid binding activity. However, mutation in this region in the case of human TERT can reduce or eliminate in-vitro activity.[35,36] The N-terminal domain contains the GQ motif and a region in this motif is required for telomere maintenance and viability but is not required for telomerase activity.

RNA –binding region:

The RNA binding domain is the region extending from the “linker” region present after the N-terminal domain to the conserved T region. This domain is required for binding the integral RNA present in telomerase. It has been found that mutations in this region can result in complete loss of RNA binding activity.

RT domain:

This domain consists of a mixture of α-helices and β-sheets and is organized in a manner similar to HIV reverse transcriptases containing a “finger” and “palm” region.[37] The motifs important for the RT function have been found to be conserved in TERT proteins by sequence analysis. All reverse transcriptases contain a signature motif “YXDD” which is found to be essential for enzymatic activity. The two aspartates have been found to be essential for their role in binding metal ions in the catalysis of RTs by the two- metal

mechanism. Three aspartates were identified in the A and C motifs of TERT proteins, two of which were present in a sequence analogous to the "YXDD" sequence of RTs. The sequence in hTERT required for enzymatic activity was "LVDD". This suggested that that telomerase mechanism of action employed the two metal ion mechanism of catalysis. It was subsequently found that replacing the aspartate residues with alanine resulted in a loss of telomerase activity. Drosoupoulous and co-workers further reported that substitution of the valine resulted in changes in enzymatic activity. [38] An effect on the nucleotide insertion rate and polymerase fidelity was also observed.

C-terminal domain:

The C-terminal domain has the same spatial position as the thumb domain of other reverse transcriptases. This domain is essential for activity in human TERT.[39] But in some species like yeast telomerase it has been reported that the CTE region promotes telomerase processivity and regulates telomerase localization but is not essential for its catalytic function.

Telomerase RNA:

Telomerase is distinct from other reverse transcriptase in that it contains an intrinsic RNA. In humans the telomerase RNA is 451 nucleotides in length and is transcribed by the RNA polymerase II.[40] The RNA contains a sequence which is complementary to the telomere sequence and hence acts as the template to extend the telomeres. The telomerase RNA has been found to vary in size and sequence among different species. However it was found that telomerase RNA from different ciliates possessed a conserved secondary structure. Chen et al. established an evolutionary conserved secondary structure for vertebrate telomerase RNA based on phylogenetic comparative analysis. [41]By this method, 35 vertebrate telomerase RNA

sequences were aligned and this revealed that the RNA has eight highly conserved regions named CR1 to CR8. The CR1 region contains the template sequence used to extend telomeres (5'-CUAACCC-3'). The conserved regions CR2 and CR3 are predicted to fold into a pseudoknot structure and this pseudoknot structure is present at close proximity to the template region. Basically the telomerase RNA contains four evolutionary conserved domains: 1. the pseudoknot domain, 2.the CR4-CR5 domain, 3.the Box H/ACA domain and 4. the CR7 domain.

The pseudoknot domain has an important role in telomerase function. In ciliates as well as humans it has found to be required for telomerase assembly in vivo. However it has reported that the pseudoknot is a transient structure in human telomerase and is only formed in a catalytically active telomerase.[42] The Box H/ACA domain is required for the 3' end processing as well as for the stability of the RNA.[43] There is reduction in telomerase assembly in cells upon deletion or mutation in this region. The human telomerase contains two regions which interact with the catalytic component of the hTERT. These two regions consist of the nucleotides 1-209 which contain the template region and the nucleotides 241-330 which contain the box H/ACA domain and the CR4-CR5 domain.

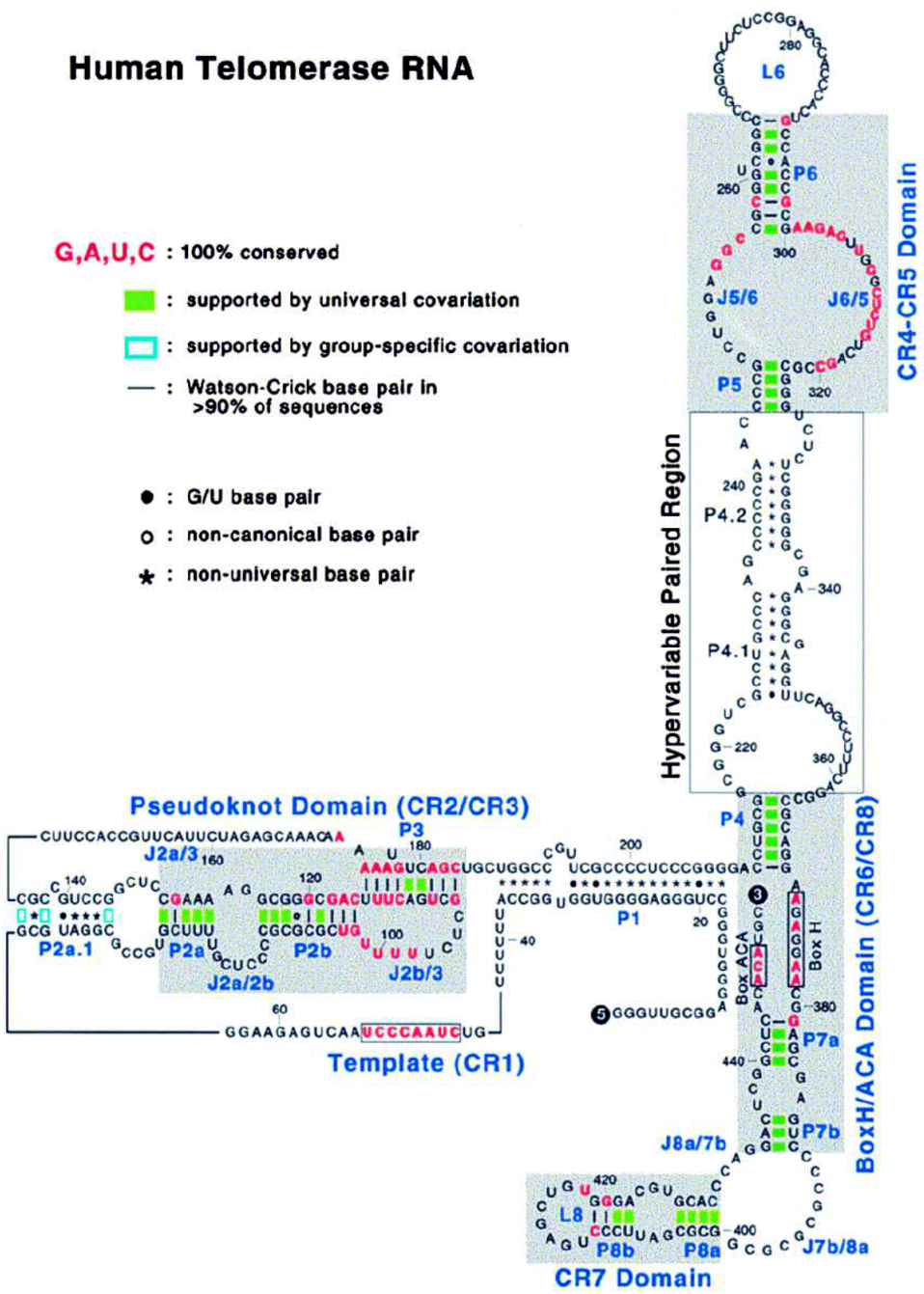


Figure 4. Schematic representation of the human telomerase RNA (hTR)[27]

Telomerase associated proteins:

Several telomerase associated proteins assist the interaction of this enzyme with telomeres. In *S. cerevisiae*, telomerase is associated with two accessory factors – Est1 and Est2 which have been shown to be important for telomere maintenance.

TERT associated proteins: In *Tetrahymena,* the proteins p80 and p95 were found to co-precipitate with telomerase. The mammalian homologue of p80 is TEP1 (telomerase associated protein 1). [44]This 2,696 amino acid long protein is found to interact with the telomerase RNA. Although TEP1 associates with both telomerase RNA and protein, its function has not yet been understood. The molecular chaperones p23 and p90 are found to be associated with telomerase and are required for the assembly of the functional telomerase. [45,46]14-3-3 proteins were also found to be associated with telomerase and these proteins play a role in signal transduction and apoptosis. The proteins act as regulators of nuclear localization of telomerase but are not required for telomerase activity.[47]

Telomerase RNA associated proteins:

In *S. cerevisiae* the telomerase RNA is found be associated with Sm proteins. [48]This is a protein which commonly binds to small nucleolar ribonucleotides (snoRNAs). Similarly in humans, proteins which commonly bind to snoRNAs have been found to be associated with telomerase RNA. Examples of such proteins include hGAR1, dyskerin, hNOP10 and hNHP2.[49] The association of these proteins with telomerase RNA suggests that these proteins are involved with the stability and localization of the hTR. Dyskerin is found to be bound to the H/ACA region of telomerase RNA and proposed to be required for ribosomal

processing.[50] Mutations in dyskerin results in a disease condition called dyskeratosis congenita[51]. This disease is also manifested due to mutations in the HTERC gene. [52,53]Other proteins found to be associated with hTR include heterogeneous nuclear ribonucleoproteins like C1, C2, A1, UP1, hStau and L22.[54,55]

Mechanism of action of telomerase:

The method by which telomerase extends telomeres was first elucidated by Blackburn et al. [56,57]Inherently telomerase behaves as a reverse transcriptase i.e. synthesizes DNA using RNA as a template. However the mechanism of action of telomerase is distinct from other reverse transcriptases because it utilizes its inherent RNA to extend the telomeres and it involves two steps: extension and translocation. In the first step telomerase binds to telomeres by means of the template region present in its intrinsic RNA. The template region has the sequence 5'-CAAUCCCAAUC- 3'. Thus it facilitates the successive addition of the telomeric sequence –TTAGGG. After this extension the enzyme translocates to the end of the telomeres and further extends it. Thus in its mechanism of action telomerase is capable of adding multiple repeats of the telomeric sequence.

Telomerase follows the two metal mechanism of of nucleotide addition as observed in all polymerases including HIV-RT.[58] The aspartate residues found near the active site bind the two magnesium ions required for the activity. It has been found that substituting the aspartate groups with alanine results in the loss of telomerase catalytic activity.[59]

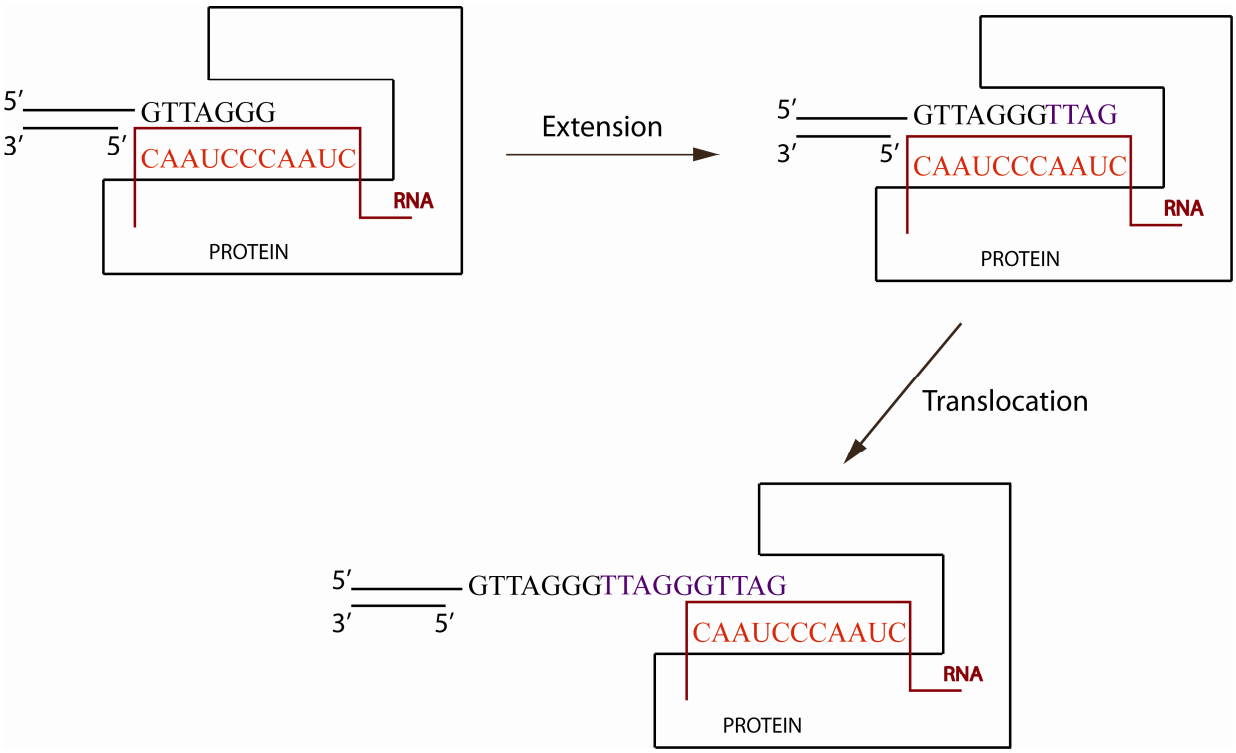


Figure 5. Schematic representation of mechanism of action of telomerase

Telomerase inhibition:

As described earlier telomerase has been implicated in a majority of cancers. Thus a number of approaches have been developed for telomerase inhibition as a strategy for cancer therapy.[60]

Telomerase inhibitors can target the

1. RNA component of telomerase:

a. Antisense oligonucleotides:

The inherent RNA of telomerase acts as a template for telomere extension. The telomere binds to a region on the telomerase RNA which has a sequence complementary to the telomere sequence. Thus anti-sense oligonucleotides which possess the telomere sequence can bind to the template region of the telomerase RNA and block telomerase activity. Telomerase activity can be blocked in a passive manner where the anti-sense oligonucleotides hinder the telomere binding to the telomerase RNA. The active method of blocking telomerase activity employs antisense DNA oligonucleotides and the RNase H enzyme.[61] The antisense DNA binds to the telomerase RNA and recruits RNase H to degrade the RNA and effectively block telomerase activity. A common problem associated with antisense oligonucleotides is stability as DNA oligonucleotides are highly labile. Hence antisense oligonucleotides with modified backbones have been developed to render higher stability to the molecules. The only caveat in using the modified antisense is that blocking of telomerase activity occurs only by the passive mechanism since RNase H is unable to cleave modified nucleic acids. Substantial research in this area has been performed at Geron corporation. They designed 2' deoxy and 2' substituted oligonucleotides targeting the 11 mer template region which were found to be effective as telomerase inhibitors. One such substituted antisense oligonucleotide, a 13 mer N3-P5 thio phosphoramidate – GRN163 was found to inhibit telomerase with an IC_{50} of 1 nM in tumor cell lines.[62-65]

b. Hammerhead ribozymes:

Hammerhead ribozymes are RNA molecules that possess ribonuclease activity. Ribozymes can be designed against the telomerase RNA active site which can block telomerase activity. These molecules possess the inherent problem of RNA stability and using

modified nucleotides can affect the ribonuclease activity. Additionally it has been observed that after over 20 passages after treatment with ribozymes the cells did not show a reduction in telomere length and exhibited uninhibited proliferation.[66,67]

c. Preventing the assembly of the telomerase RNA subunit with the protein subunit:

Keppler et al. identified two regions on telomerase which were required for the assembly of the telomerase holoenzyme. They designed oligonucleotides targeting different regions on telomerase and identified two oligonucleotides which inhibited telomerase activity by preventing the assembly of the telomerase holoenzyme. Oligonucleotides which bound to the P3/P1 pairing region or the CR 4 – 5 domain blocked telomerase activity if added prior to telomerase assembly. The P3/P1 region is located on the pseudoknot region which as described earlier is required for telomerase activity. However the addition of the oligonucleotides has to be done before the assembly of the telomerase holoenzymes otherwise this method of telomerase inhibition is ineffective.[68]

2. The telomerase protein sub-unit (TERT):

a. Catalytically inactive TERT subunit:

Mutants of the protein subunits of telomerase when introduced into cancer cells are able to sequester the telomerase RNA but are unable to extend the telomere. Hahn et al. designed a catalytically inactive telomerase protein subunit which had alanine and isoleucine instead of aspartic acid and valine at the active site.[69] Cells infected with the mutant form did not exhibit telomerase activity and had shortened telomeres.

b. Knockdown of the mRNA coding for TERT:

Dong et al. designed a siRNA targeting the mRNA coding for the protein portion of telomerase. This siRNA was administered to breast cancer cell lines, MCF-7 and MDA-MB-453 both singly and in conjunction with the anti-cancer drug Doxorubicin. The hTERT mRNA was reduced to about 40% in the cells treated with the siRNA and a decrease in the hTERT protein level was also observed. This reduction in the telomerase level was found to correlate with decreased number of viable cells. siRNA and doxorubicin when administered together caused a greater decrease in the number of viable cells.[70]

Reverse transcriptase inhibitors:

Reverse transcriptase inhibitors (RTI) are commonly used in the treatment of HIV.[71] They can be of three different types:

Nucleoside analog reverse-transcriptase inhibitors (NARTIs or NRTIs)

Nucleotide analog reverse-transcriptase inhibitors (NtARTIs or NtRTIs)

Non-nucleoside reverse-transcriptase inhibitors (NNRTIs)

The nucleoside and nucleotide reverse transcriptase inhibitors can compete with dNTPs as the substrate for incorporation into the viral DNA by HIV reverse transcriptase and inhibit the extension of the DNA. In a similar manner they can be incorporated into the telomere by telomerase and inhibit the enzyme's activity. 3'-azido-3'-deoxythymidine (AZT) and 6-thio 7-deaza -2'-deoxyguanosine 5' triphosphate (TDG-TP) are some examples of RTIs which have been tested against telomerase and have demonstrated the ability to inhibit telomerase.[72-74]

G-quartets stabilization:

Telomeres are G-rich sequences that have the capacity to form tetra-stranded structures by Hoogsteen base pairing called G-quartets. G-quartets hinder the function of telomerase. Hence molecules which are capable of stabilizing these quartets behave as inhibitors of telomerase.[75,76] Some compounds that have been tested against telomerase and have been found to be effective include porphyrin derivatives, acridine derivatives and 2, 6 diamidoanthraquinones. However molecules which target the G quartet are said to lack specificity for cancer cells due to the possibility of the presence of this structure even in normal cells.[77,78]

Assays used to determine telomerase activity:

In order to observe telomerase activity in-vitro and the subsequent loss of activity in presence of inhibitors several assays have been developed. Some of these assays include

1. Nuclease protection assays
2. RT-PCR
3. *in situ* hybridization
4. Direct method using radioactivity
5. TRAP-based assays
6. PCR-based fluorescent assays

In our laboratory two of the above mentioned assays were used extensively. The first one is the direct assay using radioactivity. This assay was developed by Morin et al. and involved the incorporation of a ^{32}P radiolabeled nucleotide into a primer by telomerase.[79] A

primer having the telomeric sequence (TTAGGG) was added to a cell lysate having telomerase activity along with dTTP, dATP and ∝-^{32}P dGTP. The products are isolated and visualized with radioimagery. This is considered a direct method as the products of the telomerase extension are radiolabeled and can be directly visualized. Further steps to increase the product signal are not used.

Sun et al. developed a modified version of the direct assay using a biotinylated telomere sequence primer. The advantage of using a biotinylated substrate was that the subsequent radiolabeled extended product could easily be isolated using streptavidin coated beads.[80]

The problems encountered with the direct assay include firstly the weak signal strength of the product. Multiple days of exposure of the gel is required to obtain a discernable signal. Secondly since gel electrophoresis is used to analyze the product, there is a limitation to the number of points that can be used. High throughput screening of inhibitors could not be performed using this assay. Finally this assay had all the hazards associated with working with a radioisotope.

Kim et al. developed a PCR based assay called the telomeric repeat amplification protocol (TRAP) assay to overcome the problem of the weak radioactive product signal. In this assay a non-telomeric oligo (TS oligo: 5'-AATCCGTCGAGCAGAGTT-3') was used as the telomerase substrate. [17]Telomerase added telomeric repeats to this oligonucleotide and the resulting products were amplified using a downstream primer and the TS oligonucleotide. A radionucleotide was incorporated during the PCR amplification and the resulting products were visualized by resolution using gel electrophoresis. This assay had certain problems associated with it such as, inhibitors of telomerase screened using this assay caused the

inhibition of the *Taq* polymerase used for PCR amplification and the hazards of using radioisotopes.

Dr. Rawle Francis, a graduate student from our laboratory, developed a variation of the TRAP assay which addressed the concerns associated with this assay. [81]A biotinylated telomeric substrate oligo was used as the primer which was extended by telomerase.

The products were then isolated using streptavidin coated PCR tubes. This allowed the removal of any inhibitors screened against telomerase and the subsequent washing to ensure non-interference of the inhibitors with the PCR amplification. The bound products were then amplified using the telomerase substrate (TS) oligo and a downstream primer and the amplified products were quantitated using a fluorescent dye which predominantly bound to double stranded oligonucleotides. Thus this assay was non-radioactive and hence could allow the high throughput screening of multiple inhibitors of telomerase.

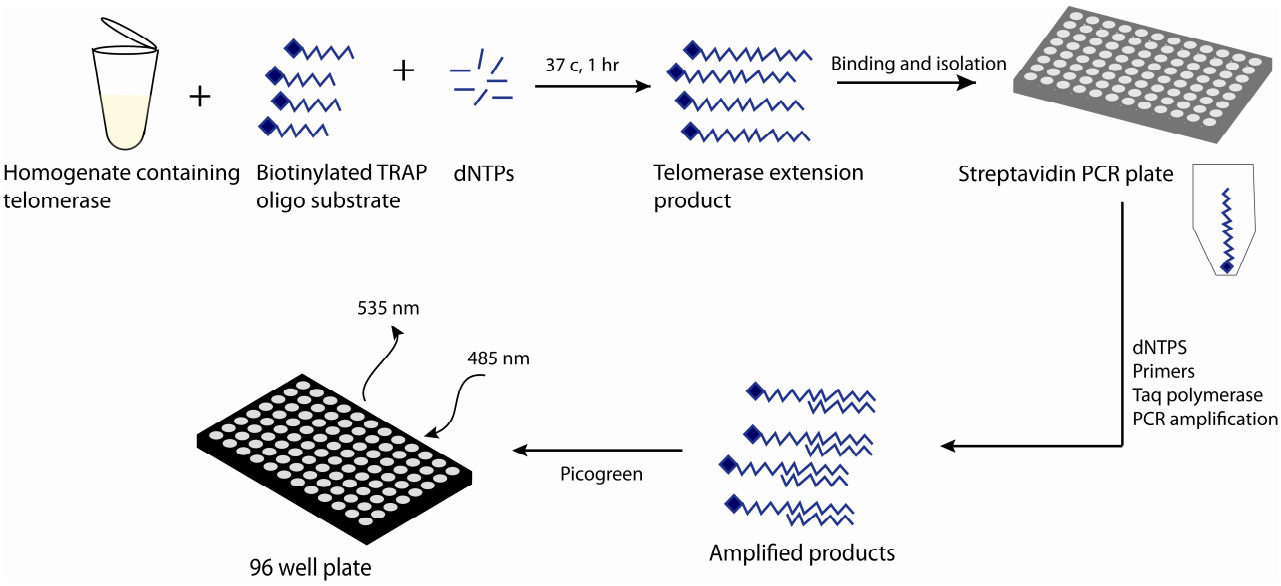


Figure 6. Schematic representation of the PCR-based assay of telomerase

Inhibition of telomerase by targeting the RNA/DNA heteroduplex:

Telomerase uses its inherent RNA to extend the telomeres. Hence the formation of an RNA/DNA heteroduplex is a key event in the mechanism of action of telomerase. Our laboratory has pioneered a method to inhibit telomerase by targeting this heteroduplex using small molecules. There are two potential ways by which an intercalator can inhibit telomerase:

1. Extension inhibition: The intercalator binds to the RNA/DNA heteroduplex and distorts it. Thereby it prevents the addition of the nucleotide to the telomere and stops extension.

2. Translocation inhibition: The intercalator binds to the RNA/DNA heteroduplex and stabilizes it. Thus prevents the detachment of the telomere from the telomerase RNA and thereby prevents translocation. A schematic representation of this idea is provided in figure 7.

The advantages of this approach are manifold:

1. The formation of the RNA/DNA heteroduplex is necessary for telomerase activity. Thus by targeting this heteroduplex it is possible to disrupt either the extension or the translocation step in the telomerase mechanism of action.

2. RNA/DNA heteroduplex are not commonly encountered in cells, making the telomerase RNA/DNA heteroduplex a rare target for cancer therapy

3. Another unique aspect associated with the telomerase RNA is the telomerase protein portion (TERT). Thus the molecules which target the heteroduplex can be used as platform to attach functionalities which can interact with the protein region. This can further enhance the specificity and the affinity of the inhibitor for telomerase.

4. Since the polymerization of the DNA strand occurs after the formation of the RNA/DNA heteroduplex, it can be inferred that the active site residues of telomerase are present at close proximity to the heteroduplex. Hence functionalities can be attached to the scaffold that interact with these residues and potentially inhibit telomerase more effectively.

5. Small molecules do not encounter the problems with stability which are associated with nucleic acid based therapies. Additionally this approach will have greater specificity towards telomerase compared to reverse transcriptase inhibitors or other generic telomerase inhibitors.

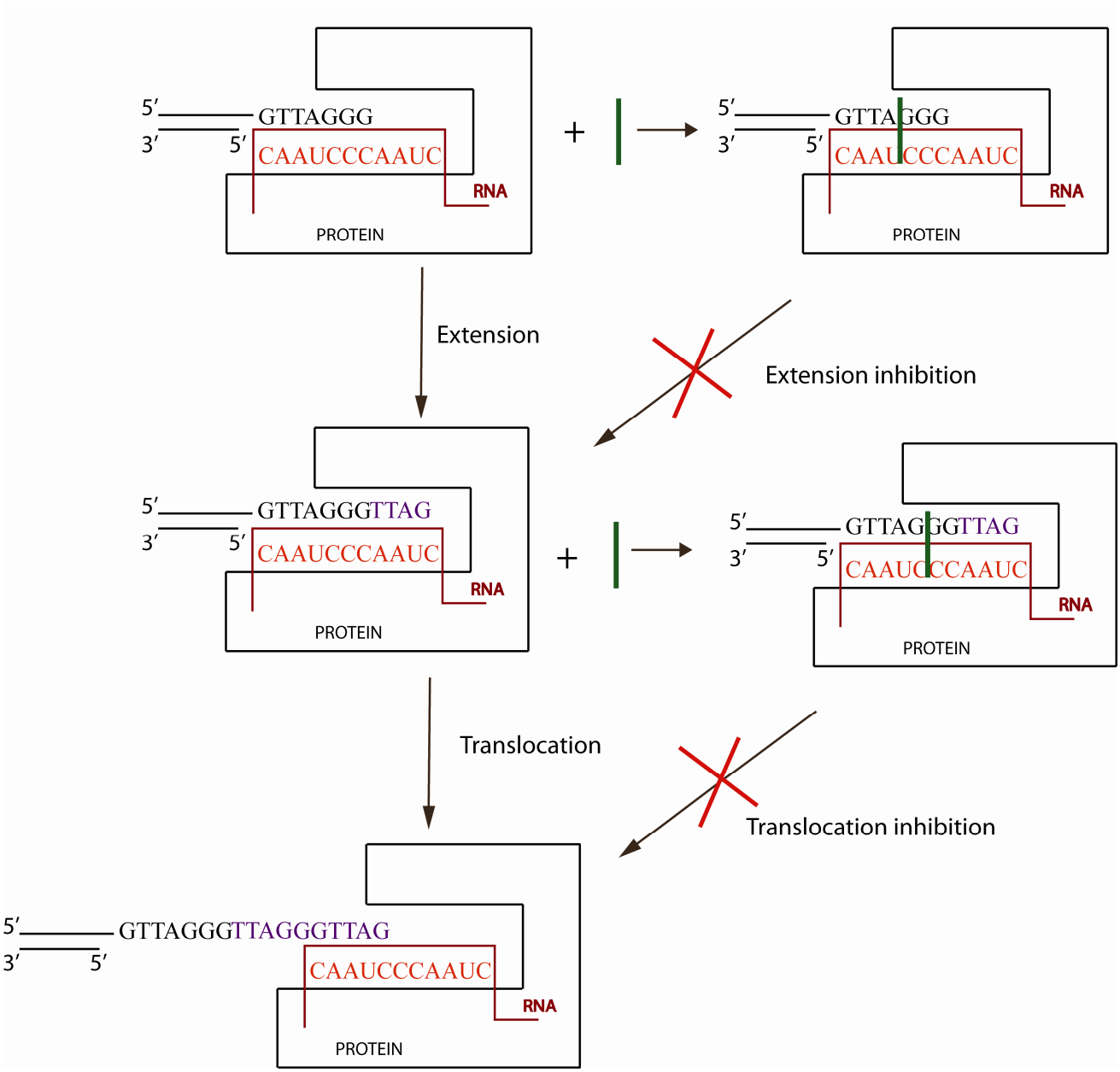


Figure 7. Schematic representation of telomerase inhibition by intercalators

Strategy to target the RNA/DNA heteroduplex:

The RNA/DNA heteroduplex could be targeted with known nucleic acid binders such as intercalators. Some intercalators which are known DNA binders are ethidium bromide, propidium iodide etc. Intercalators are characterized by a planar core which can stack between the base pairs of duplexes.[82] Although intercalators are generic nucleic acid binders, specificity and affinity for telomerase could be obtained by introducing functionalities on the intercalators which could develop interactions with the TERT region.

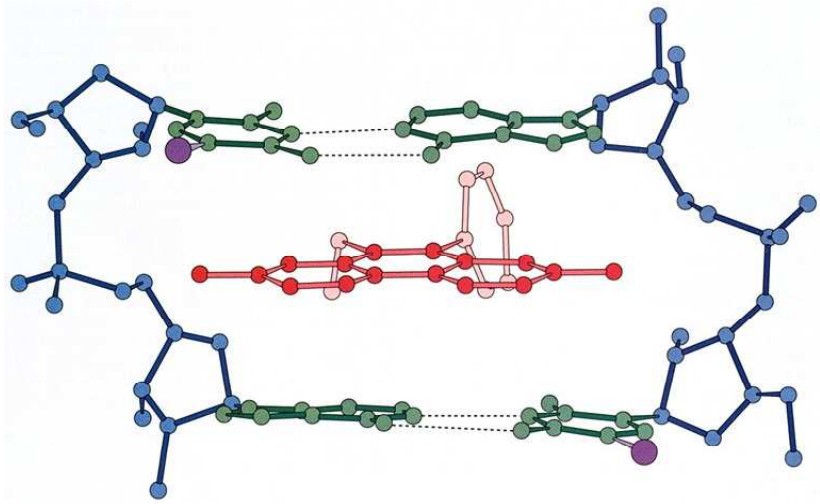


Figure 8. Ethidium bromide intercalating between base pairs[82]

Dr. Francis, a former graduate student from the Friedman laboratory screened commercially available intercalators for their ability to inhibit telomerase. The results of the screening experiment at provided in table 1. The results indicate that ethidium bromide and rivanol effectively inhibit telomerase with IC_{50} in the low micromolar range. [81,83]

There exists literature precedence that ethidium bromide preferentially binds to a RNA/ DNA heteroduplex. Ren et al. have demonstrated using a competition dialysis assay that ethidium bromide has a preference for a RNA/DNA heteroduplex over 12 other nucleic acid structures.[84] Similarly Corrina West, a former graduate student of the laboratory, demonstrated that from a mixture of nucleic acid binding molecules, ethidium bromide had the strongest affinity for a RNA/DNA heteroduplex bearing the telomerase sequence.[85] She added a mixture of intercalators to a preformed heteroduplex, allowed the molecules to bind and then washed the unbound molecules. The heteroduplex was then heated to separate the strands and the molecule which bound with the greatest affinity was identified to be ethidium bromide. Additionally it was proved in our laboratory that the inhibition of telomerase was due to binding to the RNA/DNA heteroduplex and not the G-quadruplex formed by the telomeric sequence. This was proved by using a primer which was $(TTAGGG)_2$ versus $(TTAGGG)_3$ for the telomerase assay. The primer which had only two repeats of the telomeric sequence was too short to form a G-quadruplex and yet ethidium bromide exhibited inhibition of telomerase. These different studies support the hypothesis that telomerase inhibition by ethidium bromide is due to the targeting of the RNA/DNA heteroduplex.

Table 1. Ranking of compounds inhibiting telomerase

Rank by IC_{50}	Compound name	IC_{50} (μM)
1	Ethidium Bromide	3.7
2	Rivanol	8.2
3	Acridine Orange	12.2
4	Acridine Yellow	21.7
5	Ethidium carboxylic acid	22
6	9-Acridine carboxylic acid	44
7	ACMA	52
8	Actinomycin D	99
9	Daunorubicin	112
10	2-Anthracene carboxylic acid	118
11	7-amino Actinomycin D	166

CHAPTER 2

DETERMING THE MECHANISM OF INHIBITION OF TELOMERASE BY ETHIDIUM BROMIDE

Although literature precedence strongly indicates that ethidium bromide preferentially binds to a RNA/DNA heteroduplex, we were interested in obtaining further support to the hypothesis that telomerase inhibition is due to the targeting of the heteroduplex formed during the enzyme's mechanism of action. The experiment performed by Corrina West established that ethidium bromide binds preferentially to a pre-formed heteroduplex. However the telomerase's mechanism of action is a dynamic process which involves multiple extension and translocation steps. In an in-vitro assay Blackburn et al. demonstrated that the enzyme dissociates from the primer with the telomeric sequence during the course of primer elongation. [56]Additionally it was demonstrated that RNase H does not cleave the telomerase RNA indicating that this RNA/DNA heteroduplex is not very stable. [86] If the RNA/DNA heteroduplex formed during telomere extension was transient, the possibility existed that ethidium bromide could bind to the RNA strand of the enzyme or the DNA strand and thus caused telomerase inhibition. If this was the case the applicability of the intercalator as scaffold in designing a specific inhibitor of telomerase is reduced.

Ethidium azide as photocrosslinker:

We tried to answer this question by probing the binding site of the intercalators using a structural approach. We accomplished this by using a version of the intercalator which could

be tethered to nucleic acids using light. The telomerase RNA was then probed using a primer extension assay to identify the region of intercalator binding. A primer extension assay employs a reverse transcriptase to extend a primer complementary to the RNA. The reverse transcriptase's activity is hindered by anything which is bound to the RNA, which in this experiment is the ethidium azide. The result is shorter extended products compared to the full length cDNA formed in the absence of ethidium azide. The products could then be separated using gel electrophoresis which indicated the position of the bound intercalator. Dideoxysequencing was performed on the RNA to identify the location of binding of ethidium azide.

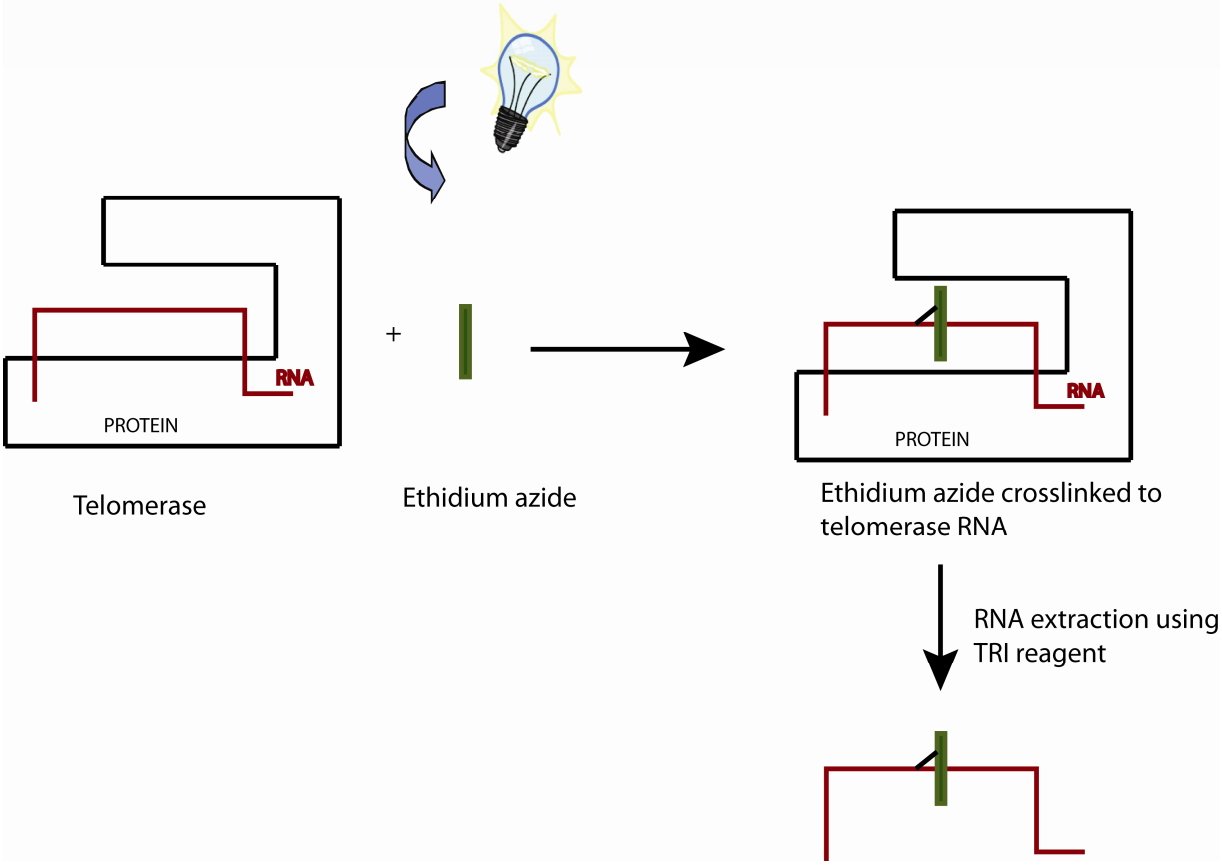


Figure 9. Schematic representation of Photocrosslinking of telomerase RNA with ethidium azide

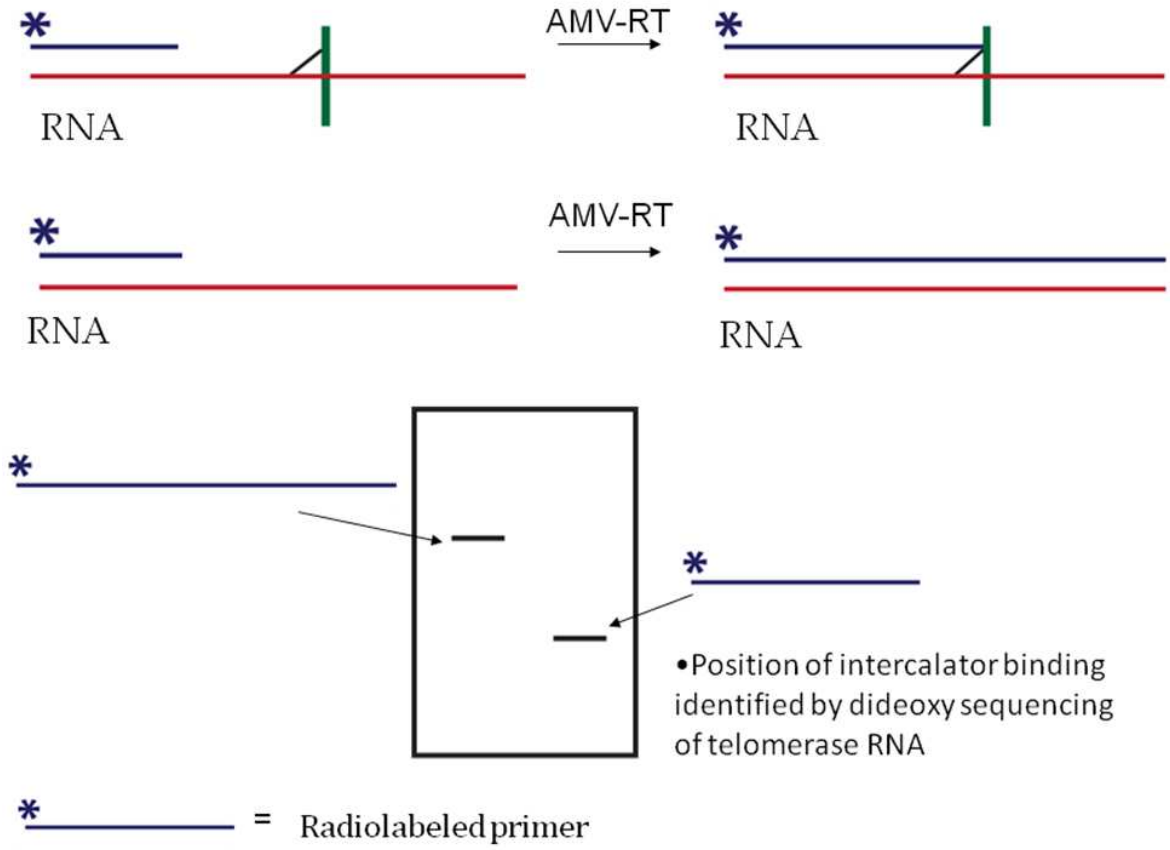


Figure 10. Schematic representation of identification of position of photocrosslinking

We used ethidium azide, a structurally similar analogue of ethidium bromide (figure 11) to photocrosslink to the telomerase RNA both in the absence and presence of the DNA substrate. In order to differentiate the affinity of the intercalator for the RNA and the RNA/DNA heteroduplex the crosslinking was performed both in the absence and presence of the DNA substrate. As described earlier the human telomerase RNA is 451 nucleotides in length and is divided into 8 regions name CR1-CR8 region. The template region is present in the CR1 region and hence we concentrated our studies on the CR1 region which is around 75 nucleotides in length.

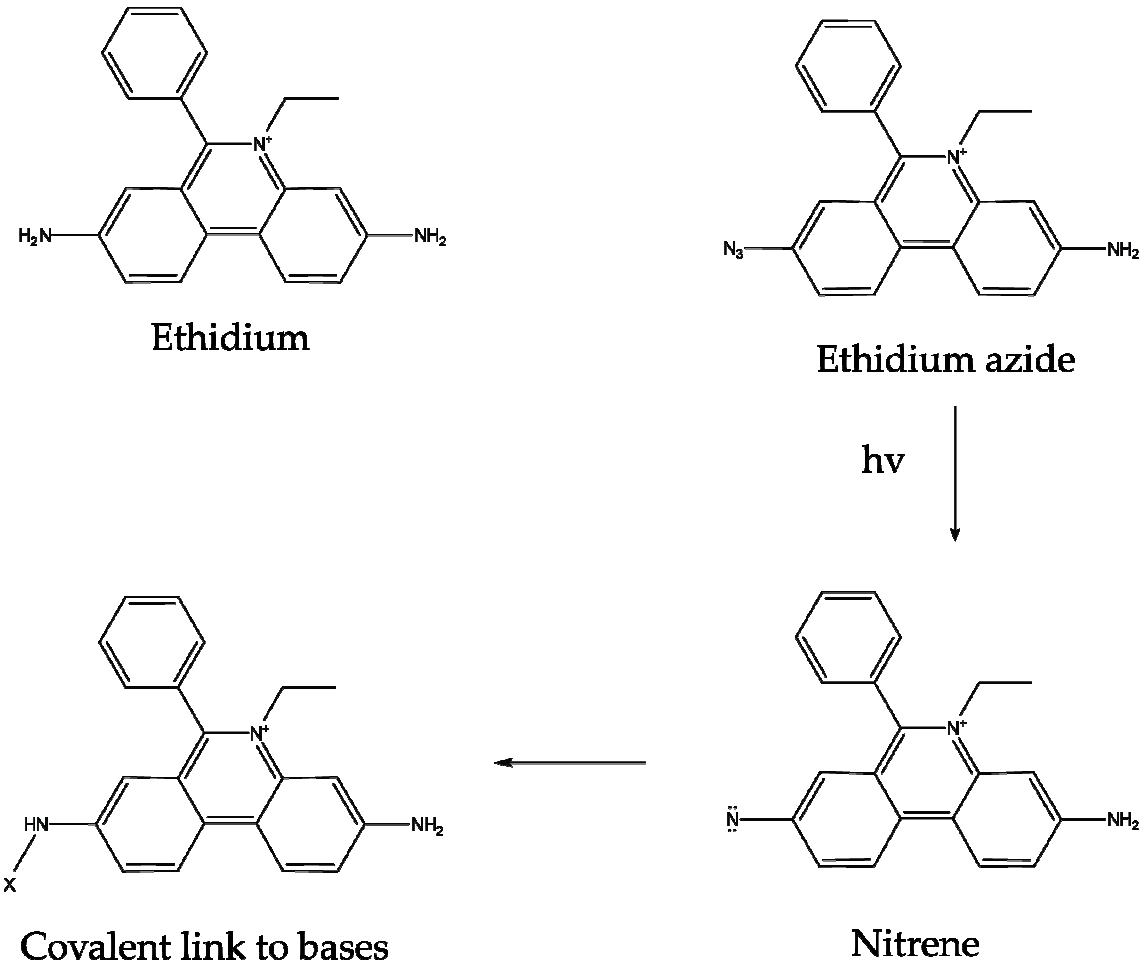


Figure 11. Mechanism of photocrosslinking of ethidium azide to nucleobases

Experimental procedure:

Preparation of HeLa cell homogenate:

Homogenate containing telomerase activity was prepared using the method as described by Kim et al.[17] HeLa S3 cells, obtained from the National Cell Culture Center and grown to an average density of 0.5 x 10^6 cells/mL, were suspended in cold washing buffer (10

mM HEPES-KOH, pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT) and pelleted at 10,000 x g for 1 minute at 4 ^oC. The pellet was resuspended in cold lysis buffer (10 mM tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM PMSF, 5 mM BME, 1 mM DTT, 0.5% CHAPS, 10% glycerol) and lysed for 60 minutes on ice. The suspension was then centrifuged at 100,000 x g for 1 hour at 4 ^oC and the supernatant containing the telomerase activity was removed, adjusted to 20% glycerol, aliquoted and stored at –80 ^oC.

Photocrosslinking of ethidium azide to telomerase RNA:

Ethidium azide at concentrations ranging from 1.25 µM to 80 µM was added to 200 µl of HeLa cell extract in a final volume of 300 µl. One assay point did not contain any inhibitor solution for the 0 µM concentration. The mixture was allowed to incubate for 30 minutes at 37°C and then radiated with F15 T8-D light bulbs for 30 min. This experiment was performed in the absence and presence of 20 µM of DNA substrate

Extraction of RNA from photocrosslinking experiments:

The RNA from the assay points was extracted using TRI reagent as per the supplier's protocol. Briefly, 500 µl of TRI reagent was added to each point and allowed to stand for 5 minutes at room temperature. 200 µl of chloroform was then added to each point and the samples were shaken vigorously and then allowed to stand for 15 minutes at room temperature. Phase separation was effected by centrifuging the samples at 12,200 g for 15 minutes at 4°C. The aqueous layer was removed and the RNA was pelleted using 850 µl of isopropanol. The samples were chilled at -20°C for 2 hours and the RNA was precipitated by centrifugation at 12,000 g for 15 minutes at 4°C. The pellet was washed twice with 75 % ethanol, allowed to dry and then taken up in RNase free water.

Primer extension:

Primer extension on the CR1 region of telomerase RNA was done using 1 nM P^{32} labeled primer(5'-CGGCGCCTACGCCCTTC-3') (Integrated DNA Technologies), 0.1 pmol of extracted RNA, 2 µl 5x hybridization buffer (50 mM tris-HCl, 60 mM NaCl, 10 mM DTT, pH 8.3) in a final volume of 10 µl. The primer was annealed to the RNA by heating at 85°C and then allowing cooling to room temperature. To 2 µl of the annealing mixture, 500 µM of dNTPs and 1 µl of AMV RT mix (AMV RT, 5x extension buffer (50 mM tris-HCl, 60 mM NaCl, 10 mM DTT, 60 mM $MgCl_2$, 50 mM magnesium acetate pH 8.3) were added in a final volume of 5 µl and the mixture was incubated for 30 minutes at 55°C. The reaction was terminated by heating at 85°C for 10 minutes. 5 µl of loading buffer was added to the samples and electrophoresis was done on a 10 % denaturing gel at 2000 V for 90 minutes.

Dideoxy sequencing for telomerase RNA:

2 µl of the annealing mixture, 500 µM of dNTPs and 1 µl of AMV RT mix (AMV RT, 5 x extension buffers, 50 mM magnesium acetate) were taken in four tubes. To each microcentrifuge tube 2 mM of each ddNTP was added for a final volume of 5 µl. Primer extension and gel electrophoresis was performed as described earlier.

Study of intercalator binding to telomerase RNA in the absence of DNA substrate:

We first wanted to learn if ethidium bromide bound to the telomerase RNA in the absence of the substrate. Varying concentrations of ethidium azide was added to HeLa cell lysate containing telomerase in the absence of the DNA substrate. In order to tether the ethidium azide to the telomerase RNA the mixture was irradiated for 30 min. Irradiation of ethidium azide results in the formation of a photoreactive nitrene which can form a covalent bond with bases present in its proximity. After the irradiation, RNA was extracted from the

mixture by a phenol-chloroform method. In order to specifically perform primer extension on the telomerase RNA we used a primer previously used by Antal et al.[87] The primer was 18 nucleotides in length and had a sequence which was complementary to the 3'end of the telomerase RNA (region containing the CR1 region).

After annealing, the primer was extended using AMV-RT and dNTPs. The dideoxy sequencing was performed in a similar manner with the exception that the primer extension mix included dideoxynucleotide along with deoxynucleotides. The dideoxy nucleotide lacks a hydroxyl group at the 2' position. Thus when a dideoxynucleotide gets incorporated into the primer during the primer extension AMV-RT is unable to add the next nucleotide and the chain terminates. When a particular dideoxynucleotide e.g. ddGTP is added to a primer extension reaction it is incorporated at the positions where there is a cytosine in the RNA. The chain does not extend further and the products can be visualized using gel electrophoresis. The bands obtained in the lane where the reaction mixture contained the ddGTP indicates the positions of the cytosine in the telomerase RNA. Thus the position of ethidium bromide bound on the RNA can be determined by matching the band formed due to the binding of ethidium azide to the respective band on the lanes containing dideoxynucleotides in the reaction mix.

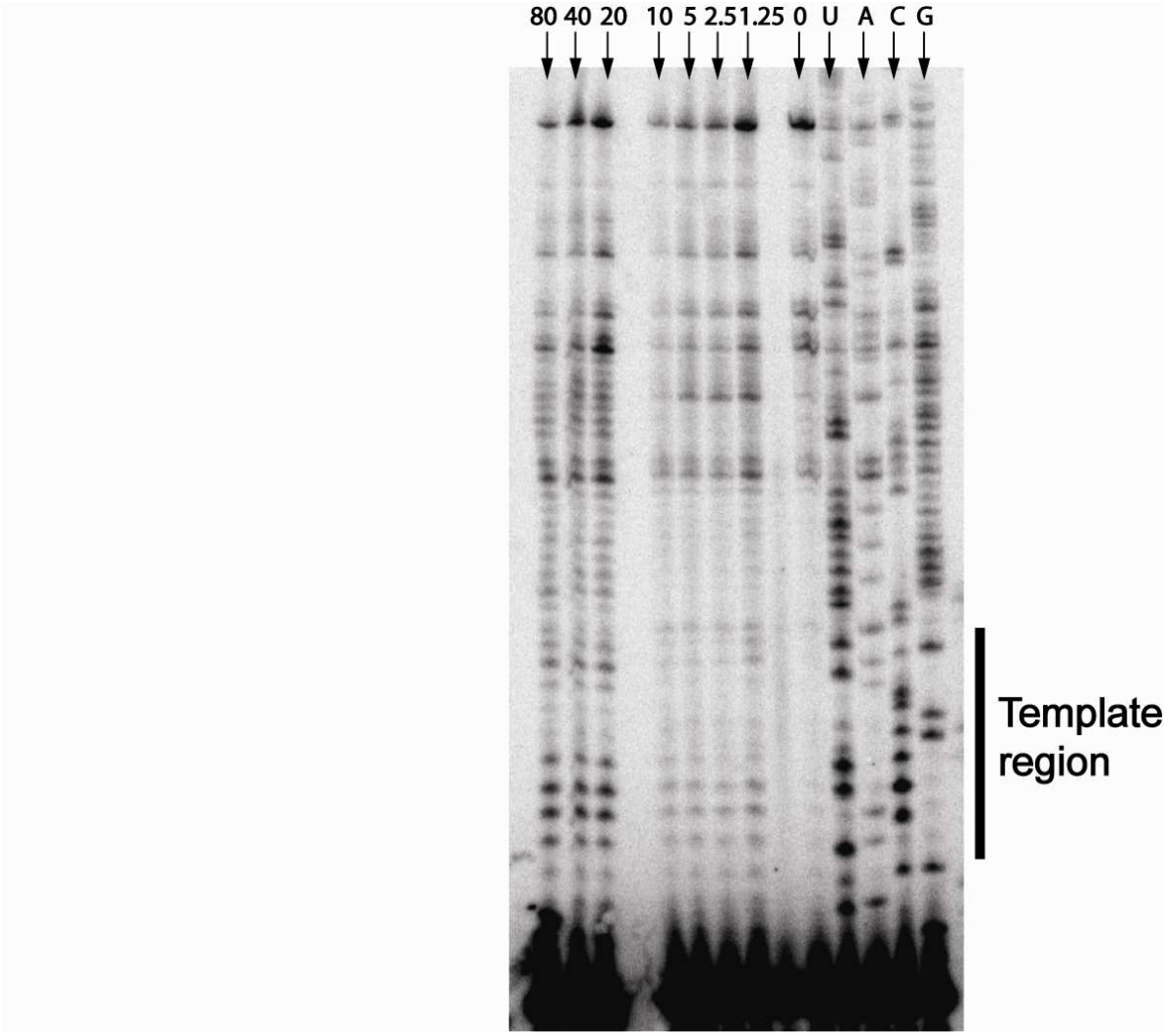


Figure 12. Gel depicting the photocrosslinking of ethidium azide to telomerase RNA in absence of DNA substrate

In the gel image (figure 12) we observed numerous bands in the lanes where the telomerase RNA was treated with ethidium azide. This indicated that the ethidium azide had bound indiscriminately throughout the length of the RNA resulting in “stop sites” for the AMV-RT. In other words the “stop sites” were regions where the AMV-RT could not extend

the primer due to the presence of the bound ethidium azide. This initial result indicated that the intercalator binds to telomerase RNA in the absence of the DNA substrate.

One explanation for this observation is that although the affinity of ethidium bromide for duplexes is well known, the intercalator has been reported to bind to single stranded nucleic acids. Rosu et al., while studying ethidium bromide's ability to bind to G-quadruplexes reported that ethidium bromide also bound to single strands of nucleic acids albeit weakly.[88] Additionally as described earlier the telomerase RNA folds into a secondary structure which forms duplexes and this world allow additional binding of the intercalator.

One interesting observation from the gel image in figure 12 is that the bands at the template region appear to be darker than the remaining portions of the lane. This suggests that although it appears that ethidium azide binds indiscriminately to the CR1 region of the telomerase RNA; its affinity for the template region appears to be greater. Forstemann et al. reported a similar observation, upon probing the template region of the telomerase RNA using DMS.[89] This could be due to the fact that the template region of the telomerase is more accessible for binding due to its role in binding to the telomeric substrate.

We then determined the change in the binding affinity for the template region with change in the concentration of the intercalator. Using the ImageQuant® software we determined the signal intensity of the bands at the template region and from the whole lane. We then calculated the proportion of the signal from the template region to the whole lane. This was done to correct for any discrepancies in the signal which could arise due to errors in loading. This proportion was then plotted against varying ethidium azide concentrations using Kaleidagraph®. The plot of proportion versus varying ethidium azide concentration sis

depicted in figure 13. We then determined the apparent binding affinity (K_{dapp}) of ethidium bromide azide for the template region by fitting the plot to the equation:

$$P = \frac{(E*(P_{max} - P_{min}))}{(E + K_{dapp}) + P_{min}}$$

where P = proportion, E = ethidium azide concentration, P_{max} = maximum proportion, P_{min} = minimum proportion.

Using this equation we obtained an apparent binding affinity or K_{dapp} of 8.9 µM with a R value of 0.89 for the binding of ethidium azide to the template region of telomerase RNA in the absence of DNA substrate.

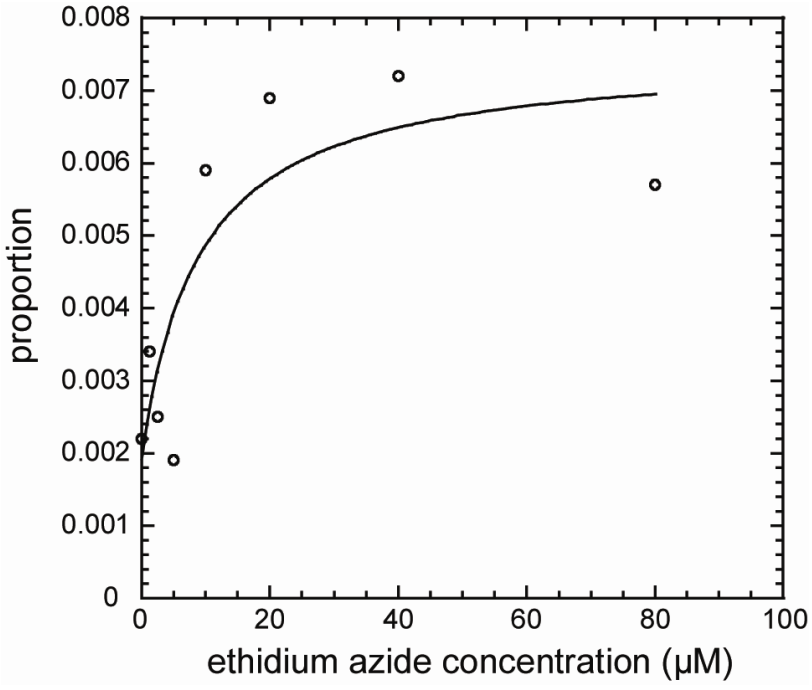


Figure 13. Binding curve to determine apparent binding affinity or K_{dapp} for ethidium azide binding at template region of telomerase RNA

Importance of the presence of DNA substrate in the intercalators' ability to bind to the telomerase RNA:

The next important question to answer was whether there was any differential affinity of the intercalator for the RNA/DNA heteroduplex. In order to ensure that every species of telomerase existed in the form of a heteroduplex we wanted to determine the saturating concentration of the DNA substrate. For this purpose we added varying concentrations of the DNA substrate to the HeLa cell homogenate containing telomerase and then added 3.3 µM (IC_{50} concentration of ethidium bromide) of ethidium azide to the mixture. We then allowed the solution to equilibrate for 30 minutes followed by irradiation for 30 minutes. The steps of RNA extraction, radiolabeling and gel electrophoresis was done as described earlier.

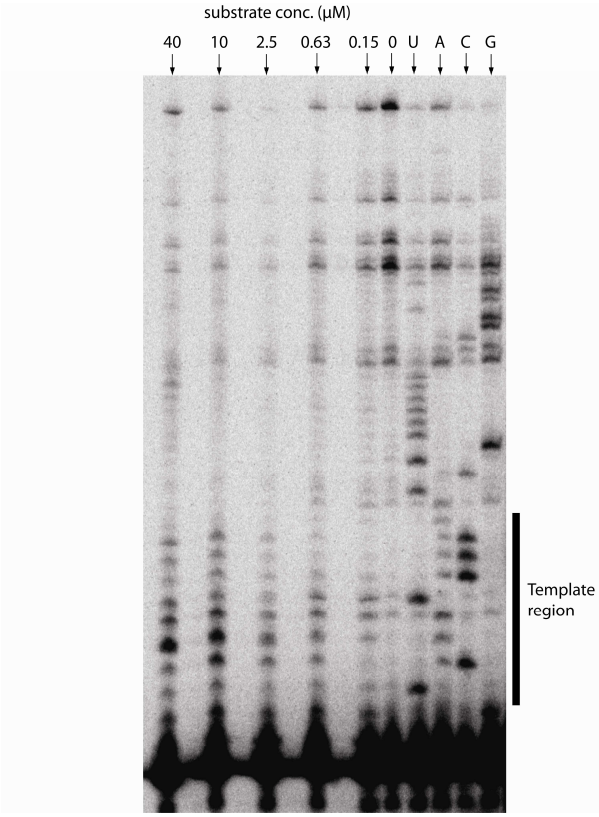


Figure 14. Photocrosslinking of ethidium bromide azide to telomerase RNA in presence of varying concentrations of DNA substrate

Result:

The gel image in figure 14 shows the binding of ethidium azide to the telomerase RNA at different concentrations of the DNA substrate. Again bands were present prominently near the template region. The signal from the bands was quantitated and the proportion of the signal in the presence of the substrate to the signal in the absence of the substrate was determined. The proportion was plotted against varying substrate concentrations using the equation

$$P = \frac{(S*(P_{max} - P_{min}))}{(S + K_{dapp}) + P_{min}}$$

where P = proportion , E = substrate concentration, P_{max} = maximum proportion, P_{min} = minimum proportion.

The plotted graph is depicted in figure 15. We obtained a K_{dapp} of 3 µM from the substrate oligo and also observed that saturation is reached around 10 µM of substrate concentration.

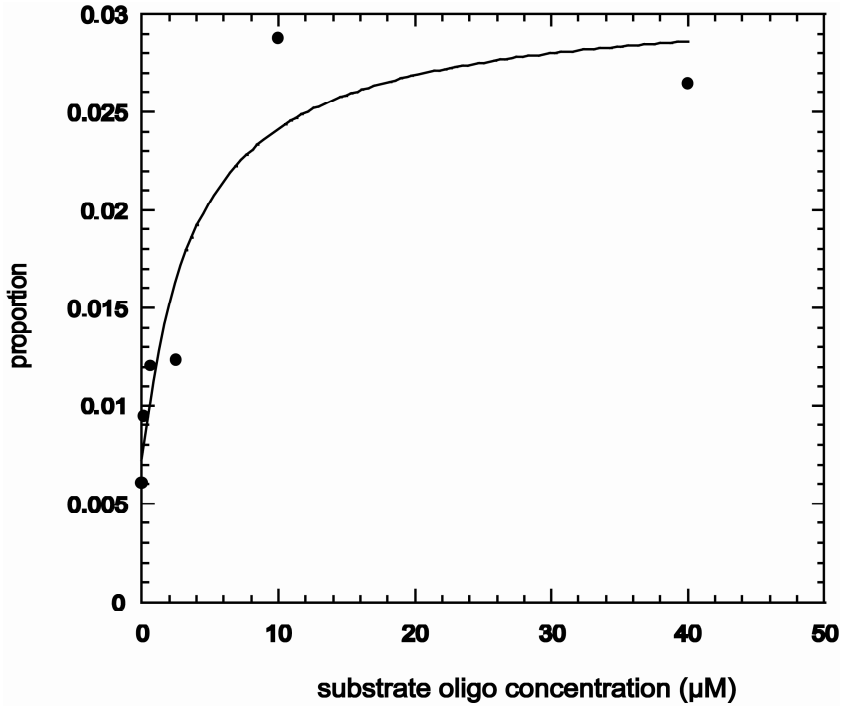


Figure 15. Binding curve to determine apparent binding affinity or K_{dapp} for DNA substrate

Crosslinking of ethidium azide to telomerase RNA in the presence of substrate DNA:

In order to probe the affinity of ethidium azide for the RNA/DNA heteroduplex, we used a saturating DNA substrate concentration (20 µM) which was above the saturating concentration observed earlier. The ethidium azide concentration was varied from 0-80 µM. We observed that the ethidium azide binding pattern in the presence of the DNA substrate was significantly different from the binding pattern observed in the absence of the DNA substrate. (Figure 16) Instead of bands being observed throughout the length of the RNA, the bands were prominently present at the template region. Bands at other locations were distinctly absent. This indicated that the ethidium azide bound only to the template region of the telomerase RNA when in the presence of the DNA substrate. A possible reason for this

pattern of bands is that the binding of the substrate to the template introduces conformational changes in the RNA structure making the other regions inaccessible to the ethidium azide binding.

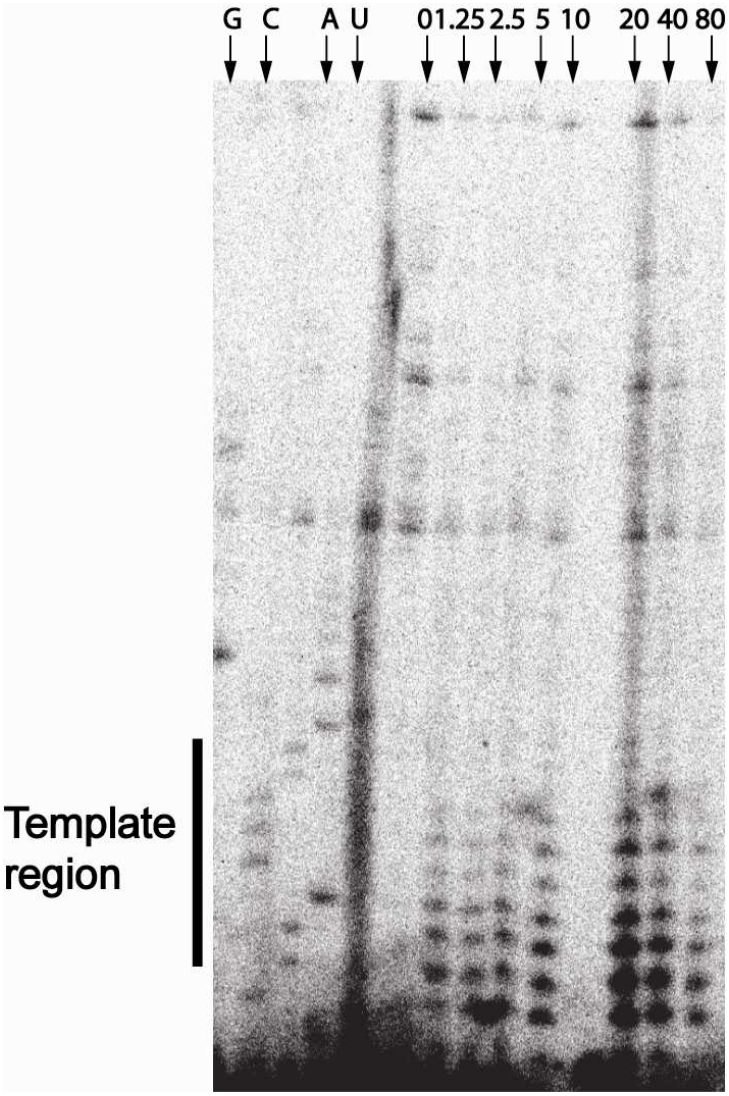


Figure 16. Photocrosslinking of ethidium azide to telomerase RNA in presence of DNA substrate

We then determined the apparent binding affinity or K_{dapp} for binding of ethidium azide to the template region in presence of the DNA substrate. We quantitated the bands at the template region and the signal from the template region was normalized to the signal from the whole lane. The proportion was plotted against varying ethidium azide concentrations and the apparent binding affinity was calculated in a similar manner to the experiment performed in the absence of DNA substrate. The data in figure 17 is an average from two determinations. The K_{dapp} was determined to be 1.3 µM with a R value of 0.93. This K_{dapp} is lower than the K_{dapp} for binding to the RNA alone suggesting that the intercalator has a greater affinity for the RNA/DNA heteroduplex compared to the RNA alone.

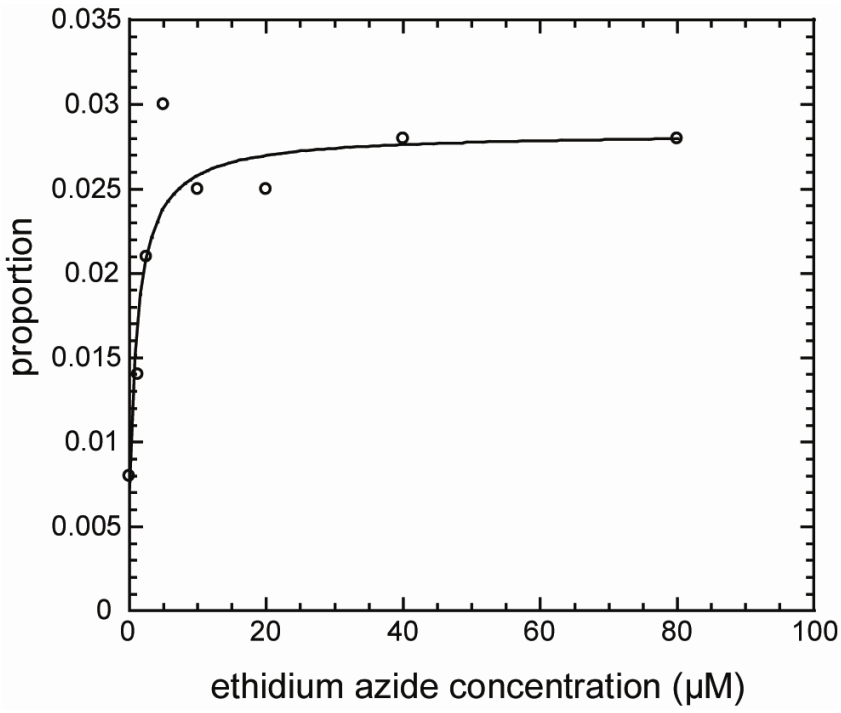


Figure 17. Binding curve to determine apparent binding affinity or K_{dapp} for ethidium azide binding at template region of telomerase RNA in presence of substrate (data is an average of two determinations)

In this work we have sought to understand the mechanism by which heteroduplex targeting inhibitors such as intercalators inhibit telomerase. We identified the location of binding of ethidium bromide to telomerase using a structural approach. Although it appeared that the ethidium bromide could bind to both the telomerase RNA and the RNA/DNA heteroduplex, the binding affinity was different for the two species. The apparent binding affinity or K_{dapp} of ethidium azide to the template region in presence of the DNA substrate to be 1.3 µM which is K_{dapp} is significantly lower than the K_{dapp} for binding to the RNA alone (8.9 µM). Hence we can conclude that ethidium bromide has a binding preference of the intercalator to the RNA/DNA heteroduplex versus the RNA alone. Thus it can be said that the intercalator causes telomerase inhibition by targeting the RNA/DNA heteroduplex and it can used as a scaffold to build more specific inhibitors of telomerase.

CHAPTER 3

DESIGNING OF PEPTIDE LIBRARIES WITH A WEAK INTERCALAT0R AS THE SCAFFOLD

In our laboratory we established that ethidium bromide was capable of inhibiting telomerase with an IC_{50} in the low micromolar range (3.3 µM). Additionally in chapter 2 we understood that the intercalator preferentially targeted the RNA/DNA heteroduplex that is formed during the mechanism of action of telomerase. Hence in order to obtain a specific inhibitor of telomerase, ethidium bromide appeared to be the most suitable scaffold to introduce functionalities which could develop interactions with the protein (TERT) region. Unfortunately strong intercalators like ethidium bromide have the disadvantage of inherent non-specificity. Ethidium bromide could potentially bind to any heteroduplex in a cell and exhibit off-target binding. Hence there was a possibility that the interactions between the introduced functionalities on ethidium bromide and telomerase would not be discernable due to the non-specific binding of ethidium bromide to the generic nucleic acids, which were present in large excess when compared to telomerase.

In order to circumvent this problem we sought to identify a new scaffold which would retain nucleic acid binding abilities but have a lower affinity. This would allow any new groups added to the scaffold to develop unique interactions with telomerase and exhibit increased affinity and specificity. The strong intercalative nature of ethidium bromide could be attributed to its three ring aromatic structure and positive charge. [90] Neutral molecules with a two ring aromatic structure would retain intercalative properties but would possess reduced affinity for nucleic acids. There is substantial literature precedence of the weak intercalative properties of two ring aromatic structures such as quinoxaline, indole, quinoline and

naphthalene derivatives.[91] Many proteins which interact with nucleic acids have intercalative amino acids (tyrosine, typtophan) at the active site. In HIV-RT the tyrosine at the active site is positioned in such a manner such that its side chain intercalates between the primer nucleotides.[38] Similarly Kaneda et al. reported that AP endonucleases specifically recognize apurinic/apyrimidinic (AP) sites in DNA by using the use the intercalative properties of tryptophan (indole side chain). These tryptophan residues were earlier reported to be essential for the activity of AP endonucleases.[92]

Since most intercalators are associated with cytotoxicity, we were interested in identifying a new scaffold which was known to be non-toxic in nature. For this purpose, we searched for the new scaffold among FDA approved drugs. We selected FDA approved drugs having a two-ring aromatic structure to test for telomerase inhibition. The structures of the selected drugs are provided in figure 18. The drugs were screened for their ability to inhibit telomerase activity using the single point PCR-based fluorescent assay. As described in chapter 1, this assay was developed by Dr. Rawle Francis, former graduate student from our laboratory.

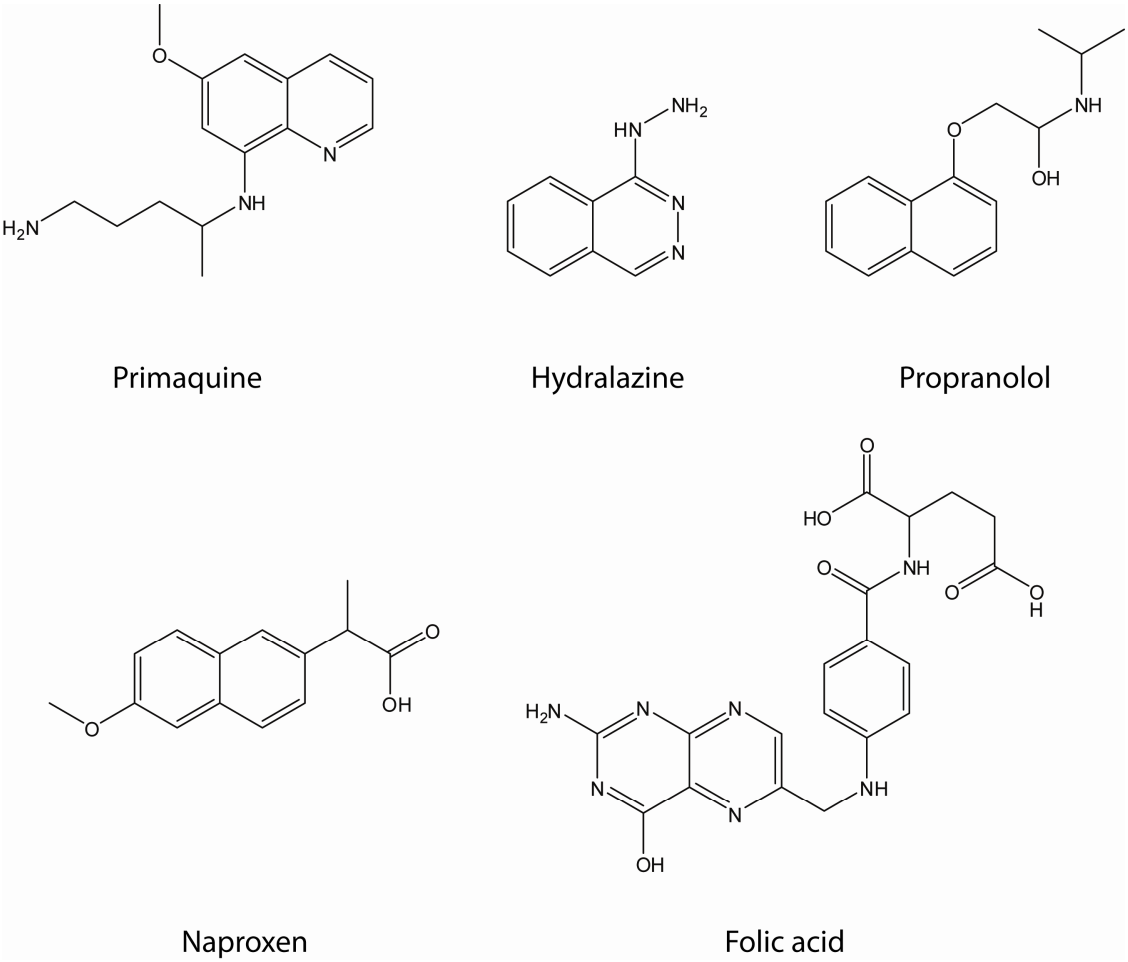


Figure 18. FDA approved drugs having a planar two ring structure

Selection of a weak intercalator as a scaffold:

Experimental procedures:

PCR based assay of telomerase: As described in chapter 1, this assay employs a fluorescent method of detection of the telomeric products. Briefly HeLa cell extract was prepared from HeLa S3 cells (National Cell Culture Center, Minneapolis, US) as described in chapter 2. The selected drugs were added to 3.2 µl of HeLa cell lysate containing telomerase

in a reaction mixture containing assay buffer (50 mM tris-OAc, 50 mM KCl, 1 mM $MgCl_2$, 5 mM β-mercaptoethanol, 1 mM spermidine, pH 7.1), 1 µM biotinylated TRAP primer substrate oligo (5'-biotin AATCCGTCGAGCAGAGTT-3') and 1 mM of each dNTP in a final volume of 20 µl. The drug concentration was maintained at 10 mM and each drug was tested in triplicates. After incubation for 1 hour at 37 ºC the biotinylated extension products were allowed to bind to streptavidin coated PCR tubes (Roche) in presence of 200 µl of reaction termination buffer (10 mM tris-HCl, 2M KCl pH 7.2) for 30 minutes. The solution was aspirated and the PCR tubes were washed five times with 200 µl of washing buffer (10 mM tris-HCl, 1 M NaCl, pH 7.5) to avoid false positives due to *Taq* polymerase inhibition by the inhibitors. Subsequent amplification of the products was done using 10 µM of biotinylated TRAP forward primer, 10 µM reverse primer (5' CCCTTACCCTTACCCTTACCCTAA-3'), 50 µM of each dNTP and 1.25 units *Taq* polymerase in a final volume of 50 µl. The PCR amplification was allowed to proceed for 22 cycles. Quantitation of the amplified dsDNA products was done by fluorescence detection (excitation 485 nm, emission 535 nm) using PicoGreen® nucleic acid stain (Invitrogen) in 1 x TE (10 mM tris-HCl, 1 mM EDTA pH 7.5). The estimated IC_{50} of the drugs was calculated using the formula $IC_{50} = [I]/(1/[(s-c)/(t-c)-1])$, where [I] is the inhibitor concentration, *s* is the fluorescence signal in presence of the inhibitor, *t* is the fluorescence signal in the absence of inhibitor and *c* is the PCR control signal which contains primers and nucleic acids but no telomerase extension products. The results of the single point assay are provided in table 2.

Table 2. Results of single point assay screening FDA approved drugs against telomerase

Name of commercially available drugs	Estimated IC_{50} (mM)
Primaquine	0.6
Propranolol	1.5
Hydralazine	2.9
Naproxen	9.2
Folic acid	> 200

The IC_{50} of the tested drugs were in the millimolar range indicating that the drugs weakly inhibit telomerase. Primaquine inhibited telomerase most effectively with an IC_{50} in the low millimolar range. It was interesting to note that there was a charge dependence on the ability of the drugs to inhibit telomerase, with the positively charged drugs inhibiting telomerase more effectively than the negatively charged drugs. This occurrence is possibly because the positively charged drugs have a greater interaction with the negatively charged phosphate backbone of the heteroduplex. This supported the idea that these drugs inhibited telomerase by binding to the heteroduplex. From the tested drugs we selected naproxen as the starting point for the synthesis of new derivatives due to following advantages: the lack of a positive charge in its structure would potentially limit the non-specific binding to nucleic acids and the carboxyl group could easily be derivatized with amino acids. We assayed for telomerase activity in presence of varying concentrations of naproxen (0-40 mM) using the PCR-based fluorescent assay. The IC_{50} of naproxen was determined by using the equation p =

1/ (1+[I]/IC_{50}) where *p* is the fluorescent signal normalized to the signal obtained in absence of the inhibitor and [I] is the inhibitor concentration. The IC_{50} of naproxen was determined to be 3.1 mM. (Figure 19)

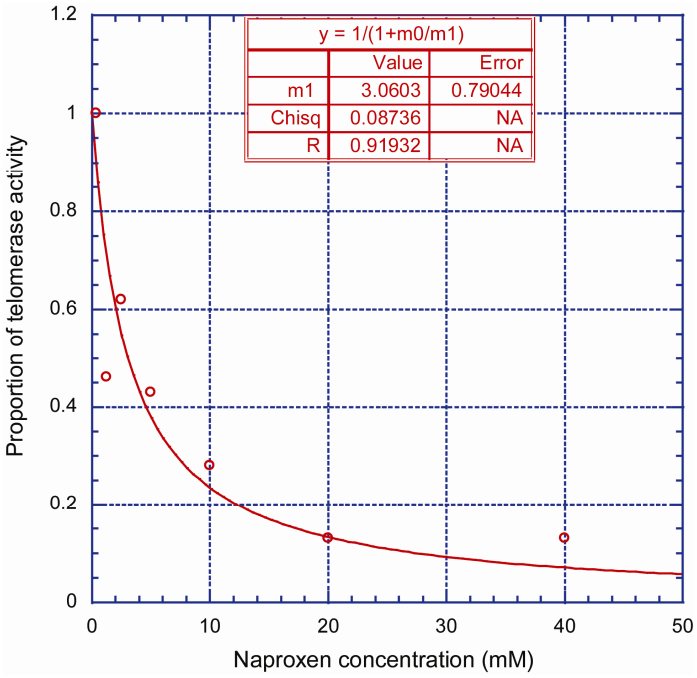


Figure 19. IC_{50} dose response curve of naproxen

Design of Naproxen based library by split and mix method:

In the earlier section we established that naproxen was a weak inhibitor of telomerase. In order to improve its affinity to telomerase we then sought to introduce specific moieties on naproxen which could interact with telomerase. The factor differentiating the RNA/DNA heteroduplex in telomerase from other duplexes in cells is the protein region of the ribonucleoprotein (TERT). Developing interactions with the protein region by attaching

amino acid groups to naproxen would potentially increase the affinity to telomerase. A schematic representation of the idea is provided in figure 20. Naproxen has the advantage of possessing a carboxy handle which could easily be derivatized by amino acids.

Initially we decided to synthesize tripeptide linear peptide libraries of naproxen using combinatorial chemistry. The advantage of using combinatorial chemistry for the synthesis of the libraries is that it allows the systematic synthesis of a large number of molecules which can then be screened against telomerase. We synthesized the combinatorial libraries using a divide-couple-recombine (DCR) process by solid phase peptide chemistry. After screening the synthesized libraries we attempted to identify the active molecule by iterative deconvolution method. Some of the techniques used in the synthesis of this library are described further below.

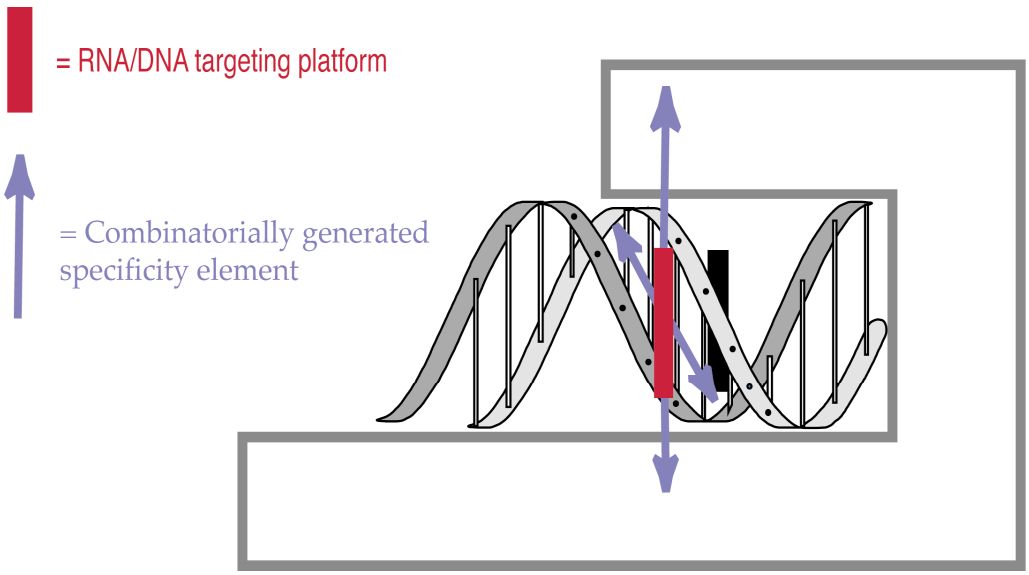


Figure 20. Schematic representation of using naproxen as a platform to introduce interactions with protein portion of telomerase

Solid phase peptide synthesis:

This method of synthesizing peptides was pioneered by Merrifield. [93]Generally the solid phase is a bead which in porous in nature and the synthesized peptide remains attached to it until cleaved. The main advantage of this technique is that the peptide remains attached to the solid surface throughout the synthesis which facilitates the manipulations in the synthetic procedure. For instance any unreacted amino acid and coupling agents at each coupling step can be easily removed and multiple steps of purification are not required during synthesis. The amino acid on the solid surface has an amine group which is coupled to the carboxy group of another amino acid whose amine group is protected. The most commonly used protecting groups of the alpha amine group are Fmoc and Boc. The Fmoc group is base labile and the Boc group is acid labile. The Fmoc group is the more commonly used protecting group as it provides an orthogonal protection from the side chain protecting groups which are generally acid labile. Thus solid phase peptide synthesis involves multiple steps of deprotection of the amine group, washing and coupling and these steps can be performed easily as the peptide remains attached to the solid surface. After the completion of the synthesis the peptide is cleaved using an acid generally 95 % TFA which simultaneously deprotects the side chain protecting groups and cleaves the peptide from the solid support.

Divide-couple-recombine method of synthesis:

This process involves division of the resin into multiple equal portions and then coupling of a single specific amino acid to each equal portion. After coupling all the resin portions with one amino acid attached to it are mixed and then further divided. Each resin

portion is then again coupled to another amino acid and this result in multiple libraries containing two random amino acids. This process can be repeated as many times as required in order to generate libraries of various sizes. The mixing of the resin allows for the synthesis of a mixture of peptide compounds. This process allows the synthesis of one bead –one compound arrays. [94]

Iterative deconvolution:

This is the most common method of identifying the active compound from a mixture of compounds in a library. This process involves the synthesis of sublibraries of the original library where one or more positions contain a fixed amino acid. If the synthesis of the library is done by the divide-couple-recombine method, the position of the last amino acid can be fixed by omitting the final pooling step. Thus each batch of resin contains a mixture of compounds which have a known amino acid in one position. Each of these sublibraries is then screened against the target and the subset showing activity determines the identity of the amino acid at a particular position. This process is then repeated by adding known amino acids at another position along with the earlier identified amino acid at the fixed position until the identity of the amino acids at each position is known. [95-97]

Synthesis of library 1:

The first library synthesized using the split and mix method was a tripeptide library with the naproxen attached to the third amino acid. In the first round of synthesis the third position had a specific amino acid instead of a mixture of amino acids. 18 of the 20 natural amino acids were used in the synthesis of the library. The amino acids which were not used

were arginine and lysine due to the presence of an amine group in the lysine side chain and a guanidine group in the arginine side chains. Both these groups had the potential of being positively charged and could potentially introduce non-specificity.

Hence the designed library could be depicted as X_1-X_2-$A_{(1-18)}$-naproxen (library 1) where X refers to a random mixture of amino acids and $A_{(1-18)}$ refers to the 18 specific amino acids. Additionally in order to impart stability to these libraries from proteases we used unnatural D- amino acids. Since the first two positions in the tripeptide library contained a mixture of amino acids, the number of compounds synthesized was too large to be characterized by mass spectrometry. Hence another dipeptide library of naproxen was synthesized simultaneously where the first position contained a mixture of amino acids and the second position contained a fixed amino acid (X_1-$A_{(1-18)}$-naproxen – library 1a). This library was used for characterization by mass spectrometry with the assumption that the successful synthesis of the X_1-$A_{(1-18)}$-naproxen library would correlate with the successful synthesis of the X_1-X_2-$A_{(1-18)}$-naproxen library since both libraries were synthesized simultaneously.

Materials and methods:

Solid phase synthesis: Briefly, 37.5 mg of rink amide resin was taken in 18 microcentrigfuge tubes and swelled for 30 minutes in NMP. The rink amide resin was deprotected by 200 µl of 20 % piperidine solution in NMP. This process was repeated twice followed by five washes using 200 µl NMP. 120 µmoles of amino acid was taken in a total volume of 200 µl to maintain a concentration of 600 mM. The carboxyl group of the amino acid was activated using the activating agent 2-(1H-7-azabenzotriazol-1-yl)-1,1,3,3-

tetramethyl uronium hexafluorophosphate (HATU) and N,N diisopropylethylamine (DIEA) for 30 minutes. The amino acid ratio to HATU and DIEA was maintained at 1:1:2. The activated amino acid was added to the resin and allowed to couple for 17 hours. The amino acid mixture was removed and the resin washed 5 times with 200 µl of NMP. Any unreacted amine groups were capped using acetic anhydride solution in NMP (10 % acetic anhydride, 5% DIEA in 200 µl of NMP). The resin was again washed 5 times with 200 µl of NMP followed by 5 washes with 200 µl DCM. The resin was dried and weighed. We observed that there was a reduction in the weight of the resin from 37.5 mg to 21.5 mg. This loss of weight could have occurred due to incomplete transferring of the resins from the mixing step. I repeated the first coupling step using 31 mg of resin in order to increase the amount of resin having a mixture of amino acids at the first position. At the end of the first coupling, I had a total of 57.6 mg of resin. This total amount of resin was divided into two equal portions for libraries 1 and 1a.

One portion was divided into 18 tubes and the coupling, mixing and dividing process was repeated with that portion to generate the second random position for library 1. The amino acid concentration in the reaction solution was maintained at 300 mM. Since the quantity of the resin was reduced by half, the amount of amino acid was reduced accordingly. Thus 30 µmoles of amino acid, 30 µmoles of HATU and 60 µmoles of DIEA in a final volume of 100 µl was used for this round of coupling. The second portion was then divided into 18 portions and specific amino acids were added to each portion for both libraries 1 and 1a. After the coupling of the third amino acid the resin was not mixed. To each of the microcentrifuge tubes, 30 µmoles naproxen, 30 µmoles of HATU and 60 µmoles of DIEA were added in a final volume of 100 µl for the final coupling. The reaction was allowed to

proceed overnight. After the final coupling the resin was washed 5 times with NMP and 5 times with DCM and then allowed to dry. The peptide library was cleaved from the solid surface by using 95 % trifluoroacetic acid. The TFA was removed using a nitrogen stream and the peptide libraries were precipitated using 400 µl of ice cold ether. The peptide libraries were then taken up in 20 µl of 100 % DMSO. The libraries were quantitated using the unique chromophore of naproxen which has an absorbance at 330 nm and a determined extinction coefficient of 1490 $M^{-1}.cm^{-1}$ at 330 nm.

Characterization of X-$A_{(1-18)}$-naproxen library by liquid chromatography and mass spectrometry:

The LC-MS conditions used were as follows: The solvent gradient in the LC method moved from 100% water / 0.1% trifluoroacetic acid – 100% acetonitrile / 0.1% TFA over 30 min through a C8 column. The separation was monitored spectrophotometrically at 212, 280 and 330 nm. The mass analysis was performed using a mass spectrometer, in the positive ion mode along with the LC system. Representative spectra of two of the 18 libraries synthesized with the motif X-$A_{(1-18)}$-naproxen are depicted below. Multiple peaks can be observed in the lower panel depicting the total wavelength chromatogram (TWC) which indicates the presence of multiple species in the libraries. The different species in the library were identified by obtaining the extraction ion chromatogram (XIC) of the mass of each species in the library.

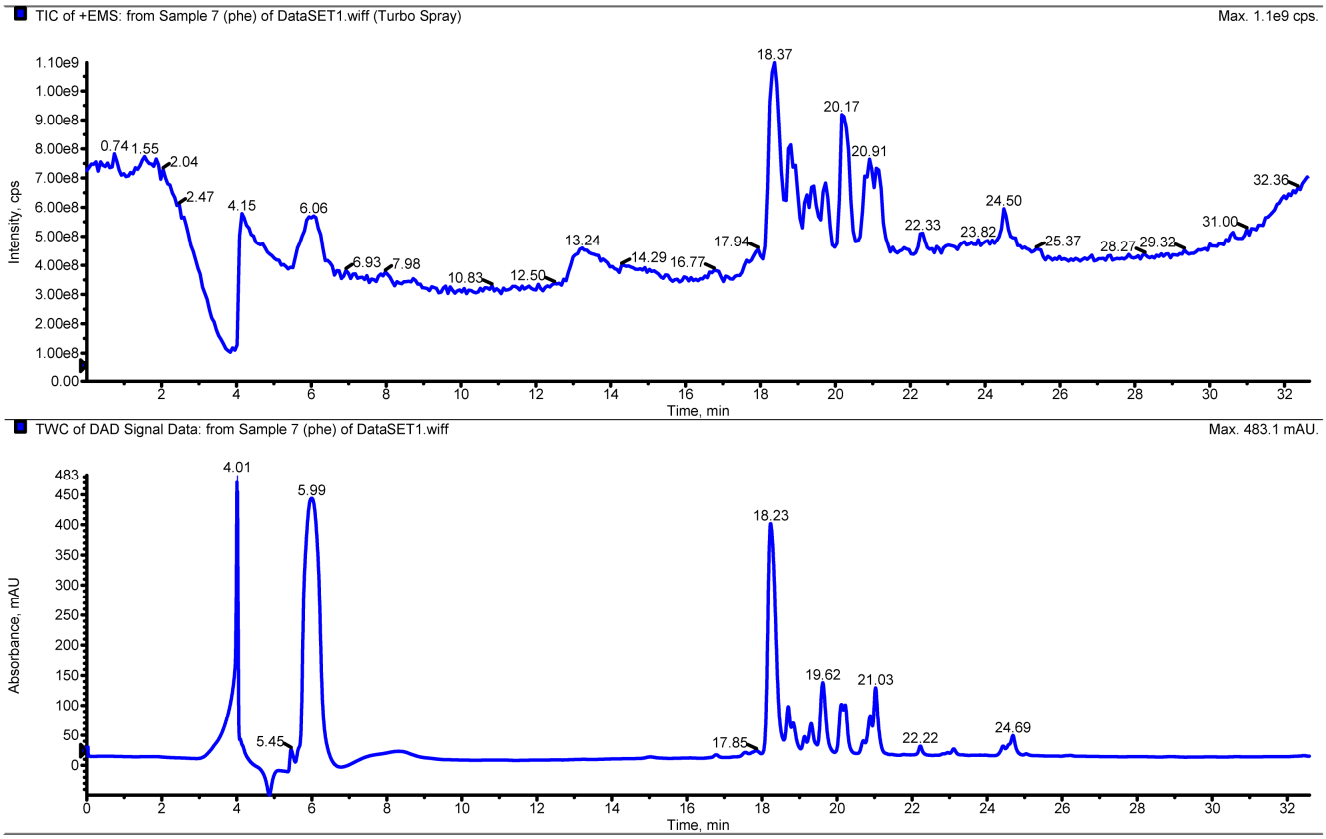


Figure 21. LC-MS spectra of X-Phe-Naproxen

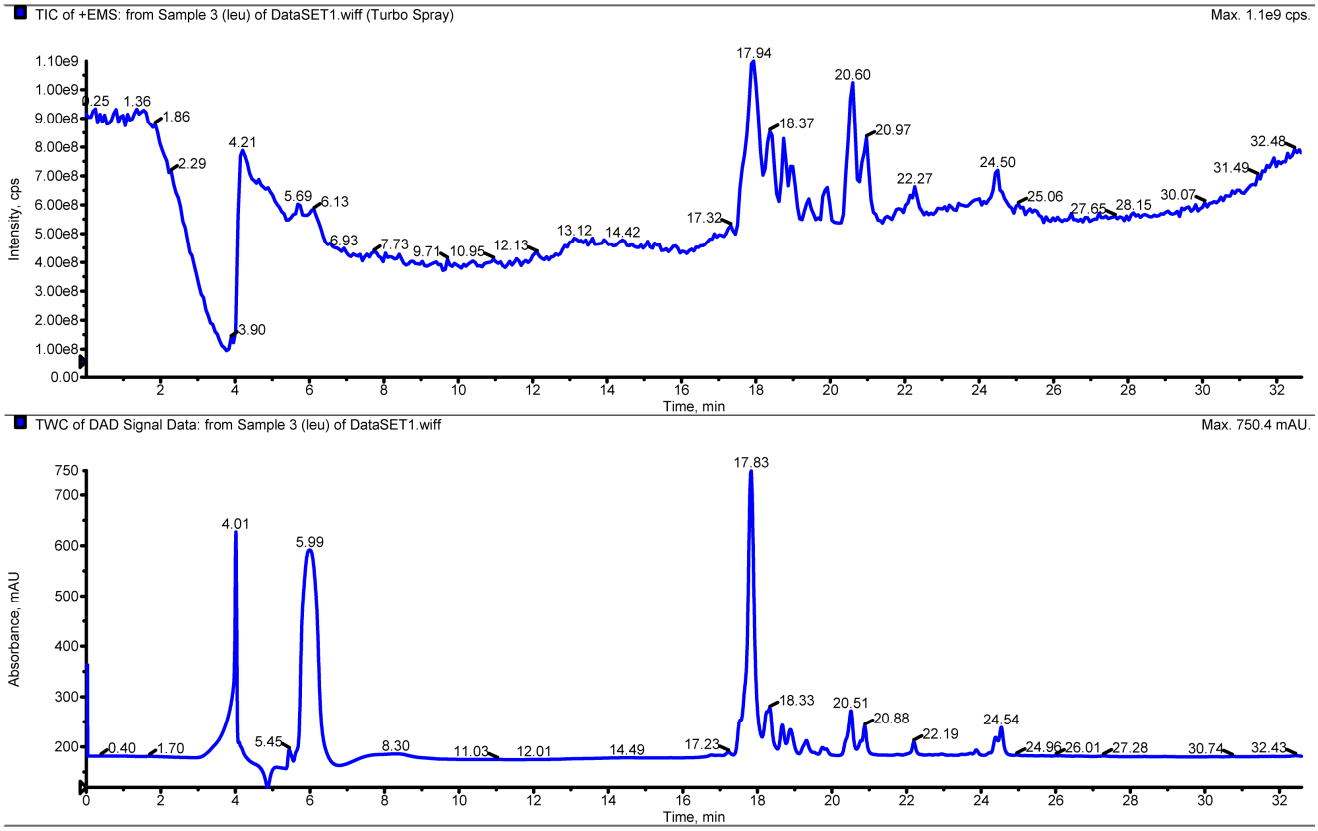


Figure 22. LC-MS spectra of X-Leu-naproxen

Screening of the library against telomerase:

The library having the sequence X_1-X_2-$A_{(1-18)}$-naproxen was screened against telomerase using the single point PCR based assay. Each fixed amino acid point was screened in triplicates and the assay concentration was maintained at 250 µM. However the library containing phenylalanine at the fixed position (X_1-X_2-Phe-naproxen) had solubility issues at 250 µM due to the hydrophobic nature of phenylalanine. Hence for this library the assay concentration was 50 µM. DMSO was maintained at 5 % in the assay. The results of the screening are depicted in table 3.

Table 3. Results of screening of library 1

Amino acid in third position	Proportion	Rank
His	-1.34	1
Asn	-0.60	2
Ala	0.03	3
Val	0.03	4
Thr	0.04	5
Tyr	0.099	6
Gln	0.18	7
Met	0.19	8
Phe	0.20	9
Trp	0.32	10
Ile	0.36	11
Gly	0.45	12
Asp	0.49	13
Pro	0.53	14
Leu	0.53	15
Glu	0.65	16
Cys	0.66	17
Ser	0.80	18

From the results we observe that the mixture of compounds having histidine, asparagine, alanine and valine at the fixed inhibited telomerase more effectively.

Synthesis of library 2:

For the next round of synthesis we had four possible libraries:

1. X-$A_{(1-18)}$-His-naproxen
2. X- $A_{(1-18)}$-Asn-naproxen
3. X- $A_{(1-18)}$-Ala-naproxen
4. X- $A_{(1-18)}$-Val-naproxen

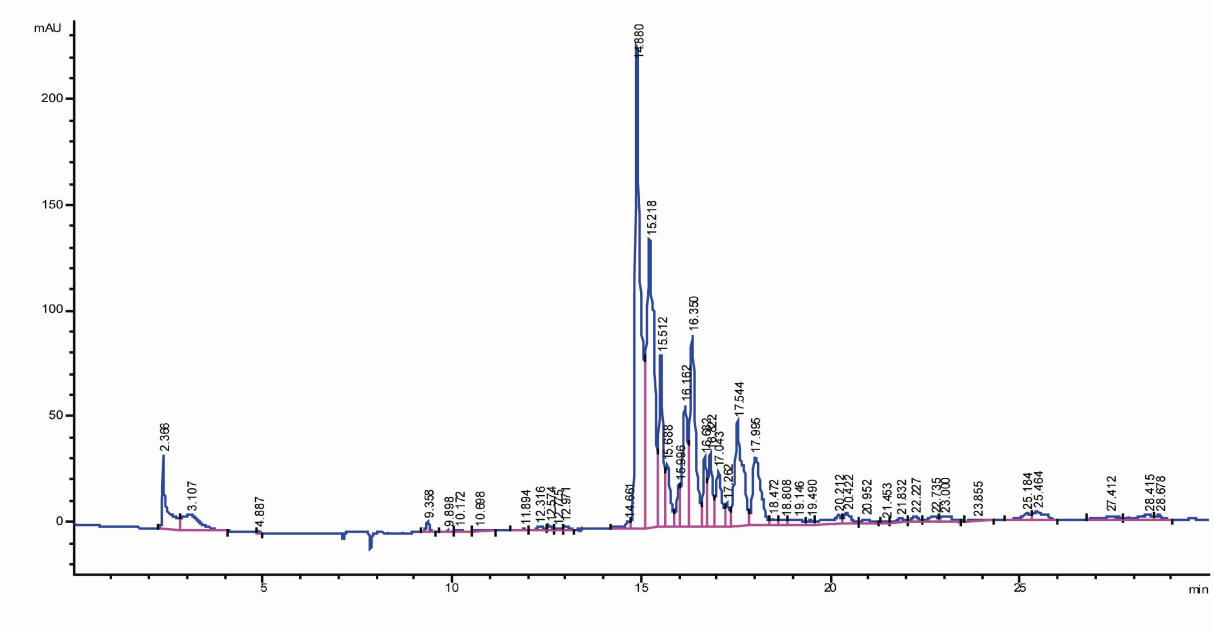
We opted to initially select the top hit from the earlier round of synthesis which is the library which had histidine in the fixed position. Again in this round of synthesis the first position contained a random mixture of amino acids but the second position had a specific amino acid and finally histidine was at the third position. Thus the design of the library was X- $A_{(1-18)}$-His-naproxen.

The random position was generated by the divide-couple-recombine method as described earlier. The resin after the first coupling was divided into 18 portions and single amino acids were added to each portion followed by histidine and naproxen to all the 18 positions. For each round of coupling 30 µmoles of amino acid, 30 µmoles of HATU and 60 µmoles of DIEA was used to maintain a solution concentration of 300 mM. The library was cleaved from the resin using 95 % TFA, precipitated using ice-cold ether and then taken up in DMSO.

Characterization of the library was performed using both liquid chromatography and mass spectrometry. The liquid chromatography was performed using the HP 1090 system on a C8 column. The gradient used for the mobile phase was 0-100% acetonitrile in 30 minutes and a flow rate of 0.6 ml/min was used. Representative spectra of two libraries (X-Asn-His-naproxen and X-Phe-His-naproxen) analyzed using liquid chromatography is depicted in figure 23. The spectra show multiple peaks representing the different species in the library which have different retention times. Since it was not possible to identify the species by liquid chromatography alone, mass spectrometry was also performed on the libraries.

Mass spectrometry was performed on these libraries using the C8 column and a mobile phase gradient of 0-100% acetonitrile in 30 min. The flow rate in this case was 0.3 ml/min. The various molecules within a library were then identified using XIC. The spectra of X-Asn-His-naproxen is given in figure 24 and the identified species are listed in table 4. In spite of some species not being observed in the XIC we decided to proceed with testing of the libraries. The libraries were the screened against telomerase using the single point PCR assay at a final concentration of 250 µM. The DMSO concentration was maintained at 5 %. Ethidium bromide at the IC_{50} concentration (3.3 µM) was tested simultaneously as a positive control. The results of the screening are depicted in table 5.

a.



b.

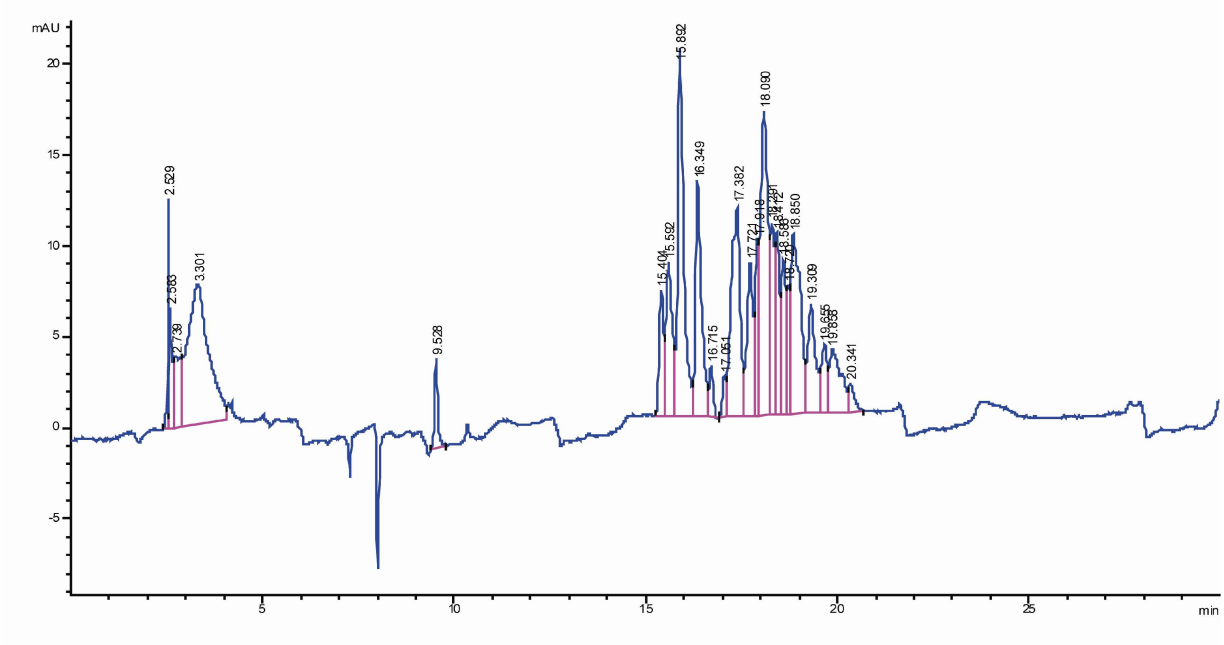


Figure 23 a. LC spectra of X-Asn-His-naproxen b. LC spectra of X-Phe-His-naproxen

Table 4. Species observed by LC-MS

S.no.	Name	MH+	Observed mass
1	ALA	552.25	551.9
2	ASN	595.26	594.8
3	GLN	609.25	608.9
4	GLY	538.23	537.8
5	HIS	618.27	
6	ILE	594.3	594.8
7	LEU	594.3	594.8
8	PHE	585.27	584.9
9	SER	568.24	567.8
10	THR	582.26	581.9
12	TYR	644.28	643.9
13	VAL	581.26	580
14	MET	569.25	567.8
15	GLU	610.25	609.8
16	CYS	584.22	
17	ASP	596.24	595.9
18	PRO	535.26	534.8

Table 5. Results of screening against telomerase

Amino acid in second position	Proportion	Rank
Thr	0.45	1
Ser	0.60	2
Ile	0.60	3
Cys	0.66	4
Asn	0.73	5
His	0.79	6
Gln	0.80	7
Pro	0.83	8
Asp	0.95	9
Phe	1.15	10
Leu	1.18	11
Met	1.26	12
Ala	1.27	13
Glu	1.29	14
Trp	1.30	15
Tyr	1.30	16
Gly	1.39	17
Val	1.40	18

In order to confirm our results of screening against telomerase we re-tested the six libraries which displayed telomerase inhibition and found that the results were a little dissimilar. In the first round of testing the library X-Thr-His-naproxen was the most effective in inhibiting telomerase while in the second round of testing X-Asn-His-naproxen emerged as the lead library. The ordering of the libraries was similar in both the rounds of testing except for this one discrepancy.

Table 6. Results of screening against telomerase

Amino acid in second position	Proportion	Rank
Asn	0.64	1
Thr	0.72	2
Ser	0.76	3
Ile	0.78	4
Cys	0.81	5
His	0.81	6

Synthesis of libraries 3 and 4:

Since we obtained two lead libraries in two rounds of screening we decided to synthesize two libraries, one with asparagine in the second position and another with threonine in the second position. These libraries had a single specific amino acid in the first position.

Libraries:

1. $A_{(1\text{-}18)}$-Thr-His-naproxen

2. $A_{(1\text{-}18)}$-Asn-His-naproxen

Synthesis of the libraries:

15 mg of rink amide resin was weighed out for each library which was split into 18 different tubes. To each tube 30 µmoles of a specific amino acid was added along with the coupling agents. After each coupling any uncoupled amines were capped by 10% acetic anhydride, 5% DIEA in NMP. To one set of 18 tubes, 30 µmoles of threonine was added and to another set 30 µmoles of asparagine was added. After this coupling step 30 µmoles histidine was added to all the points followed by 30 µmoles of naproxen. Cleavage of the libraries from the resin was performed as described earlier. The libraries were taken up in 20 µl of 100 % DMSO and quantitated using the extinction coefficient of naproxen. The synthesized molecules were analyzed by infusion in the mass spectrophotometer. Representative spectra of some of the molecules from both the libraries are depicted below. Testing of the molecules for telomerase inhibition was done by the single point PCR based assay by maintaining the inhibitor concentration at 250 µM. The results of the screening of the libraries are in table 7.

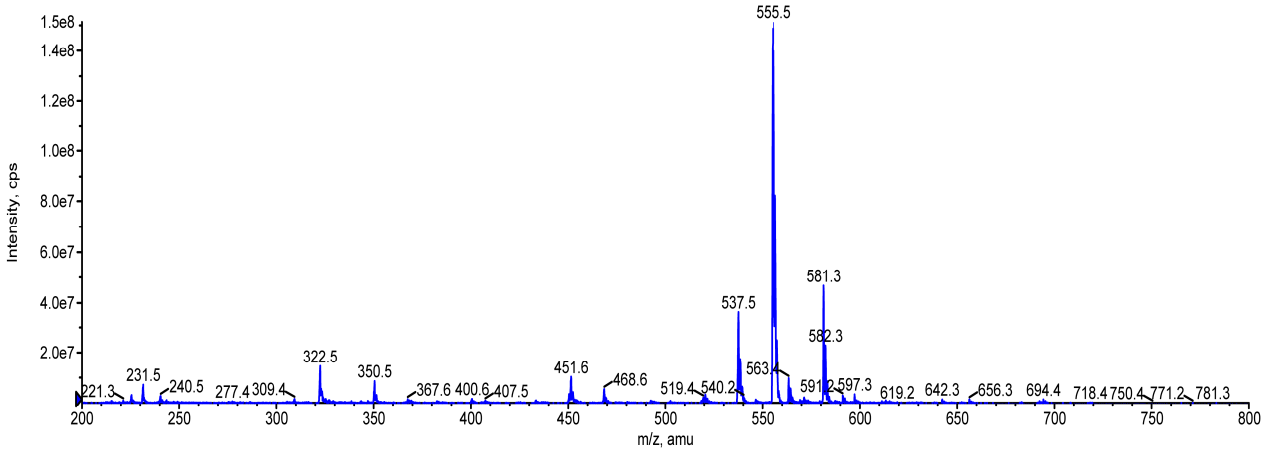


Figure 24. MS spectra of Ser-Thr-His-Naproxen (expected mass – 555.25)

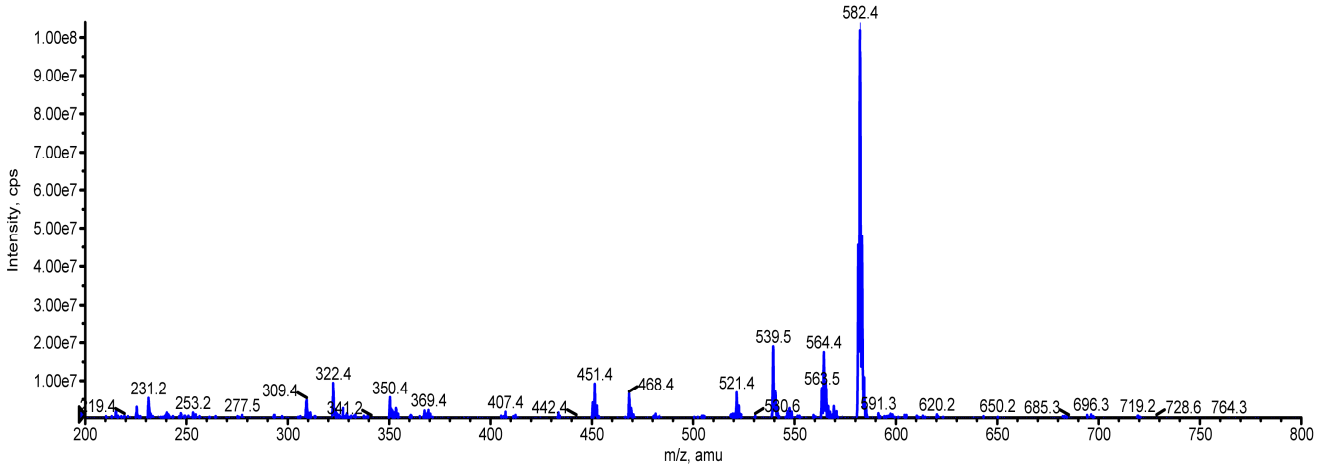


Figure 25. MS spectra of Ile-Thr-His-Naproxen (expected mass – 582.2)

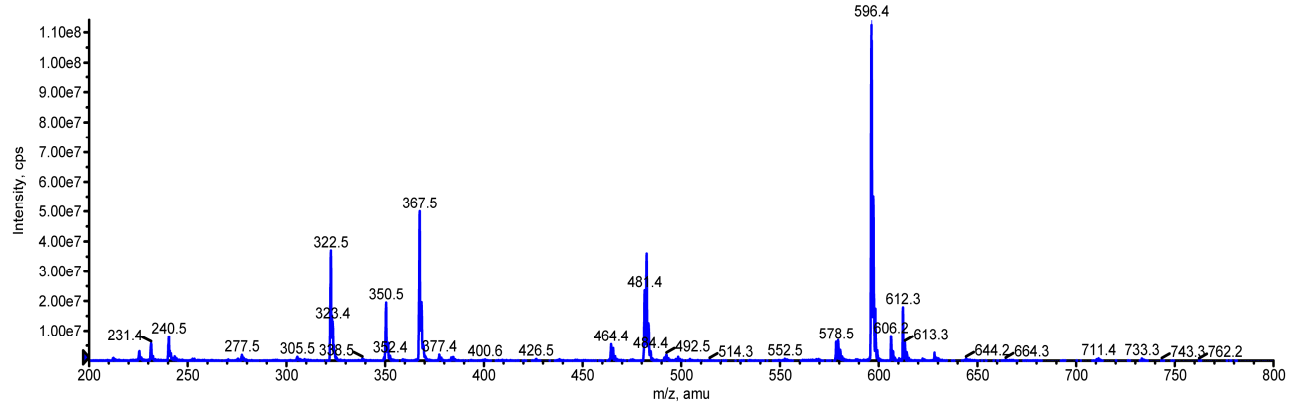


Figure 26. MS spectra of Asp-Asn-His-Naproxen (expected mass – 596.2)

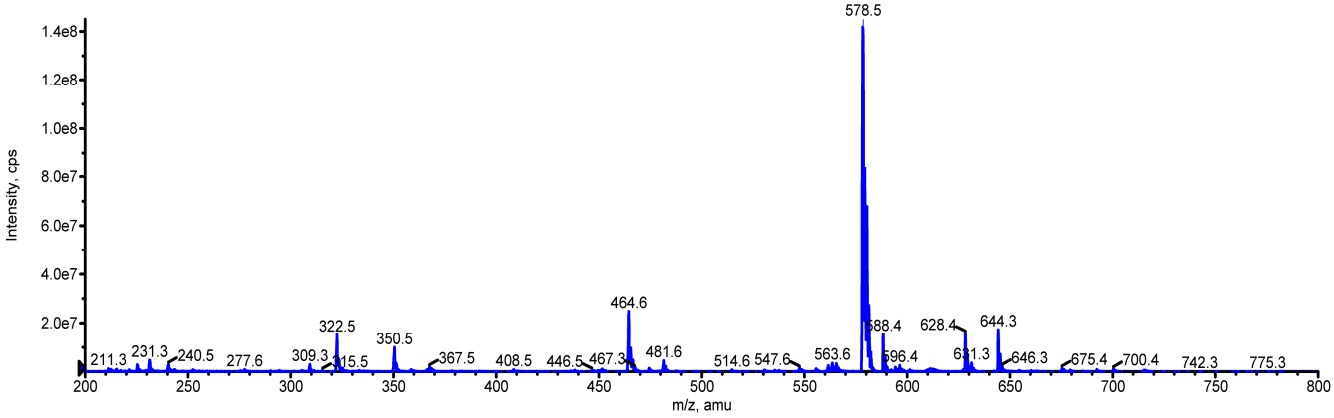


Figure 27. MS spectra of Pro-Asn-His-Naproxen (expected mass – 578.3)

Table 7. Results of screening against telomerase

a. $A_{(1-18)}$-Thr-His-Naproxen library

Amino acid in first position	Proportion	Rank
Asn	0.58	1
Ala	0.84	2
Ser	0.93	3
Glu	1.03	4
Cys	1.06	5
Val	1.08	6
Met	1.13	7
Asp	1.22	8
Leu	1.22	9
His	1.25	10
Thr	1.25	11
Tyr	1.26	12
Pro	1.28	13
Phe	1.30	14
Gln	1.32	15
Ile	1.32	16
Gly	1.45	17
Trp	1.56	18

b. $A_{(1-18)}$-Asn-His-Naproxen library

Amino acid in first position	Proportion	Rank
Thr	0.7	1
Asn	0.77	2
Ser	0.78	3
Val	0.8	4
Pro	0.88	5
Gln	0.85	6
Leu	0.91	7
Met	0.92	8
Asp	0.93	9
Gly	0.93	10
Phe	0.93	11
His	0.97	12
Trp	0.98	13
Ala	1.05	14
Cys	1.08	15
Tyr	1.09	16
Glu	1.1	17
Ile	1.6	18

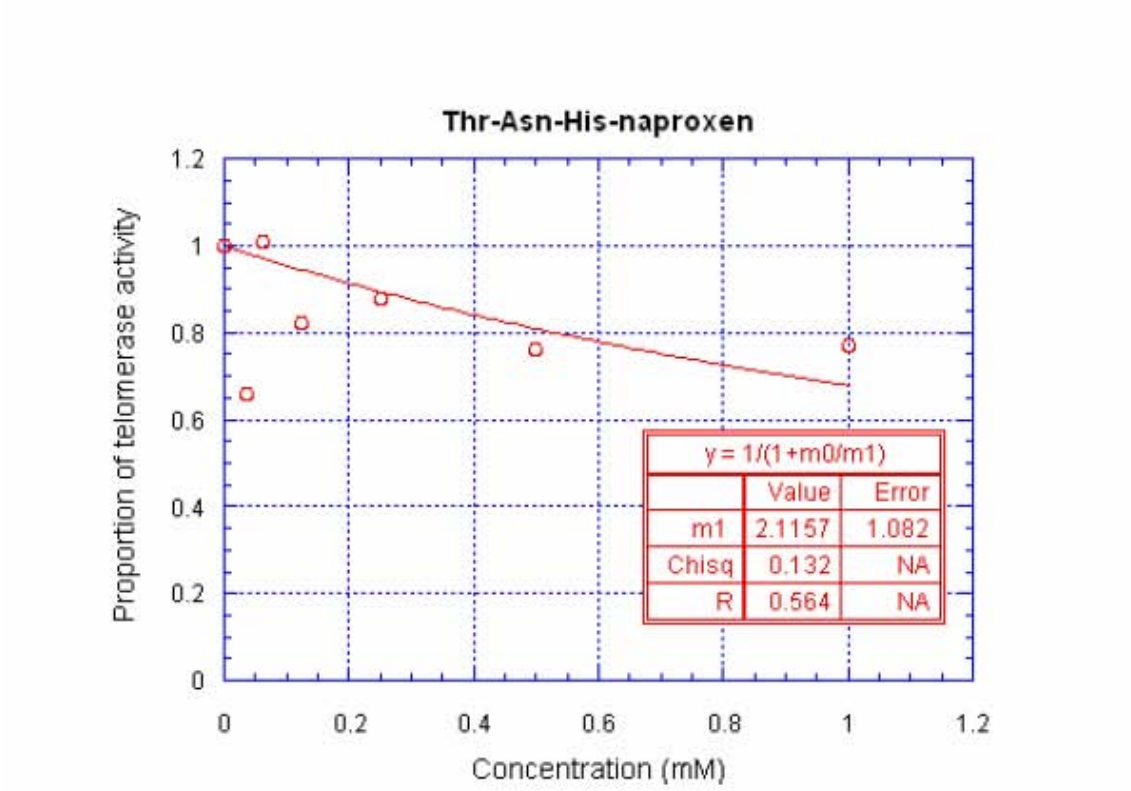
Results:

Table 7a lists the ranks of the the different molecules in the A -Thr-His-naproxen library and table 7b lists the ranks of the molecules in $A_{(1-18)}$-Asn-His-naproxen library. The lead molecule from the library $A_{(1-18)}$-Thr-His-naproxen was Asn-Thr-His-naproxen. The lead molecule from the library $A_{(1-18)}$-Asn-His-naproxen was Thr-Asn-His-naproxen. It was interesting that the hits from both the libraries had the same amino acid residues but with different arrangement.

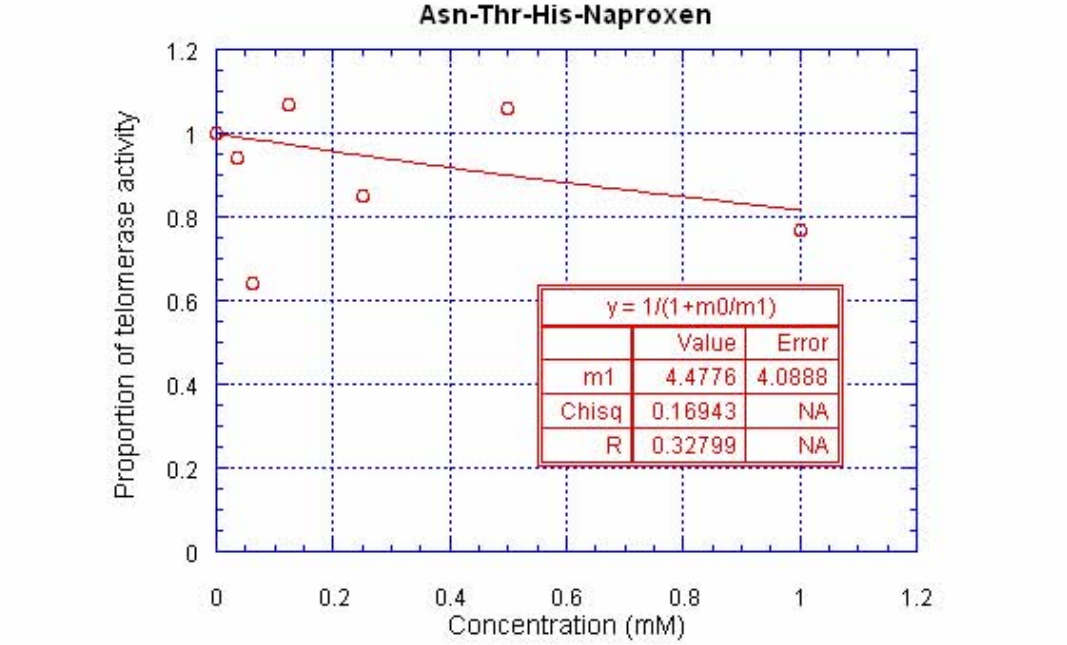
Determination of IC_{50} of the lead molecules:

The single point screening using the PCR based assay of telomerase does not provide the true IC_{50} value. In order to determine the true IC_{50} value of telomerase the lead molecules were tested for their ability to inhibit telomerase over a range of concentrations. The assay concentration of the molecules was varied from 1mM to 31.25 µM. As a control the IC_{50} of ethidium bromide was also determined in this assay. The DMSO level in the assay was maintained at 1%. The IC_{50} plots are depicted in figure 28.

a.


Thr-Asn-His-naproxen
Proportion of telomerase activity
Concentration (mM)
0
0.2
0.4
0.6
0.8
1
1.2
y = 1/(1+m0/m1)
Value
Error
m1
2.1157
1.082
Chisq
0.132
NA
R
0.564
NA

b.


Asn-Thr-His-Naproxen
Proportion of telomerase activity
Concentration (mM)
0
0.2
0.4
0.6
0.8
1
1.2
y = 1/(1+m0/m1)
Value
Error
m1
4.4776
4.0888
Chisq
0.16943
NA
R
0.32799
NA

.

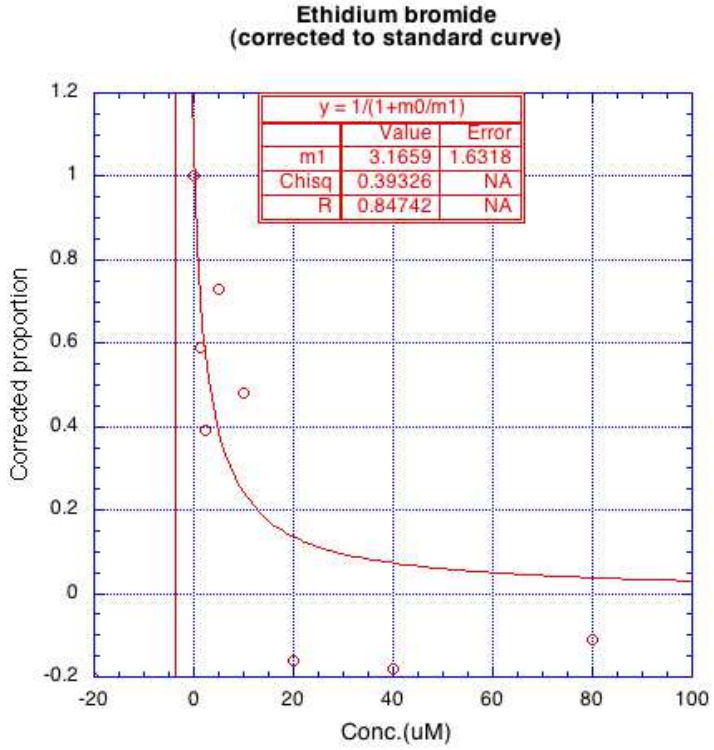


Figure 28. IC_{50} dose response curve of a. Thr-asn-his-naproxen b. Asn-thr-his-naproxen c. ethidium bromide

It was very disappointing to observe that the lead molecules obtained through the split and mix library synthesis and screening did not exhibit telomerase inhibition activity. However this phenomenon of observing false positives while screening combinatorial library is not uncommon. Shoichet et al. have extensively studied the artifactual hits observed during the screening of inhibitor libraries against enzymes and have postulated that the molecules in the combinatorial libraries form aggregates due to their nature of being organic molecules in aqueous solutions.[98,99] These aggregates sequester the enzyme and thus inhibit the enzyme

activity. It is possible that some species in our combinatorial libraries with naproxen formed aggregates and thus appeared to falsely inhibit telomerase. Additionally the combinatorial libraries are generally screened at a high concentration (250 μM) which further increases the chances of aggregate formation.

CHAPTER 4

DESIGN OF NAPROXEN BASED PEPTIDE LIRARIES WITH VARYING LENGTHS AND POINT OF ATTACHMENT OF NAPROXEN

Our next approach in the development of specific inhibitors of telomerase involved the designing of peptide libraries differing in peptide lengths and point of attachment of naproxen. The lengths and motifs were varied to synthesize molecules of different sizes and orientations which would maximize the possibility of interacting with the telomerase protein region. In the earlier chapter we observed that the synthesis of peptide libraries by combinatorial chemistry is associated with problems of aggregation and identification of false positives. Hence we decided to avoid the synthesis of combinatorial libraries and instead tried to synthesize single molecules within each designed motif.

Selection of amino acids:

Instead of randomly selecting amino acids to incorporate into the peptide libraries we sought to identify key telomerase residues which are located near the RNA/DNA heteroduplex. Since the crystal structure of telomerase TERT was unavailable, we attempted to identify residues of the TERT region which have been reported in the literature by homology study to other reverse transcriptases. As described in chapter 1, telomerase contains several conserved motifs which are important for the reverse transcriptase function. All reverse transcriptases contain a signature motif “YXDD” which is found to be essential for enzymatic activity. This motif in telomerase consists of the amino acid residues “LVDD” in its active site which is analogous to the active site residues of other reverse transcriptases.

Due to the important role played by these residues in extending the DNA strand we assumed that they are present at close proximity to the RNA/DNA heteroduplex. In order to support our hypothesis we studied the crystal structure of HIV-RT, a reverse transcriptase similar to telomerase. The active site residues in HIV-RT have the sequence "YMDD". [100] Upon studying the crystal structure of HIV-RT (PDB id – 1 HYS) using the Chimera software from UCSF, we found that the active site residues are present within 10 Å of the RNA/DNA heteroduplex. (Figure 29)

Thus there were two reasons which made these residues attractive targets: these residues were essential for enzymatic activity and these residues were at close proximity to the RNA/DNA heteroduplex. In addition, telomerase has been reported to utilize a magnesium ion present at its active site for its mechanism of action. Amino acids which have been known to interact with this metal ion could potentially be selected to incorporate in the libraries. To identify amino acids which would have the greatest interaction with the above mentioned residues, we utilized the *Atlas of protein side chain interactions* by Singh and Thornton.[101] This atlas enumerates the most likely binding partner of an amino acid in any given protein. An example of the possible interactions of amino acid is depicted in the table 8 for aspartate. Using this atlas we selected the most prevalent binding partner of the amino acids in the active site region (leucine, valine and aspartic acid). Aspartic acid had the maximum interaction with arginine and lysine. In accordance with the aim of avoiding non-specific binding, any positively charged amino acids were not selected in the library synthesis. Hence for aspartic acid we selected the second and third most prevalent binding partners. The magnesium ion has been reported to bind mainly to acidic residues.[102,103] The list of selected amino acids is provided in Table 9. Six representatives of each motif were synthesized

utilizing the selected amino acids. The sequence of the molecules within each motif is given in Table 10 and a schematic of the synthesized motifs is depicted in figure 30.

Table 8. Amino acids interacting with aspartic acid

Selected amino acids	Interacting amino acids	Number of contacts
Aspartate	Arginine	1999
Aspartate	Lysine	1845
Aspartate	Tyrosine	1090
Aspartate	Leucine	1085
Aspartate	Serine	965
Aspartate	Glycine	905
Aspartate	Asparagine	880
Aspartate	valine	849
Aspartate	Histidine	743
Aspartate	Isoleucine	740
Aspartate	Alanine	711
Aspartate	Aspartate	670
Aspartate	Phenylalanine	665
Aspartate	Glutamine	662
Aspartate	Proline	522
Aspartate	Glutamate	500
Aspartate	Tryptophan	426
Aspartate	Methionine	310
Aspartate	Cysteine	185

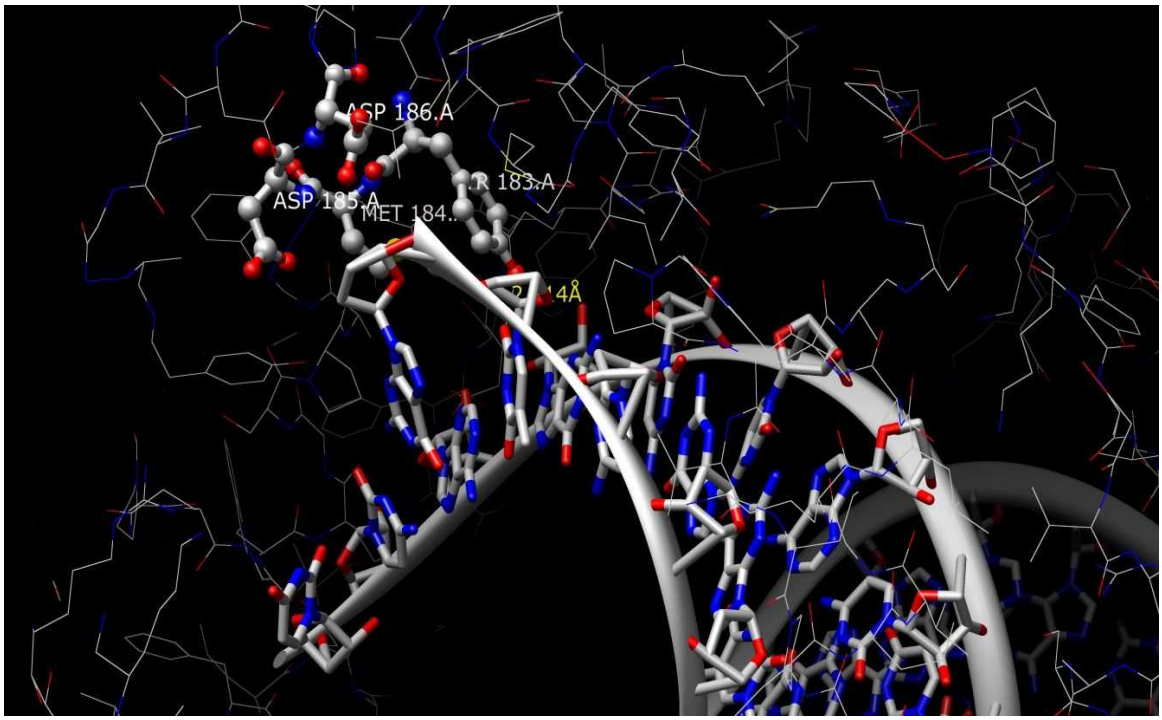


Figure 29. Active sites residues in HIV-RT present at close proximity to the RNA/DNA heteroduplex (modeled using Chimera software)

Table 9. List of amino acids selected for the synthesis of the libraries

Amino acids selected
Phenylalanine
Isoleucine
Tyrosine
Histidine
Leucine
Asparagine
Aspartic acid

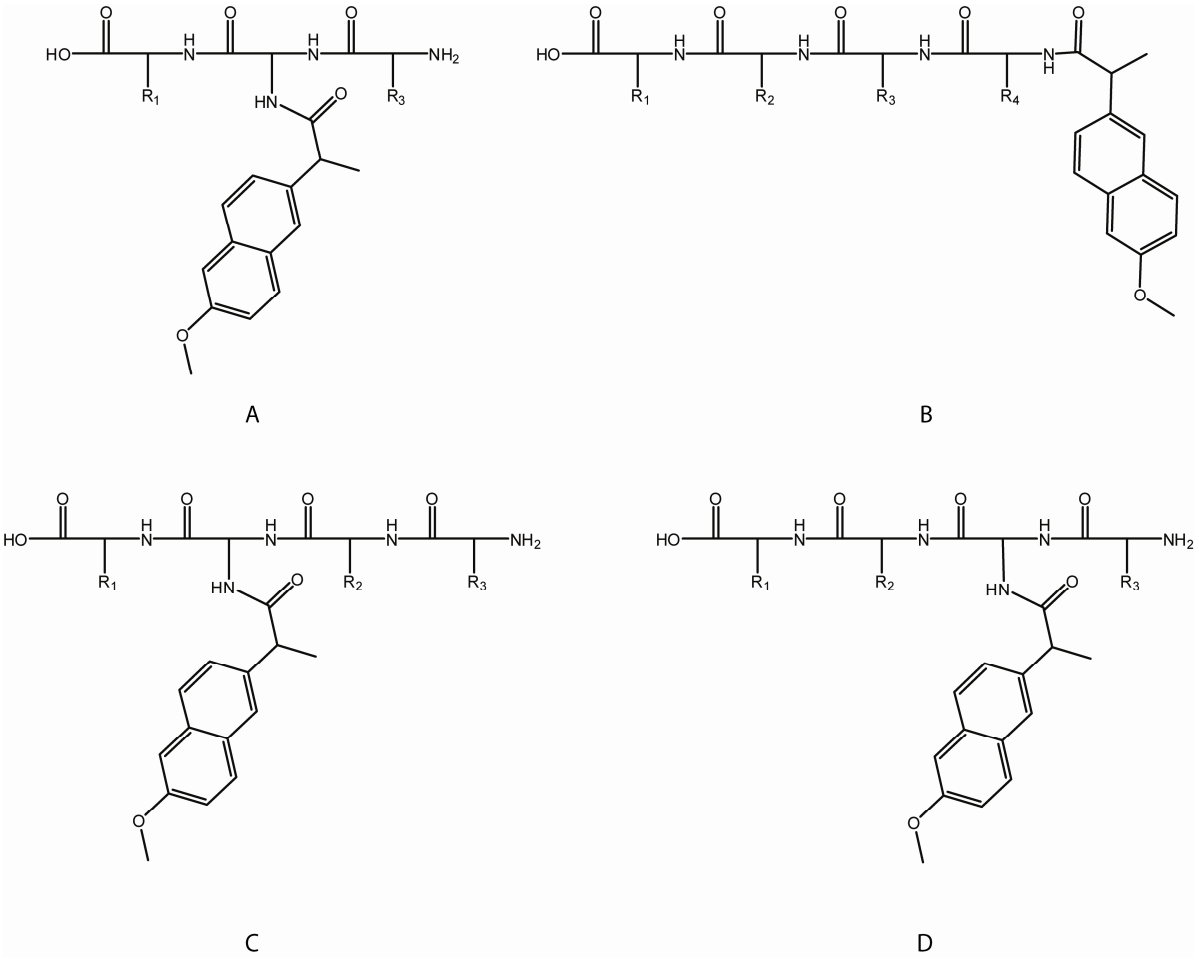


Figure 30. Motifs of peptide based libraries of naproxen. A. Tripeptide branched library; B. Tetrapeptide linear library; C. Tetrapeptide branched library with naproxen at 2nd position; D. Tetrapeptide branched library with naproxen at 3rd position

Table 10. Sequence of synthesized libraries

Tripeptide branched library			
Molecule name	R_1	Linker to attach naproxen	R_2
A1	Asp	Dap	Tyr
A2	Tyr	Dap	Asp
A3	Phe	Dap	Tyr
A4	His	Dap	Tyr
A5	Phe	Dap	His
A6	Phe	Dap	Asp

Tetrapeptide linear library				
Molecule name	R_1	R_2	R_3	R_4
B1	Phe	Asp	Leu	His
B2	Asp	Leu	Tyr	Tyr
B3	Phe	Tyr	Asn	Tyr
B4	Leu	His	Tyr	Asp
B5	His	Tyr	Phe	Asn
B6	Asp	His	Phe	Tyr

Tetrapeptide branched library (Naproxen in the 2nd position)				
Molecule name	R_1	Linker to attach naproxen	R_2	R_3
C1	Phe	Dap	His	Tyr
C2	Tyr	Dap	Leu	Tyr
C3	Asp	Dap	Tyr	Leu
C4	His	Dap	Leu	Asp
C5	Asp	Dap	Leu	His
C6	Phe	Dap	Asp	His

Tetrapeptide branched library (Naproxen in the 3rd position)				
Molecule name	R_1	R_2	Linker to attach naproxen	R_3
D1	Phe	His	Dap	Tyr
D2	Tyr	Leu	Dap	Tyr
D3	Asp	Tyr	Dap	Leu
D4	His	Leu	Dap	Asp
D5	Asp	Leu	Dap	His
D6	Phe	Asp	Dap	His

Experimental procedures:

Solid phase synthesis of peptides: The synthesis of the peptide based naproxen libraries was performed using standard solid phase chemistry as described in chapter 3. The amino acids used were D-amino acids to make the drugs more resistant to proteases. Synphase PS rink amide discs (D series) containing Fmoc protected amine were used as the solid phase for the synthesis. The loading capacity of each disc was 7.8 µmol for 9 discs. 2 discs (total loading capacity – 1.73 µmol) were used for the synthesis of each peptide. 20 % piperidine in NMP was used to deprotect the amine functionality and HATU/DIEA was used as the activating agent for the carboxy group. The amino acid to HATU and DIEA ratio was maintained at 1:1:2. For every coupling step the amino acid concentration was maintained at 300 mM.

Deprotection of the side-chain protecting 4-methyltrityl group: The branched peptide libraries have the naproxen attached to a side chain. For this purpose diaminopropionic acid was used to provide the handle for attaching naproxen. Diaminopropionic acid has the alpha amine protected by a Fmoc group which is base labile and the side chain amine protected by a mtt (4-methyltrityl) group which is acid labile. The mtt group provides orthogonal protection during the peptide synthesis. The mtt group can be removed by 1% TFA versus the 95 % TFA required to cleave the peptide from the solid support.[104] Hence during the synthesis the mtt group can be specifically removed to attach the naproxen while the peptide still remains on the solid support. After the peptide sequence was synthesized, the mtt group was deprotected using 4 rounds of 1 % TFA in 200 µl DCM. This was followed by the attachment of naproxen. The peptide libraries were cleaved from the solid support using 95 % TFA.

Analysis and screening of synthesized libraries:

The analysis of the synthesized libraries was performed using LC-MS technique. A characteristic spectrum of FYNY-naproxen is depicted in figure 31. In the LC MS spectra depicted below the lower pane represents the total wavelength chromatogram and the uppermost pane represents the extracted ion chromatogram (XIC) of the mass of the molecules. The lower pane shows multiple peaks in the total wavelength chromatogram. This indicated that there were impurities in the library. However the extraction ion chromatogram or the XIC indicated that the major peak was the synthesized product. This was confirmed by the presence of a peak of 817 amu which is the expected mass of FYNY –naproxen. The 800 amu peak corresponds to the loss of a water molecule and the 839 amu peak corresponds to the sodium adduct. We unfortunately observed impurities in several other synthesized species of the libraries. Since the major peak consisted of the synthesized product in all the cases, we performed the first round of screening with the unpurified products.

The crude synthesized molecules were tested for telomerase inhibition by using the single point PCR based assay as described earlier. It was observed that several molecules in the library were very hydrophobic and were insoluble in water at higher concentrations. Hence the assay concentration was maintained at 60 µM and the DMSO level was maintained at 5%.

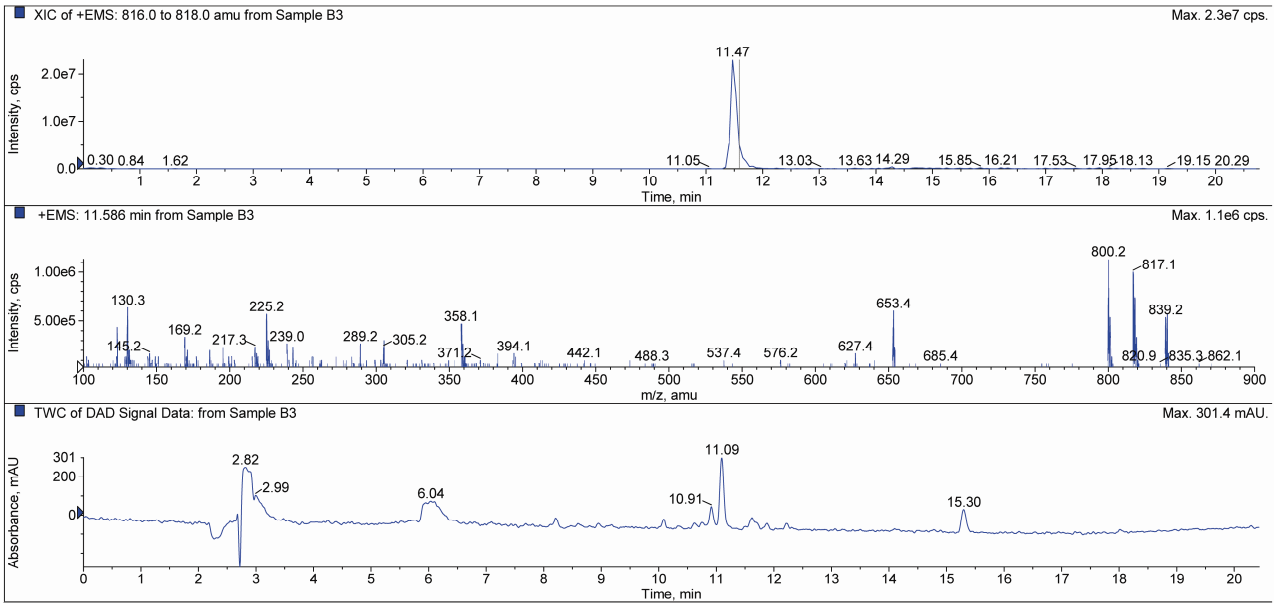


Figure 31. LC-MS spectra of FYNY-naproxen

Results:

We synthesized a total of 24 molecules based on our current strategy. The single point assay indicated that out of the synthesized molecules, 5 molecules inhibited telomerase with IC_{50} values in the micromolar range. The molecules which exhibited a greater inhibition efficacy have been listed in Table 11. Among these 5 molecules the molecule named 'B3' exhibited the strongest inhibition towards telomerase. This molecule was a linear tetrapetide with naproxen and has the sequence Phe-Tyr-Asn-Tyr-naproxen (FYNY-naproxen). (Figure 32) The telomerase activity was assayed at varying concentrations of this molecule to more accurately determine the IC_{50} for telomerase inhibition. For this purpose, the molecule FYNY-naproxen was resynthesized in a more purified form. In the fluorescent based PCR assay the concentrations ranging from 0 µM to 240 µM were used and the IC_{50} was determined as

described earlier. FYNY-naproxen inhibited telomerase with an IC_{50} of 0.25 mM. (Figure 33). The IC_{50} of FYNY-naproxen represents a 10 fold improvement in the ability to inhibit telomerase in comparison with naproxen alone.

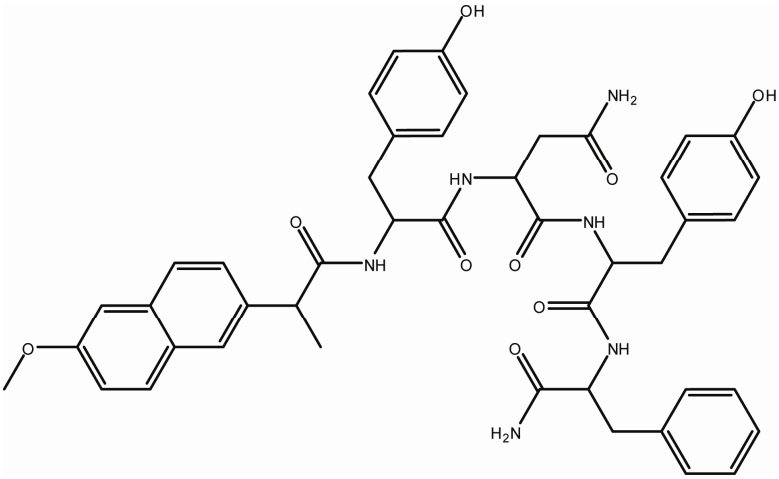


Figure 32. Phe-tyr-asn-tyr-naproxen (FYNY-naproxen)

Table 11. Results of screening against telomerase

Rank	Name	Estimated telomerase IC_{50}
1	B3	105 µM
2	D1	138 µM
3	D6	255 µM
4	C2	257 µM
5	C5	444 µM
6	A6	1981 µM

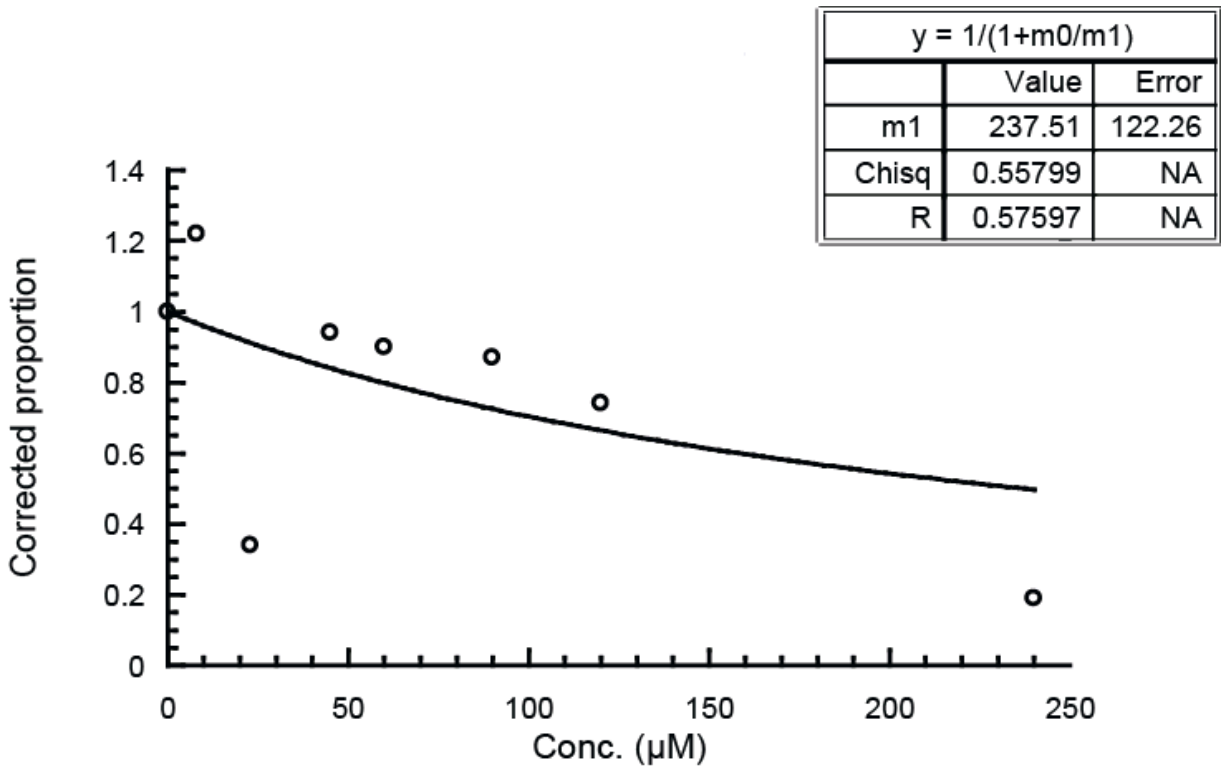


Figure 33. Dose response curve of FYNY naproxen

In this chapter, we have described a novel strategy for the development of compounds which inhibit therapeutically important protein-nucleic acid complexes. Using this strategy we have identified weak inhibitors of telomerase eg. naproxen with an IC_{50} of 3 mM. This was then used as a scaffold to introduce new interactions for the purpose of increasing the affinity to telomerase. The best of the resulting molecules had an IC_{50} similar to a strong intercalator. The essential advantage of this approach is that the scaffold is a weak binder of nucleic acids and hence the improved IC_{50} can be attributed to its increased affinity to telomerase. Additionally, since this approach utilizes the FDA approved drug naproxen as the scaffold, it reduces the chances that the derivatives will be toxic. We have synthesized and tested 24 derivatives of naproxen which is small fraction of the possible combinations of

molecules that can be synthesized using this approach. It is possible that a stronger inhibitor of telomerase can be generated by exploring other combinations of peptide sequences and motifs. We are also interested in exploring the application of this approach to weak inhibitors targeting other therapeutically important protein-nucleic acid complex, for example: HIV-RT.

CHAPTER 5

INTRODUCTION: MICRO RNA PROCESSING, ROLE IN DISEASES AND DETECTION TECHNIQUES

In 1993, Ambros et al. discovered a small RNA encoded from the gene *lin 4* which regulated the *LIN 14* gene.[105] This was the first reported regulatory function of small RNAs. In 2000 the let-7 small RNA was discovered in *C.elegans* which regulated the LET 7 gene. It was also found that the let-7 was conserved in many organisms including humans. [106] Over the years several small RNA have been identified which possess multiple regulatory roles and these have been named microRNA. MicroRNA or microRNA are 21-25 nucleotide RNA which regulate gene expression by targeting mRNA and either cleaving the mRNA or blocking translation by preventing the ribosome from binding to the mRNA.

MicroRNA processing:

MicroRNA are mainly transcribed by RNA polymerase II (Pol II) and occasionally by RNA polymerase III (Pol III).[107,108] Transcription by RNA polymerase results in the formation of primary microRNA or pri-microRNAs. [109] These can be hundred to thousand nucleotides in length, consist of a stem loop structure and possess a 5' cap and a 3' poly (A) tail. The pri-microRNA is then cleaved to produce precursor microRNAs or pre-microRNAs. Cleavage of pri-microRNA occurs at the stem of the hairpin by a protein termed Drosha. Drosha teams with DGCR8 in humans to form a complex called microprocessor complex. [110,111]DGCR8 is required for guiding Drosha towards cleaving the pri-microRNA. [111]

Multiple microRNA precursors (pre-microRNAs) can be present in a single pri-microRNA. Pre-microRNAs are approximately 70 nucleotides in length; consist of a 5'-phosphate and a 3'overhang of approximately 2 nucleotides. Further processing of the pre-microRNAs occurs in the cytoplasm. The pre-microRNAs are exported to the cytoplasm by the nuclear transport protein exportin 5 via nuclear pore complexes.[112] [113]In the cytoplasm the pre-microRNAs are acted upon by the endonuclease RNase III enzyme or Dicer. [114]

MicroRNAs silence their target mRNAs by being incorporated into a complex of proteins called Argonaute (Ago) protein to form the RNA-induced silencing complex (RISC). The proteins are characterized by the presence of a piwi-argonaute-zwille (PAZ) domain and a PIWI domain and are 100 kDa in weight. [115]The processing of the microRNA in the cytoplasm and loading into the RISC is mediated by the RISC loading complex (RLC). This complex consists of Dicer, double stranded RNA binding proteins TRBP (Tar RNA binding protein) and PACT (protein activator of PKR) and mainly of the Argonaute-2 protein.[116,117] Dicer is a highly specific enzyme and is conserved in almost all eukaryotic organisms. The enzyme identifies the stem portion of the pre-microRNA by the presence of the 5' phosphate group.[118] It cleaves the pre-microRNA about 22 nucleotides from the terminus of the pre-microRNA to release the loop joining the stem of the pre-microRNA and the terminal base pair. This results in the formation of a duplex approximately 22 nucleotides in length consisting of two strands termed microRNA: microRNA* arms. The duplex formed have characteristic 5' phosphate and a two nucleotide 3' overhang. The proteins TRBP and PACT facilitate the cleavage of the microRNA by Dicer by stabilizing the enzyme. [119,120]

The affinity of the Ago proteins for the double stranded RNA is low. Thus the dsRNA is unwound and only a single strand associates with the proteins. This strand is

complementary to the target and is called the guide strand. The other strand in the duplex is called the passenger strand (microRNA*) and it is degraded. A helicase which is responsible for the unwinding of the duplex has not been identified but several helicases such as p68, p72, RNA helicase A and human Mov10 have been found to associate with the RNAi machinery.[121,122] The selection of the strand to be loaded into the RISC complex is determined by the thermodynamic stability of the 5' end. The strand preferentially incorporated into the Ago proteins is the one whose 5' end is less tightly paired.[123,124]

The microRNA then targets the mRNA with which it has complementary base pairing and induces gene silencing by either blocking translation, by preventing the binding of ribosomal proteins or by cleaving the mRNA using the Ago proteins. The mechanism of gene silencing depends on the degree of complementarity between the microRNA and the target mRNA. It has been found that microRNA which bind to its target with perfect or near perfect complementarity, causes the degradation of the mRNA by the ribonucleases present in the RISC complex. [125]The other method by which microRNA blocks gene expression is by binding to the 3' untranslated regions (UTRs) of the mRNA targets with imperfect complementarity.[126] This blocks translation by hindering ribosome binding onto the mRNA. In this case the levels of proteins have been found to be reduced while the mRNA levels remain intact. The processing of microRNA in the nucleus and cytoplasm is illustrated in figure 34.

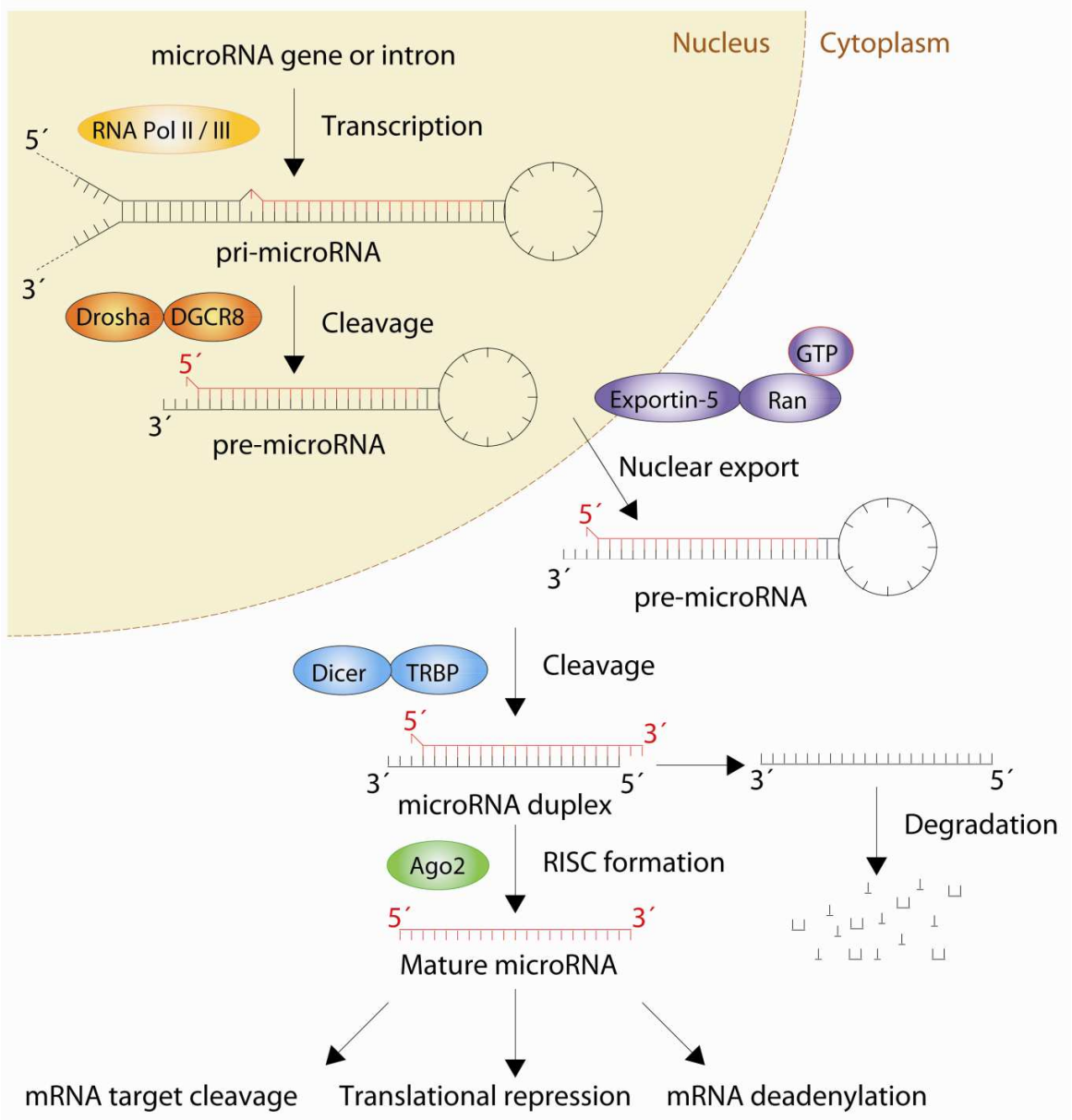


Figure 34. MicroRNA biogenesis[127]

Micro RNA and cancer:

Since microRNA plays an important role in silencing of genes, they subsequently regulate numerous cellular processes. For instance in vertebrates, microRNA regulate a varied number of processes such as pancreatic islet cell development (miR-374), adipocyte differentiation (miR-143), heart development (miR-1) and differentiation of hematopoietic cells (miR-181).[128-131] The processing of microRNAs is strictly regulated during the transcription and post-transcription processing. Any deregulation of the microRNA processing can result in increased or decreased levels which can subsequently result in disease conditions. MicroRNA have been found to have a major role in diseases like neurodegenerative disorders, diabetes, heart conditions and cancer.

Cancer is disease which is characterized by the upregulation of several oncogenes and down regulation of several tumor suppressor genes. Subsequently it was established that microRNAs regulate many genes which have been implicated in cancer. Cancer cells are characterized by uninhibited proliferation, a loss in cellular identity and a defect in the mechanism controlling apoptosis and cell death. In *C. elegans* it has been found that mutations in the microRNA genes *lin-4* and *let-7* results in discrepancies in the cell cycle and the cells undergo terminal differentiation.[132] Similarly in *Drosophila* the pro-apoptotic gene *hid* is blocked by the *bantam* microRNA. Overexpression of this microRNA caused excessive growth of wing and eye tissue. [133]

It was also observed that human microRNA genes were located at sites in the genome which are either amplified or deleted in cancer. The first report of microRNAs being involved in cancer came from Calin et al.[134] They studied a deletion on chromosome 13 which is commonly observed in chronic lymphocytic leukemia and found that two microRNA genes,

miR-15 and *miR-16* were located in this deletion. Subsequently they found that these microRNA were downregulated in patients suffering from chronic lymphocytic leukemia. Cimmino et al. reported that miR-15a and miR-16-1 negatively regulate an anti-apoptotic gene BCL 2. This gene is over-expressed in many cancers and this is due to the down regulation of the microRNAs which regulate it. [135]This finding supports the tumor suppressor role of microRNAs.

Many microRNA which are associated with cancer have been found to be located in regions of the genomes which have been called “fragile sites”. miR 125b-1 is a homologue of *C. elegans* lin 4 and is found to be located on a fragile site on chromosome 11q24. This is deleted in patients with breast, ovarian and cervical cancer.[136] The first family of microRNA found to regulate a proto-oncogene RAS was *let-7*. RAS proteins regulate cell growth and differentiation and are membrane associated signaling proteins. In approximately 15-30% of cancers the RAS oncogene was found to be overexpressed resulting in higher cellular proliferation. Johnson et al. demonstrated that this correlated with a reduction in expression of *let-7* microRNA.[137] Thus it was ascertained that microRNAs can behave a tumor suppressors.

He et al. observed that that the microRNA from the cluster miR-17-92 were overexpressed in lymphoma cell lines. It was also found that this cluster is located in a region in chromosome 13 which is found to be amplified in human B-cell lymphomas. Thus there was a correlation between the overexpression of the microRNA in this cluster and lymphomas. Using a mouse model of human B cell lymphomas, He et al. demonstrated that overexpression of the miR-17-92 microRNA increases c-Myc-induced tumorigenesis in mice. C-Myc is encoded by the Myc oncogene and regulates cell proliferation and growth and is commonly found to be overexpressed in cancer. [138]Another microRNA which is associated

with MYC overexpression is miR-155. Metzler et al. reported that miR-155 is upregulated by 100 fold in Burkitt lymphoma, Hodgkin lymphoma and B cell lymphoma. [139]

Extracellular microRNA:

The first report of the presence of microRNAs in extracellular fluid was published by Valadi et al. where they studied the exosome mediated transfer of nucleic acids between cells.[140] They observed that mRNA and microRNA were released from both mouse and human mast cell lines packaged in small vesicles of endocytic origin called exosomes. Exosomes have been found to be released from various cells such as B cells, T cells, epithelial cells, dendritic cells and tumor cells and earlier had been reported to carry proteins.[141-143] These small vesicles have been demonstrated to participate in signaling events by binding to cells via receptor-ligand interactions and being internalized by endocytosis. This indicated that the cargo packaged in the exosomes are capable of cell-cell communication. Valadi et al. demonstrated that the mRNA present in the exosomes were functional by an in vitro translational assay. Next they found that approximately 121 microRNAs were present in the exosomes released from the mast cells. Additionally some microRNAs were found in higher levels in exosomes than within the cells, suggesting that these microRNA were specifically packaged into exosomes. Some of the identified microRNA were let-7, miR-1, miR-15, miR-16, miR-181 and miR-375. These microRNA have been found to play major roles in important biological processes like angiogenesis, haematopoesis and tumerogenesis. These findings strongly suggest that exosomes behave as carriers of nucleic acids which may be involved in intercellular communication.

Another report of the presence of extracellular microRNA in serum was published by Mitchell et al.[144] They observed stable microRNA circulating in the human plasma and serum and suggested that these microRNA can be used as biomarkers. They observed that exogenously introduced microRNA were not stable in serum and were degraded in less than 2 minutes. This suggested that the extracellular microRNA were either packaged in vesicles like exosomes or were associated with proteins which imparted the stability.

Wang et al. further pursued the idea of the extracellular microRNA being involved in cell-cell communication.[145] They hypothesized that for microRNA to behave as signaling factors, the cells have to selectively identify and export the microRNA in a manner in which they remain stable. Further the microRNA should be taken up by the cells. They serum deprived cells and then investigated the presence of extracellular microRNA in the growth medium. By centrifuging at different speeds, they were able to separate cell pellets, microvesicles and exosomes. The authors reported that microRNA were released from cells packaged in vesicles or exosomes as reported by Valadi et al. but a significant amount of microRNA were present outside these vesicles. The most interesting fact was that although there were several microRNA which were present both in the vesicles and free in the supernatant, some microRNA were present specifically in the supernatant. This negated the possibility that the microRNA observed in the supernatant were due to ruptured vesicles.

The authors treated the cells with a respiratory chain inhibitor, rotenone, which reduces the cellular ATP level and found that this reduced the levels of microRNA present extracellularly but not within the cells indicating that the export of microRNA is an active process which requires energy. Interestingly, the levels of extracellular microRNAs was not affected by the treatment with 5-N, N- dimethyl amiloride (DMA) which is a known inhibitor

of exosome secretion. This further supports the fact that the extracellular microRNA are not necessarily packaged before release. Wang et al. additionally observed a substantial number of proteins being released into the extracellular media. One of them was the nucleolar RNA-binding protein, nucleophosmin 1 (NPM1). It was found that a majority of the protein was present in the supernatant but not in the vesicles. This suggests that this protein is exported with the microRNA and could be the reason for the stability of the microRNA. In order to test whether NPM1 imparts stability to microRNAs, the authors incubates a synthetic microRNA with the protein and treated it with RNase A. They found that NPM1 made the RNA resistant to RNase cleavage which supports the theory that this protein is involved in the microRNA export and stability.

Circulating microRNA and diseases:

As mentioned earlier, microRNA was found to play a major role in the development and progression of cancer. Lawrie et al. were the first to study if the microRNA overexpressed in cancer was found to exist extracellularly and if there was difference in the extracellular levels between cancer patients and healthy subjects. They studied the extracellular levels of three microRNAs: miR-155, miR-21 and miR-210 which were found to overexpressed in patients suffering from diffuse large B-cell lymphoma (DLBCL). The sera of patients suffering from DLBCL showed higher levels of the microRNA.[146] This was the first report which showed the presence of microRNA in biological fluids and suggested that microRNA could be utilized as non-invasive biomarkers of cancer.

Mitchell et al. inoculated human prostate cancer cells (22Rv1) cells into mice and were able to detect the human cancer miR-629* and miR-660 in the plasma of the mice.

These human derived microRNA did not have any orthologs in mice indicating that they were generated from the cancer cells. Further by comparing the serum of metastatic prostate cancer patients and healthy human serum, they observed that miR-141 expression was significantly higher in patients with prostate cancer and it correlated with the levels of prostate-specific antigen (PSA).[144]

Chen et al. compared the microRNA from different types of sera, healthy serum, serum from patients with lung cancer and patients with colorectal cancer using both qRT-PCR and Solexa sequencing. [147] Firstly they observed that the microRNA present in the blood and sera of healthy patients were stable and consistent among the patients. Compared to healthy patients, in patients with lung cancer 28 microRNAs were not observed while 63 new microRNAs were observed. Similarly in patients with colorectal cancer 69 new microRNA species were observed. Another interesting observation was that certain microRNA species were common for both patients with lung cancer and colorectal cancer (ex. miR-134, miR 221, miR-222 etc.)

These studies establish the fact that microRNA are present extracellularly and the microRNA profile differs in normal and disease conditions. Although the microRNA observed in disease conditions are treated as biomarkers it is highly likely that the presence of microRNA in extracellular fluids is for some specific purpose. We hypothesize that the microRNA exported from the cells have certain physiological roles.

Hypothesis:

The earlier paragraphs have established the role of microRNA as regulators of cellular development and as the causative reason of several diseases including cancer. We propose a

new role for microRNA, that as mediators of intercellular communication. Support of this hypothesis comes in the form of literature indicating the presence of microRNA in extracellular fluid. It has also been reported that the levels of the extracellular microRNA are sometimes significantly higher than intracellular levels. In some cases the microRNAs observed extracellularly are not observed within the cells suggesting a specific reason for the cells to export the microRNA into the extracellular fluid. Additionally extracellular microRNA have been found to be upregulated in disease conditions like cancer. Further the profile of extracellular microRNA has been found to be very different in normal people and cancer patients.

The roles of these extracellular microRNA have not been elucidated and they have been treated as biomarkers of disease conditions. [148,149]Our hypothesis is that these small RNAs are released as signaling factors and can play a major role in progression of diseases like cancer. We hypothesize that in cancer, the microRNA are released extracellularly as signaling factors for proliferation. To elaborate we believe that specific microRNA are released from cancerous cells and are taken up by normal cells in the vicinity which then causes those cells to display increased proliferation.

Dr. Subhashree Rangarajan and Dr. Samit Shah, prior graduate students from our laboratory, initially designed a method to capture microRNA which existed extracellularly. They immobilized ethidium benzylic acid, an intercalator which binds nucleic acids, onto a resin. This resin was then introduced into the media of culturing cells via a transwell. We observed some promising but inconsistent results where the cells exposed to resin modified with the intercalator had fewer cells which were alive.

Additional support of our hypothesis is the reports of RNases which have been found to be quite effective in the treatment of cancer. Pouckova et al. reported that polymer bound RNase effectively inhibited tumors in mice. [150,151]Bovine serum RNase or BS-RNase was modified with hydrophilic poly[N-(2-hydroxypropyl) methacryl-amide] (PHPMA) to prevent its degradation or fast elimination. These polymer coated RNases were more cytotoxic when administered intravenously compared to native BS-RNase. Experiments using RNase radiolabeled with 125-I exhibited that the native BS-RNase was internalized by tumor cells within 1 hour while polymer conjugated RNases was not internalized. Even after 24 hours of administration 40% of the conjugated RNases remained in the blood stream while 98% of the native RNase was eliminated.[152,153]

We intended to test our hypothesis by:

1. Determining the presence of microRNA in the media of culturing HeLa cells
2. Identifying the extracellular microRNA
3. Generically removing nucleic acids (specifically RNA) from the extracellular media of culturing HeLa cells (a cervical cancer cell line) and observing effect on proliferation
4. Capturing the extracellular microRNA and observing the effect on proliferation

Methods of detecting microRNA:

The detection of microRNAs is challenging due to the short length, limited number in cells and the lack of a poly (A) tail to allow PCR amplification. Most methods of microRNA detection depend on hybridization. There exists two main methodologies of detecting microRNA namely the solid phase (where hybridization of sample microRNA occurs to probes bound on a solid surface) and solution phase (hybridization occurs in solution). Solid

phase techniques are generally useful in case of high throughput screening and solution phase is more applicable where in-vivo determination of microRNAs are required.

Solid phase techniques:

Northern blotting: In the method, the microRNA are separated via gel electrophoresis. This is followed by transferring the microRNA on a nitrocellulose membrane and then soaking it in a solution containing the microRNA probe. The probe has a sequence complementary to the microRNA sequence and is fluorescently labeled or radiolabeled. After hybridization, the unhybridized probes are washed away and the microRNA detected. The disadvantage of this method is that it is a long process requiring over 24 hours for completion. The sensitivity of this technique was improved by Varallay et al. by using LNA modified probes. Due to the greater stability of an LNA modified probe the hybridization could be performed at the higher temperature of 50 ºC. This reduced the assay time from 16 hours using DNA probes to 2 hours when using LNA modified probes. [154,155]

Microarrays:

This is the most common method used to perform a high-throughput detection of microRNAs. [156]In this method the probe having a sequence complementary to the microRNA is bound to a solid surface. The cDNA of the microRNA of the sample is made and is fluorescently labeled or biotinylated. The cDNA is allowed to bind to its complement on the solid surface and the unbound species are washed away. To the biotinylated cDNA a streptavidin labeled fluorescent probe is added. Detection of the fluorescent signal intensity aids in understanding the expression level of the microRNA. This method is very useful in the

detection of multiple microRNAs in a very expeditious manner. The only disadvantage of this technique is that microarrays are still expensive to fabricate and there is a lower threshold of RNA sample required in order to allow detection. Hence microRNA which are expressed in very low amounts can miss detection by this technique.

Microarrays using electrochemical method of detection:

This method was developed by Gao et al. where the target microRNA were conjugated to electrocatalytic species such as OsO2 nanoparticles or $Ru(PD)_2Cl_2$. DNA probes with sequence complementary to the target are immobilized on a solid surface. After hybridization, the target is detected by observing an increase in current. The advantage of this system is that is has very high sensitivity.[157,158]

Label free detection of microRNA:

The above discussed methods of microRNA detection employ a label (fluorescent or electrochemical). Driskell et al. developed a label free method of microRNA detection using silver nanorods which are adsorbed on a glass slide. The microRNA are allowed to then adsorb onto the silver nanorods. This is followed by detection of the surface enhanced Raman scattering spectra. This method is very reproducible and shows very high specificity. But the disadvantage of this method is that individual spectra of the microRNA have to be taken before the assay in order to record the representative spectra and there is a problem that sequences which have overlapping peaks cannot be differentiated.[159]

Solution phase methods of microRNA detection:

qRT-PCR: The most popular method of microRNA detection in the solution phase is reverse transcriptase polymerase chain reaction (RT-PCR).[160]Performing qRT-PCR on microRNA includes a few extra manipulations since the lack of a poly (A) tail on the microRNA poses a challenge in the cDNA synthesis. Thus in this method the mature microRNA which lacks a poly (A) tail is poly adenylated using poly (A) polymerase. A poly dT primer is then added which anneals to the poly (A) tails and gets extended by means of a reverse transcriptase resulting in a cDNA. PCR is performed by using two primers in which the forward primer is specific to the microRNA sequence and the reverse sequence is standardized. The PCR amplification is monitored in real time by using a fluorescent dye like SYBR green which binds specifically to dsDNA. The main advantage of this system is that RT-PCR can quantitatively detect very small amounts of RNA.

Another method of solution phase detection of microRNA is by using fluorescence correlation spectroscopy developed by Neely et al. In this method two fluorophore labeled oligonucleotides are added to the microRNA sample. The labeled oligonucleotide hybridizes to its complementary microRNA sequence. The unbound labeled oligonucleotides are bound with an oligonucleotide with its complementary sequence but containing a quencher. Thus the background fluorescence is significantly reduced and this allows the detection of very low concentration of RNA (up to 500 fM). [161]

Cissel et al. developed a method of RNA detection by employing the luminescent enzyme Rluc. Two complementary sequences of oligonucleotides were conjugated to the N-terminal and C terminal fragments of the enzyme. When the sequences hybridize the two fragments are brought to close proximity resulting in the formation of the active enzyme

which displays luminescence. In the presence of the microRNA, the sequence competes with the microRNA sequence for hybridization. Hence there is a decrease in luminescence which indicates the presence of the target microRNA. The advantages of this method is that it is rapid and Rluc based assays have high sensitivity.[162]

CHAPTER 6

EXPERIMENT TO OBSERVE AND IDENTIFY SMALL RNA IN THE EXTRACELLULAR MEDIA OF CULTURING CELLS

The presence of extracellular microRNAs has been reported by several researchers as described in chapter 5. However we wanted to ascertain that microRNA were being released during the culturing of HeLa cells and further identify the released microRNA. In order to determine the presence of microRNA in media, we performed RNA extraction on the media of culturing cells, radiolabeled the RNA and then visualized them by radioimagery. An illustration of the scheme of the experiment is depicted in figure 35.

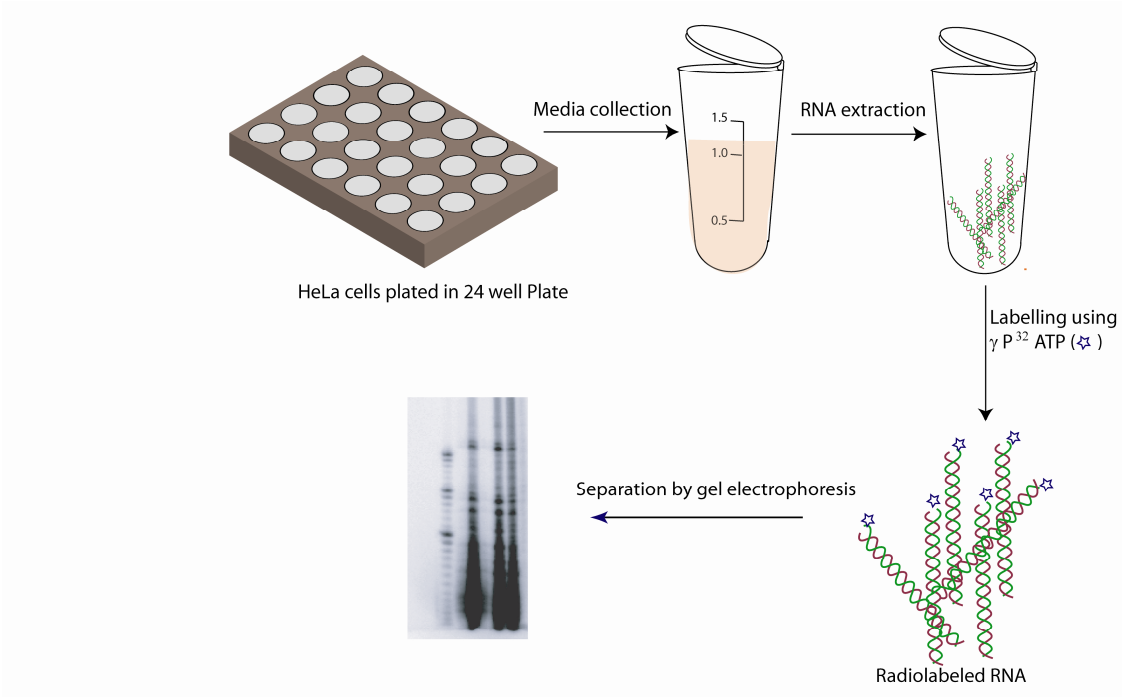


Figure 35. Schematic representation of experiment to determine the presence of microRNA in the media of culturing HeLa cells

Materials and method:

Cell culture: I was assisted in the initial cell culture studies by Piyush Jain. HeLa cells were plated by Piyush Jain at 5%, 30 % and 100% confluency in a 24 well plate in 480 µl media (DMEM with 10% FBS) and cultured for 24 hours. After 24 hours the media was collected and RNA extraction was performed on the collected media. As a control RNA extraction was also performed on media in which cells had not been cultured. This was done to ensure that any microRNA observed was not from the fetal bovine serum present in the media.

RNA extraction: 500 µl of TRI reagent was added to the tubes containing media. The tubes were vortexed and then allowed to stand for 5 minutes at room temperature. Then 200 µl of chloroform was added to the tubes and the tubes were gently shaken. The tubes were then allowed to stand for 15 minutes at room temperature in order to allow for phase separation, followed by centrifugation at 12,000 g for 15 minutes. The colorless aqueous layer was collected in a separate tube and the RNA was precipitated using 500 µl of isopropanol. The tubes were stored at -20 ºC for 12 hours. The RNA was precipitated by centrifugation at 12000 g for 10 minutes at 4 ºC. The pellet was then washed 3 times with 75 % ethanol and resuspended in 5 µl of RNase free water.

Dephosphorylation of the 5' phosphate of the extracted RNA: In order to visualize the extracted RNA, the nucleic acids were radiolabeled using the the enzyme polynucleotide kinase (PNK) and γ-P^{32} ATP. This enzyme catalyzes the transfer of a phosphate group from the γ-P^{32} ATP to the 5' hydroxyl group of the nucleic acid. Thus the 5' end of the nucleic acid had to be dephosphorylated for this enzyme to act. This was accomplished using the enzyme calf intestinal alkaline phosphatase.

Briefly, 4 µl of the extracted RNA was dephosphorylated by adding 4 µl of RNase free water, 1 µl of 10 x dephosphorylation buffer (1x composition - 50 mM tris-HCl (pH 8.5), 0.1 mM EDTA) and 1 µl of calf intestinal phosphatase (Invitrogen) and incubating at 37 ºC for 1 hour. The reaction was terminated by the addition of 1 mM of EDTA and heating at 65 ºC for 15 minutes.

PNK labeling : To the microcentrifuge tube containing RNA, 2.5 µl of 10 x PNK buffer (0.5 M tris-HCl, pH 7.5, 100 mM $MgCl_2$, 100 mM β-ME**)** , 2µl of γ-P^{32} ATP (10mCi/mmol) and 1.55 µl polynucleotide kinase in kinase dilution buffer (50 mM tris-HCl, pH 8.0) (USB) was added. The solution was incubated at 37 ºC for 30 minutes. The reaction was terminated by heating at 65 ºC for 5 minutes in presence of 170 µl of 5 M guanidine HCl. The radiolabeled RNA was precipitated using 6.6 µl of 20 mg/ml glycogen, 75 % ethanol and centrifuging at 17400 g for 30 minutes at 0 ºC. The pellet was washed twice with 75 % ethanol and allowed to dry for 20 minutes at room temperature. We also radiolabeled DNA molecular weight markers in a similar manner. The RNA was taken up in 5 µl of RNase free water. 1 µl of the RNA was added to a loading buffer consisting of 80% formamide, 10 % TBE and loaded on a 8 % poly acrylamide gel. Gel electrophoresis was done for 1 hour, followed by drying of the gel for 1 hour. The gel was exposed for 1 day using phosphoimager plates (Kodak phosphor storage screen) and then read using a gel reader (Molecular Dynamics 640 Storm Phosphorimager).

Results:

The image of the gel is depicted in figure 36. Prominent bands which corresponded to the length of microRNAs (18-24 nucleotides) were observed in all the lanes where RNA had

been extracted from the media of culturing cells. These bands were absent in the lane of media which had not been in contact with culturing cells indicating that the RNA were released from the cells and were not from the serum present in the media. This gel image supported our hypothesis that RNA which appeared to correspond in size to microRNAs were released into the media from culturing HeLa cells. However, it was interesting to note that the bands appeared identical in nature even though the cells had been cultured at different confluencies.

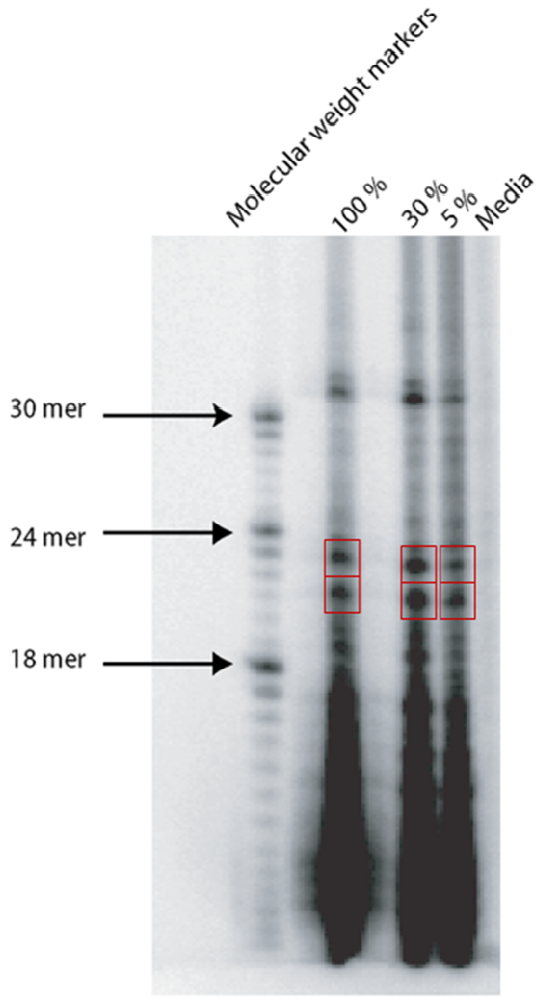


Figure 36.Gel image showing bands which correspond to microRNA

Quantitation of the extracellular microRNA:

We were required to have an estimate of the amount of microRNAs that were being released into the media. This was necessary for selecting the method of identification of the microRNAs (microarray versus qRT-PCR). Typically microarrays require a larger amount of microRNA compared to qRT-PCR method of identification. In order to accomplish this we labeled known quantities (40 – 0.625 attomoles) of an oligonucleotide while labeling the extracted RNA. The known quantities of radiolabeled oligonucleotide and the extracted RNA were separated using gel electrophoresis. The radioactive signal obtained from the known quantities of oligonucleotide was quantitated and plotted against the number of moles to generate a standard curve. The signal intensity from the extracted RNA was converted into moles using this standard curve.

For this experiment RNA was extracted from the media of cells cultured at 5% and 100 % confluencies. The media was collected in 1.5 ml microcentrifuge tubes and the tubes were centrifuged at 120,000 g for 1 hr at 4 ºC. The hard centrifugation was done in order to remove any cells which might have been collected inadvertently. More importantly it had been reported by Valadi et al., that microRNA were released encapsulated in exosomes from cells. We were interested in the microRNA which were present free in the media. The hard centrifugation had been reported to effectively precipitate exosomes. We removed the top 200 µl of the media to avoid the precipitated exosomes and then performed RNA extraction on the media as described earlier. The RNA was radiolabeled as described earlier along with varying moles of an 18 nucleotides long oligonucleotide. These species were then resolved by gel electrophoresis. The signal from the varying moles of the oligonucleotide was quantitated and

the signal intensity was plotted against the number moles to generate a standard curve (Figure 38). Upon quantitating the signal intensity of the bands and using the standard curve, we found that the amount of microRNA in the extracellular media ranged in attomoles (11-23 attomoles). This is an amount that would not be sufficient for identification using the microarrays. Hence we opted for using the qRT-PCR method of identification of the microRNA.

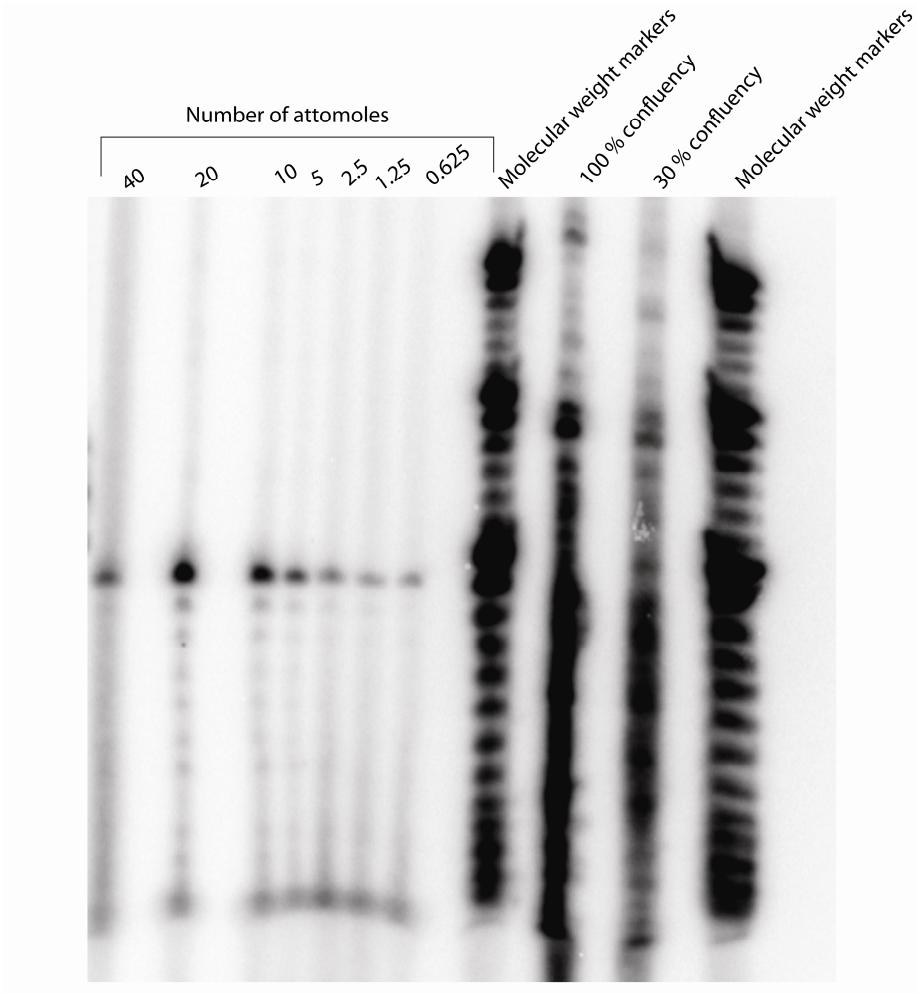


Figure 37. Gel depicting different concentration of labeled 18 mer oligonucleotide and characteristic bands obtained from media of culturing cells.

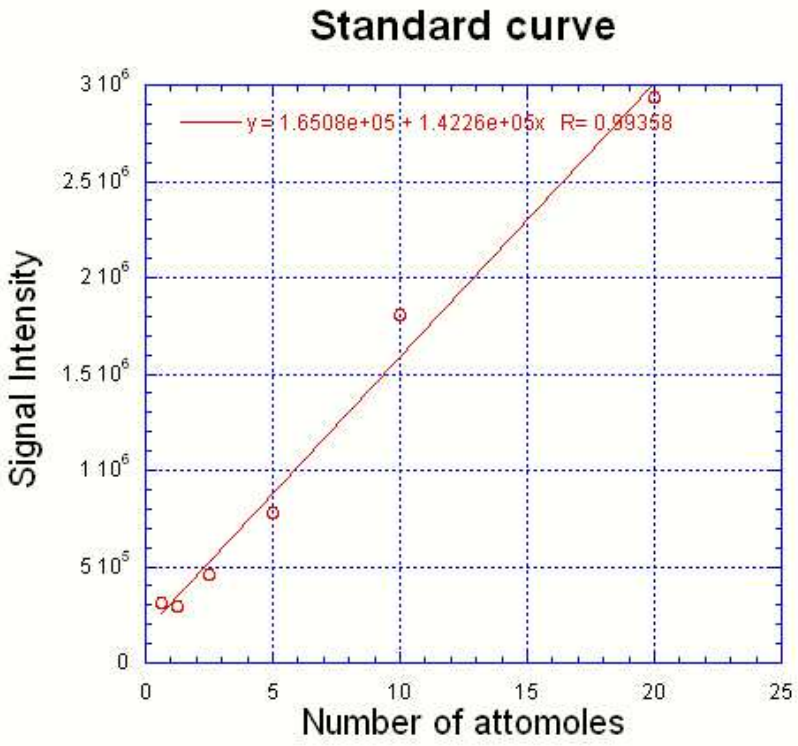


Figure 38. Variation of signal intensity of a radiolabeled oligonucleotide with change in concentration

Identification of extracellular microRNA:

The qPCR method of microRNA identification has been described in chapter 5. The identification of the extracellular microRNA released from culturing HeLa cells was done using the Genome - wide qPCR microRNA expression profiling performed by System biosciences. The primer complementary to all the known microRNAs listed in Sanger's database were present in three 384 well plates. An illustration of the procedure in provided in figure 39. Briefly in this procedure the microRNA is poly adenylated at the 3' end using the enzyme poly (A) polymerase and ATP. The microRNA are then converted to the cDNA using

an oligo dT primer attached to a known sequence of oligonucleotides and a reverse transcriptase enzyme. Hence the cDNA synthesized by this technique is specific due to the microRNA sequence but has a common sequence at the 3' end. This cDNA is then amplified using polymerase chain reaction. The reverse primer in the reaction is the complementary to the 3' end of the cDNA and is common to every well. The forward primer is specific to each microRNA.

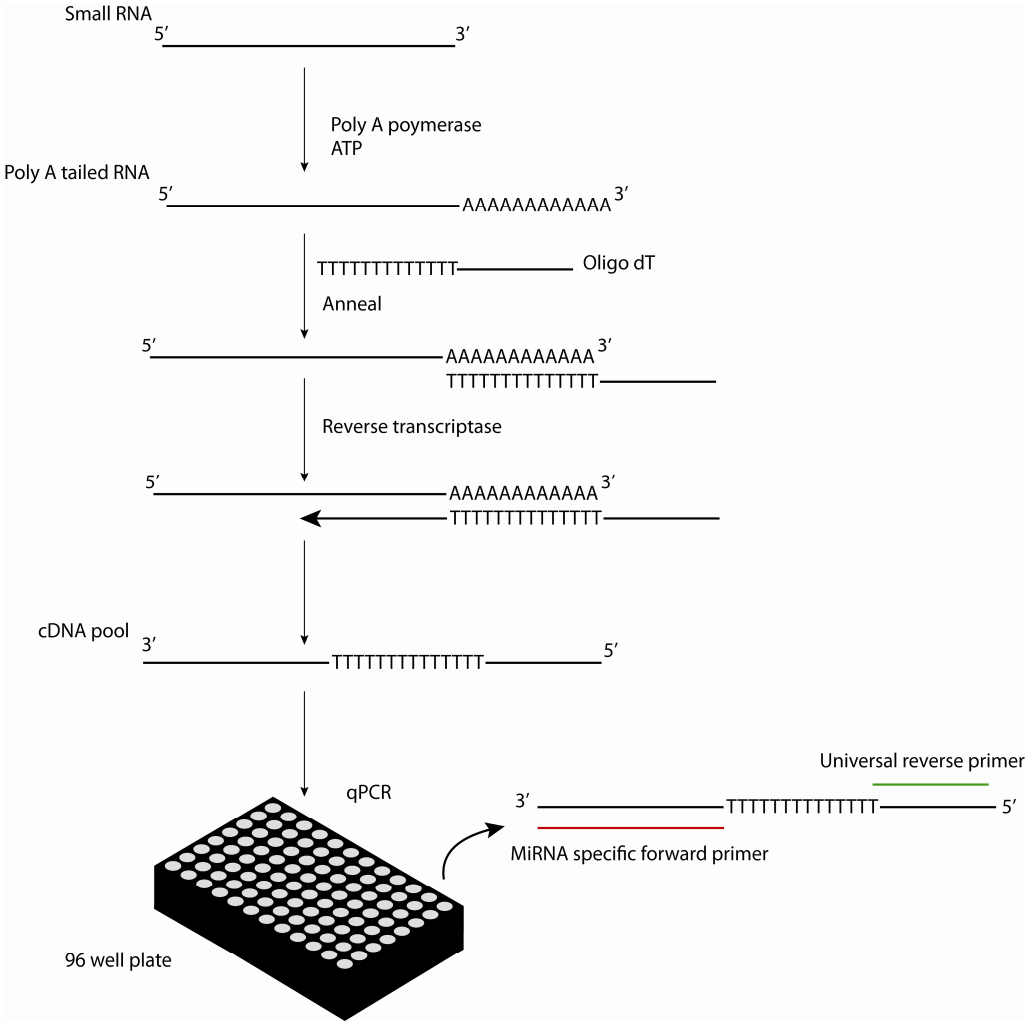


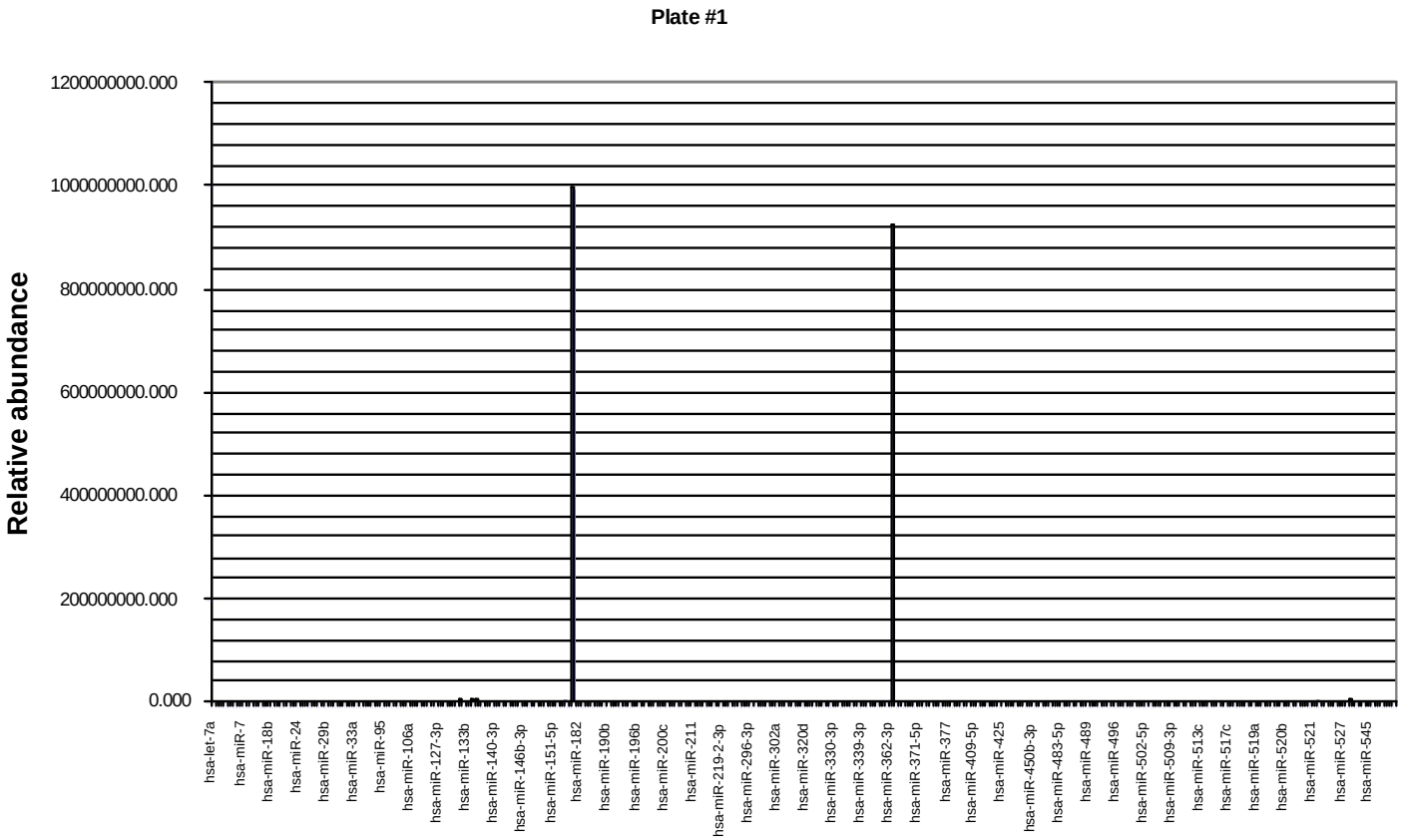
Figure 39. Schematic representation of qRT-PCR method of microRNA detection

In order to identify the extracellular microRNA, HeLa cells were plated at 30 % confluency in a 24 well plate in Optimem (serum free media). This confluency was selected based on two assumptions: firstly that at this stage the cells would be proliferating which would induce them to release microRNA and secondly that at 30 % confluency there would be sufficient cells to generate adequate amounts of microRNA for detection. The plating was done in serum free media in order to reduce any possibility of interference with any serum ribonucleases. After culturing the cells for 24 hrs, the media was collected and spun at 100,000 g for 1 hour. The upper portion of the media was taken and RNA extraction performed as described earlier. A portion of the RNA was radiolabeled and visualized by gel electrophoresis to confirm the presence of small RNA. The remaining portion was frozen on dry ice and then shipped to Systems Biosciences.

Results:

In the three plates used for identifying the microRNA, plate 1 and plate 2 contained the primers complementary to the microRNA strands which were the guide strands. As explained earlier these are the strands which block gene expression. Several of these microRNA were observed in the media removed from culturing cells. Since this media had been subjected to a hard centrifugation and only the upper layer had been used, these microRNA were not present bound within exosomes. In plate 3, the primers complementary to the passenger strand (microRNA*) were present. Interestingly one such strand (miR 125b-1*) was also observed in high amounts in the media while the miR 125b-1 strand was not observed.

The names of the microRNA present in high concentrations are listed in Table 12. Among all the observed microRNA miR-181c and miR-362-5p are present in significantly higher amounts.



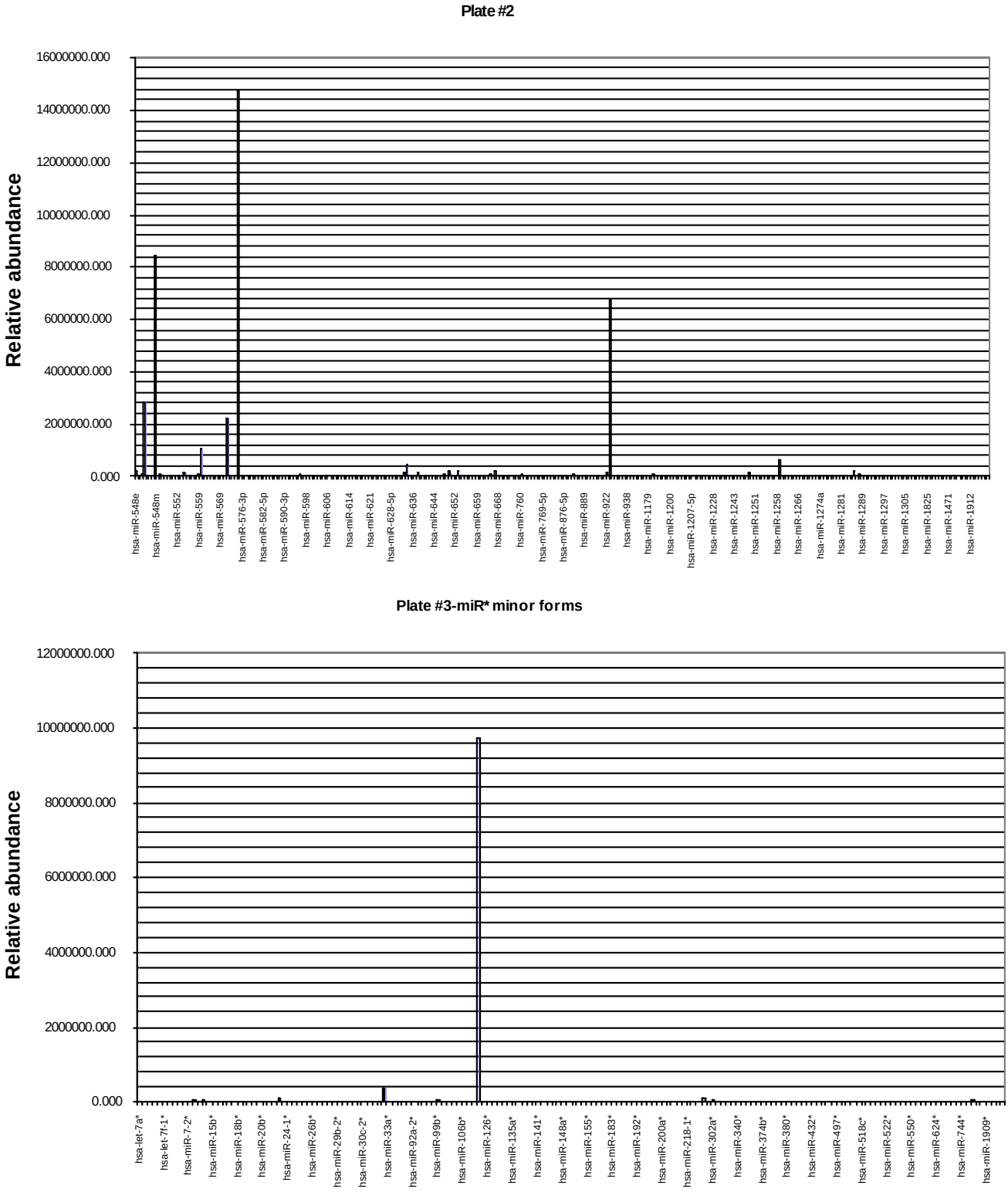


Figure 40. Graphical representation of relative abundance of extracellular microRNA

Table 12. List of microRNA observed in the media of culturing HeLa cells

MicroRNA sequence	Relative abundance (Million)
hsa-miR-181c	996
hsa-miR-362-5p	923
hsa-miR-125b-1*	9.7
hsa-miR-548l	8.5
hsa-miR-923	6.7
hsa-miR-135a	7.5
hsa-miR-539	6
hsa-miR-135b	3.8
hsa-miR-132	3.8
hsa-miR-181a	3
hsa-miR-574-5p	1.5
hsa-miR-523	1.3

It was interesting to observe that some microRNAs which were observed in the media of HeLa cells had already been implicated in cancer. The microRNA 181c which is the most abundant species observed in our analysis is a derivative of the miR-181 precursor. Depending on the position of cleavage during processing this precursor generates different mature microRNAs named miR 181a-d. Ji et al. found that hepatocellular carcinoma (HCC) cells in which the Wnt/catenin pathway is activated behave as hepatic cancer stem cells. It

was also observed the all four miR 181s (a-d) were overexpressed in these cells.[163] Two other microRNA from the list, miR-135 a and b have been implicated in colorectal cancer. It had been reported that a major initiating event of colorectal cancer was the inactivation of the adenomatous polyposis coli (APC) gene. Nagel et al. demonstrated that miR-135 a and b target the APC gene and suppress its expression. Thus an upregulation of these two microRNAs correlated with a low APC gene expression.[164] These reports indicate that it would be interesting to observe if the removal of these microRNA from the extracellular fluid had any effect on the characteristics and behavior of the cells.

CHAPTER 7

DESIGN OF METHOD TO GENERICALLY CAPTURE EXTRACELLULAR MICRO RNA

The role of the extracellular microRNA observed in blood, serum etc. have not been elucidated and the presence of microRNA in the extracellular fluid has often been treated as biomarkers of diseases. Our hypothesis is that these extracellular microRNA behave as signaling factors. This hypothesis could be tested by observing the effect on a certain cellular function upon removal of the extracellular microRNA. In order to test our hypothesis we decided to observe the effect of removing microRNA from the extracellular media on the proliferation of cancer cells.

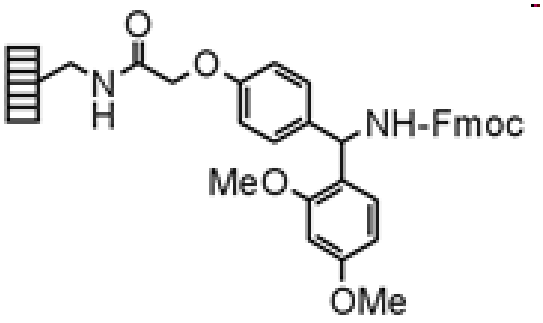
In the earlier chapter we established the presence of microRNA in the media of culturing cancer cells. In preliminary studies, Dr. Subhashree Rangarajan and Dr. Samit Shah demonstrated that introducing an agent which binds nucleic acids exclusively in the media of culturing cells causes a reduction in cellular proliferation. Their experimental design involved the covalent immobilization of an intercalator onto a resin. However any unreacted intercalator could potentially cause the observed proliferation reduction.

In order to address this concern we designed a different system by which an agent capable of binding to nucleic acids was suspended into the media of culturing cells. SynPhase PA rinkamide discs (Mimotopes) were used as the substance used to capture nucleic acids. These discs were grafted with polyamide which allowed the use of these discs in both organic and aqueous solutions. The discs contained an amine group which was protected by the base labile Fmoc group. Removing the Fmoc group resulted in the discs having a free amine group which would be positively charged at physiological pH. Hence the disc would be cationic in

nature and could potentially bind the negatively charged nucleic acids. As a control a disc was designed which would have all the amine groups capped with acetic anhydride and thus would potentially not bind to nucleic acids. We decided to test for the change in proliferation of HeLa cells (a cervical cancer cell line) in presence of the discs. Since we believed that the extracellular microRNA were behaving as proliferative signals, by removing these microRNA from the media of culturing HeLa cells we expected to observe a reduction in cellular proliferation.

a.

b.



c.

Figure 41. Schematic depiction of a. image of polyamide coated lanterns; b. disc with rinkamide linker and fmoc protected amine; c. disc whose amine functionality has been capped with acetic anhydride and a disc with a free amine functionality

Before testing this system in cells, we wanted to ascertain that a deprotected disc (which is cationic in nature) was capable of removing nucleic acids from a solution and a capped disc would not bind nucleic acids. Hence initially two discs were modified and placed in microcentrifuge tubes containing nucleic acids and the reduction in the nucleic acid concentration in the solution was monitored.

Materials and methods:

Deprotection and capping of discs: Two polyamide coated discs (SynPhase, Mimotopes) were swelled in NMP for 30 minutes. The Fmoc group was removed by treating with 20 % piperidine in 200 µl NMP twice for 30 minutes each. The discs were then washed 5 times with NMP. One disc was then capped using 10 % acetic anhydride, 5% DIEA in 200 µl NMP for 1 hour and then washed 5 times with NMP. Both the discs were washed 5 times with water to remove the organic solvents. The discs were blocked with 1 mg/ml of BSA for 12 hours to block any non-specific binding of nucleic acids.

Testing discs for ability to bind nucleic acids:

Experimental procedure: 200 nM of a 22 nucleotide ssDNA was taken in two microcentrifuge tubes. To one microcentrifuge tube the cationic disc was added and to the other the capped disc was added. 2 µl aliquots were removed at different time intervals, diluted to a final volume of 200 µl (assay conc. 10 nM) and the concentration of nucleic acid remaining in solution was determined using Quant-iT™ OliGreen ® ssDNA (Invitrogen) reagent. This is an ultrasensitive, green-fluorescent nucleic acid stain used for quantitating oligonucleotides in solution. OliGreen® gives a characteristic fluorescence (excitation 485

nm, emission 535 nm) upon binding to single stranded nucleic acids. A standard curve was prepared using different concentrations (0 nM – 10 nM) of the single stranded DNA. The fluorescence of the solution was recorded at different time points and the concentration was determined from the standard curve.

Results:

The results of the binding study are in figure 42. Firstly the standard curve depicts a linear change in fluorescence intensity with change in oligonucleotide concentration. 2 µl of oligo solution was taken from each of the microcentrifuge tubes over a period of 24 hours, diluted with OliGreen® in 1 x tris-EDTA buffer (200 µl final volume) and the fluorescence intensity was recorded. This fluorescence intensity was converted into oligonucleotide concentration using the standard curve. The oligonucleotide concentration was plotted against time using the Kaleidagraph® software. The results (depicted in figure 43) indicated that there was a reduction in the concentration of nucleic acid in the microcentrifuge tube containing the cationic disc. There was no change observed in the oligonucleotide concentration in the microcentrifuge tube containing the capped disc. This indicated that the cationic disc effectively removed single stranded nucleic acids from a solution while the capped disc (blocked with BSA) was incapable of binding to nucleic acids.

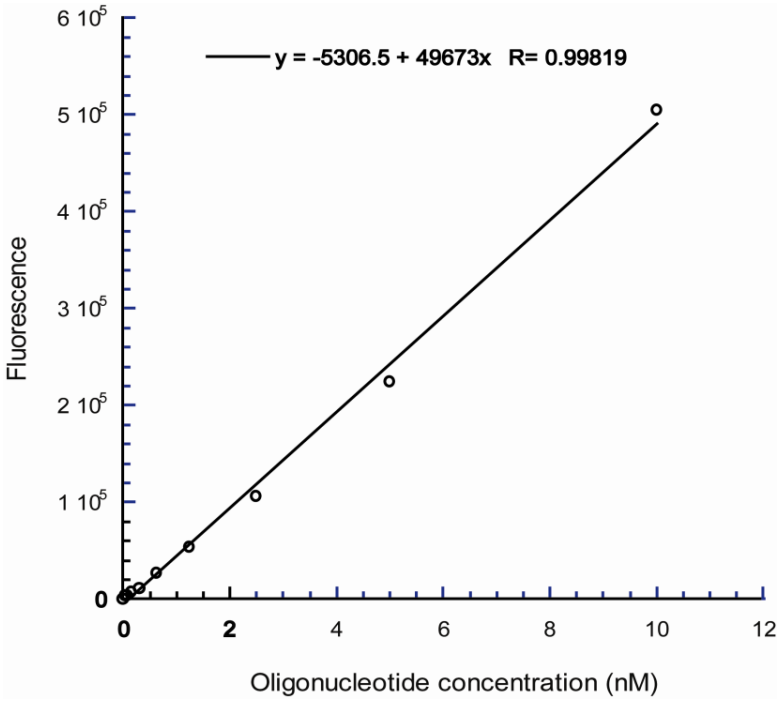


Figure 42. Standard curve with OliGreen®

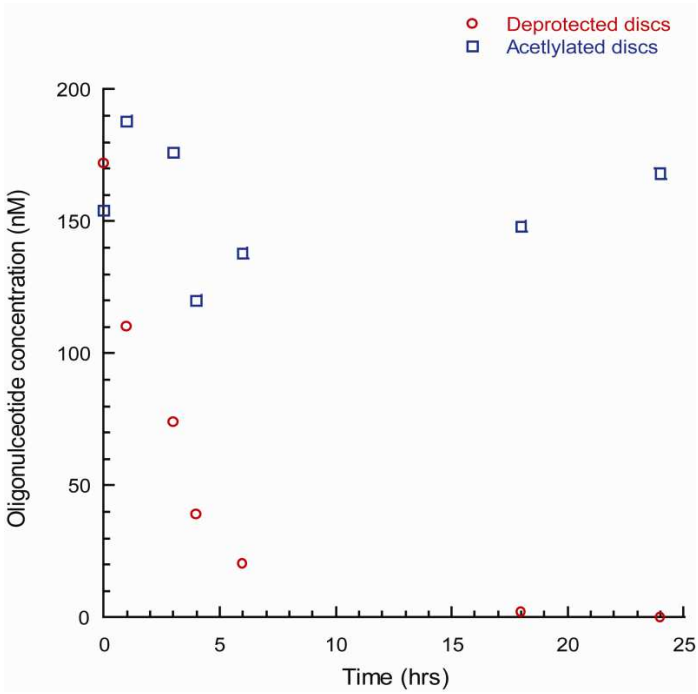


Figure 43. Capture of nucleic acids using cationic discs

Biological study using disc:

It was ascertained in the earlier experiment that the cationic disc was capable of capturing nucleic acids. We initially wanted to observe the effect of removing extracellular microRNA from the extracellular media on cellular proliferation. In order to determine the effect, it was required to introduce the cationic disc into the media of culturing cells. A method of suspending the cationic discs in the media on culturing cells was designed by Dr. Friedman by screwing pins on the lid of a 96 well plate. The discs were suspended into the media of the culturing cells using the pins at a distance of 0.2 mm from the base. Pictures of the 96-well plate lid modified with pins and discs suspended using pins in depicted in figure 44. HeLa cells were cultured in the 96 well plate. The effect on cellular proliferation was observed by performing MTT assay.

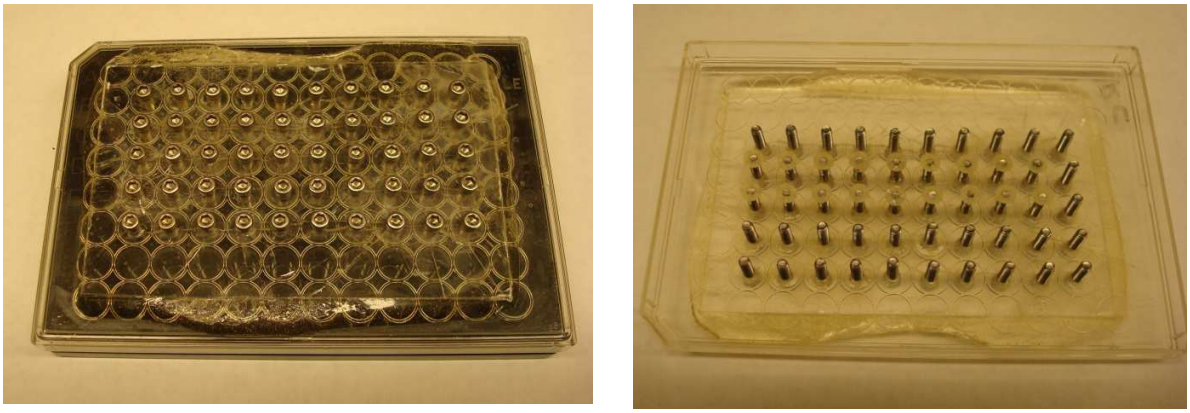


Figure 44. A. Lid of a 96 well plate in which screws had been inserted. B. discs suspended from screws inserted through the lid of a 96 well plate.

Materials and methods:

Twenty replicates of polyamide discs were deprotected as described earlier. Ten discs were then capped using 10 % acetic anhydride. Capping of the deprotected amine groups made the disc neutral and was used as a control. Cells not exposed to discs were used as another control. The discs were soaked overnight in RNase free water. HeLa cells were plated at 5 % confluency in DMEM (Invitrogen) supplemented with 10% FBS. The media was changed to 10 % serum containing colorless media (no phenol red) after 12 hours. Media without phenol red was used because it was observed that the cationic disc bound to phenol red which in turn had an adverse effect on the cells. At this point the discs were suspended from the pins screwed onto the lid. The lid was placed over the 96 well plate containing cells and the cells were allowed to culture for 4 days. At the end of 4 days the discs were removed from the media of the cells and MTT assay to test for cellular proliferation was performed on the cells.

MTT assay for cellular proliferation: The MTT assay is a colorimetric assay which indicates the number of viable cells. MTT is a tetrazole which is yellow in color and is reduced to purple colored formazan by mitochondrial reductase present in living cells. The purple formazan has a characteristic absorbance maximum at 490-500nm. Thus the greater number of viable cells the greater in the purple color formation which would be indicated by a higher absorbance at 490 nm. We used the CellTiter 96® Non-Radioactive Cell Proliferation Assay from Promega for the assay. The assay involved the addition of 20 µl of MTT solution to 200 µl of colorless DMEM in each well of the 96 well plate. The plate was then incubated at 37 ºC for 1-4 hours. The plate was read at an absorbance of 450 nm using the plate reader.

Results:

The graph depicted in figure 45 is an average of 10 repeats to the experiment. The results indicate a decrease in absorbance in the row of cells exposed to deprotected discs, compared to the row of cells not exposed to discs. It can be assumed that the deprotected discs were having an effect on the cellular proliferation possibly by removing nucleic acids from media. It was interesting that row of cells exposed to the capped disc were also having an effect on cellular proliferation. It was possible that the acetylated discs were also removing nucleic acids from media.

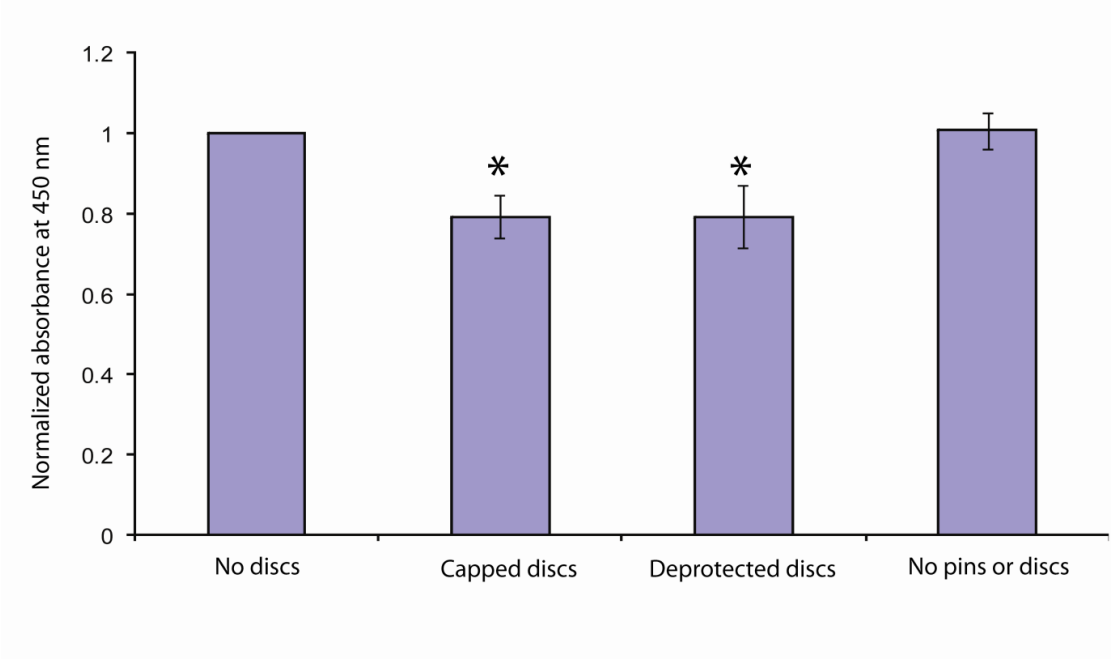


Figure 45. Results of MTT assay

We have observed earlier that discs which have not been blocked by BSA are also capable of binding nucleic acids. In the in-vitro study the discs were blocked with BSA in order to prevent any non-specific binding of nucleic acids. Hence the capped discs did not

remove any nucleic acids from the solution. However in the in-vivo study we did not block the discs with BSA in order to not introduce other foreign substances in the cell culture studies.

Potentially there could have been another explanation for the reduction in the cellular proliferation that we were observing in the presence of discs. It was possible that the discs were removing essential nutrients from the media or were undergoing degradation and thus effecting the growth of the cells. In order to control for this phenomena, we allowed deprotected discs and capped discs to stand in media for 1 day. This would allow the discs to either bind to components of the media or undergo degradation and leach into the media.

In order to mimic our earlier experiment we allowed media to stand in well without any discs. As another control media also was placed in wells not containing any pins or discs. This pre-treated media was then added to cells plated at 5 % confluency in a 96 well plate. The cells were allowed to culture for 3 days and then MTT assay for performed on these cells. The results in figure 46 indicate that there is no reduction in cellular proliferation when the cells were exposed to the preconditioned media. Thus this indicates that the reduction in the number of viable cells is due to the removal of some species which is released from the culturing cells.

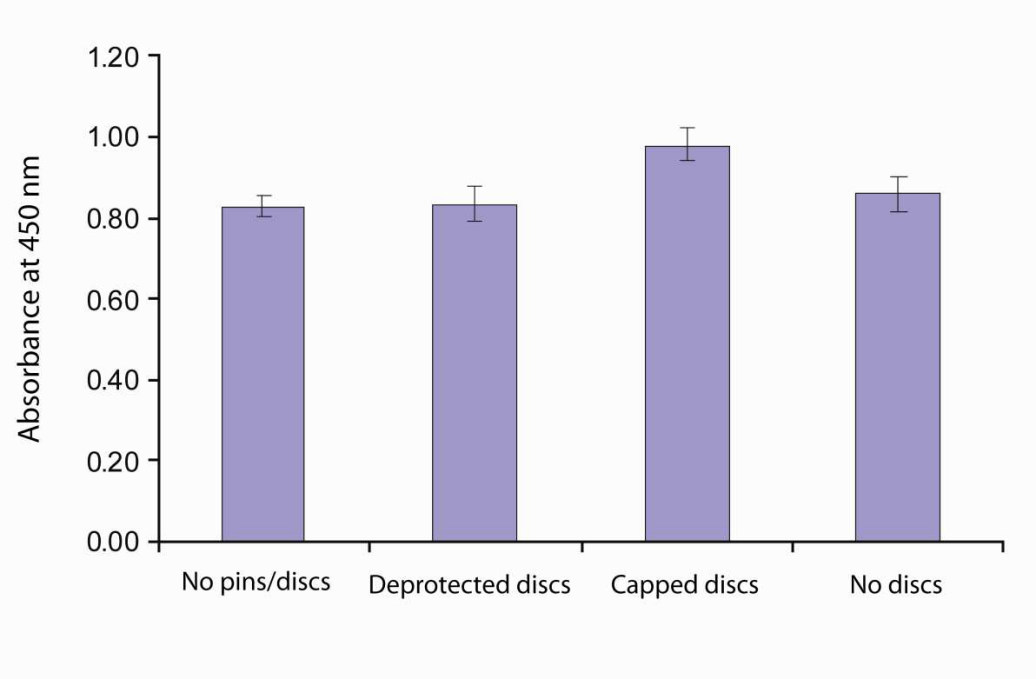


Figure 46. Results of MTT assay performed on cells cultured in conditioned media.

Immobilization of RNases on a solid support:

We demonstrated earlier, that discs capable of binding nucleic acids caused a decrease in the number of proliferating cancer cells. In order to further support our hypothesis that the effect on proliferation is due to extracellular microRNA, we decided to subject the microRNA to cleavage by RNases. Ribonucleases or RNases are nucleases which cleave RNA and thus render them inactive. Puckova et al. demonstrated that polymer bound RNases were more effective as anti-cancer therapy.[152] We wanted to observe whether solid bound RNases were capable of cleaving extracellular microRNA and whether there was a correlation with the reduction in cellular proliferation as described earlier.

We again used polyamide grafted discs as our solid support, which have an amine group protected with a Fmoc group. As described earlier these discs have a free amine group

upon deprotection. In order to ensure that there was no interference due to the binding of nucleic acids by the disc we capped the amine group with glutaric anhydride. The RNase was then reacted to the carboxy handle of the glutaric acid by the formation of an amide bond with the amines present in the protein.

Numerous ribonucleases exist which cleave different forms of RNA. Some examples include:

1. RNase A: it specifically cleaves single stranded RNA near the 3' end of cytosines and uracils.

2. RNase T1: it also cleaves single stranded RNA near the 3' end of guanosines.

3. RNase V1: this ribonuclease cleaves double stranded RNA non-specifically

4. RNase H: this ribonuclease cleaves RNA which is in the form of a RNA/DNA heteroduplex resulting in single stranded DNA.

At this point we were not aware whether the microRNA were exported in the extracellular media in the form of single strands or duplex. Hence we selected two RNases: one which cleaved single stranded RNA (RNase A) and the other which cleaved duplexes (RNase V1) for our experiments.

Experimental procedure:

Modification of the polyamide grafted discs with glutaric anhydride: The discs were soaked in NMP for 30 minutes followed by deprotection of the amine group with 200 µl of 20% piperidine in NMP. The deprotection was performed twice for 30 min each. The discs were washed 5 times with NMP and then allowed to react with 300 mM glutaric anhydride, 600 mM DIEA for 12-15 hours. The discs were then washed 5 times with NMP. Any

unreacted amine group was capped using 10% acetic anhydride, 5% DIEA in NMP for 1 hour. The discs were washed 5 times with NMP followed by 5 washes with RNase free water.

Reaction of the discs with RNase: The discs were reacted with EDC (1M) and NMI (100 mM, pH 6) in 200 µl for 1 hour. The reaction solution was aspirated and the discs washed with RNase free water. We activated the carboxyl group with the water soluble carbodiimide prior to the addition of the RNases in order to prevent any formation of amide crosslinks between proteins. RNase A was obtained from Sigma-Aldrich and RNase V1 was obtained from Ambion Inc. We also found that the reaction of the RNases to the solid support was dependent on the pH of the solution. We reacted the discs to the RNases in phosphate buffered saline (sodium phosphate 10 mM, NaCl 138 mM, KCl 2.7 mM) at pH 4, 6 and 8. 1 mg of RNase A (conc. 0.58 mM) and 5 µl of RNase V1 (0.1 U/ µl) was taken in a final volume of 200 µl. The disappearance of the ribonuclease was characterized by UV-Vis spectrophotometer. RNase A has a λ_{max} at 280 nm and an extinction coefficient of 9700 $M^{-1}.cm^{-1}$ at 280 nm. The extinction coefficient of RNase V1 was not reported. Hence the number of moles reacted on the discs was calculated using the extinction coefficient of RNase A.

Results:

It was determined that the reaction of RNase to the solid support occurred most efficiently at pH 8. The UV-Vis spectra in figure 47 depicted a characteristic peak at 280 nm before the start of the reaction (0 hour) and after 17 hours of reaction at pH 8. A decrease in absorbance at 280 nm was observed after 17 hours. This indicated a decrease in the RNase concentration in solution due to the reaction with the solid support. The concentration of

RNase remaining in solution was determined using RNase A extinction coefficient. The initial concentration in solution at 0 hour was 0.58 mM and the final concentration after 17 hours of reaction was 0.44 mM. We calculated that 33nmoles of RNase had reacted onto the solid support. This indicated that 28% of the starting number of moles of RNase had reacted to the disc.

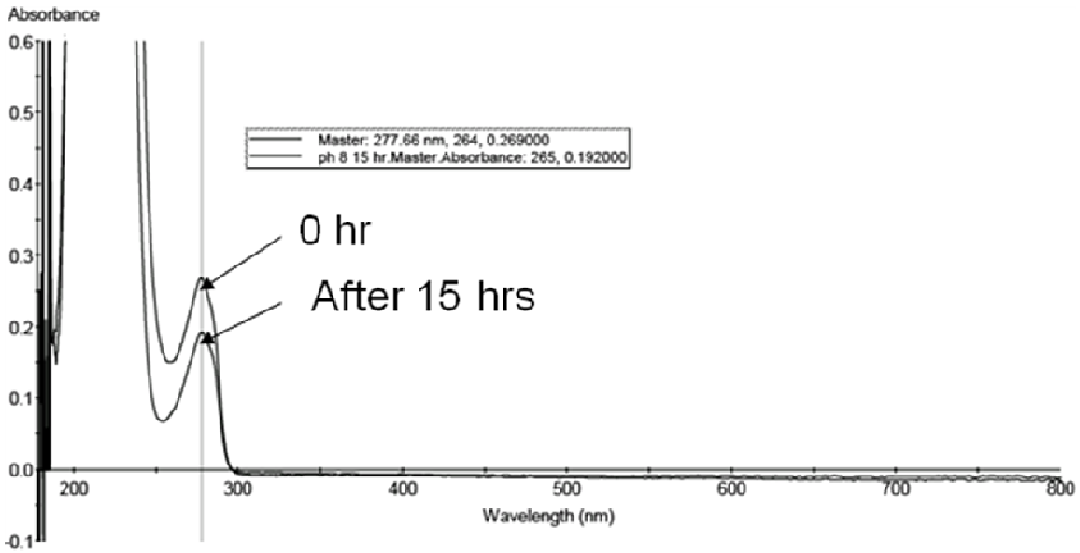


Figure 47. UV-vis spectra depicting the absorbance spectra of the RNases at different time points of reaction.

Testing the ability of solid support bound ribonucleases to cleave RNA:

Before introducing the RNases which were covalently bound to a solid support into the biological system it was necessary to confirm that the RNases retained their ability to cleave RNA. In order to test the activity of the RNases, the discs were introduced into a solution of ssRNA and a solution of dsRNA. We tested RNA cleavage using the dye OliGreen®. This dye exhibits fluorescence upon binding to nucleic acids. As the nucleic acids

are cleaved to shorter fragments, the binding of the dye is hindered and hence a reduction in fluorescence is observed.

Experimental procedures:

500 nM of a ssRNA was taken in 200 µl of 1 x tris-NaCl buffer and 20 nM of dsRNA was taken in 200 µl of 1 x tris-NaCl buffer in two separate microcentrifuge tubes. RNase modified discs were added to the microcentrifuge tubes. From the tube containing dsRNA, 20 µl of solution was removed at regular intervals and 2 µl of solution was removed from the tube containing ssRNA. OliGreen® was added to the samples to make a final volume of 200 µl. The fluorescence was detected using a spectrofluorometer and the fluorescence was converted in to concentration using a standard curve as described earlier. In order to ensure that the RNA cleavage is not due to the inadvertent introduction of RNases into the buffer we allowed the RNA (single stranded and double stranded) to remain in the buffer in microcentrifuge tubes in the absence of the RNase modified discs. Samples were taken from these tubes over a period of time and the integrity of the RNA was checked using OliGreen®.

Results:

The graph in figure 48 represents the proportion of ssRNA remaining in the solution as a function of time. There is a reduction in the concentration of ssRNA with time in the microcentrifuge tube containing RNase modified discs. The ssRNA from the microcentrifuge tube containing buffer alone did not exhibit any change in concentration. This indicated that

the reduction in the amount of full length RNA to which OliGreen® could bind is due to the RNases bound to the discs.

Similarly the graph in figure 49 represents the decrease in proportion of the dsRNA with time. It was interesting to observe that the cleavage of the dsRNA was a slower process compared to the cleavage of the ssRNA. Again the dsRNA stored in the buffer in the absence of the RNase modified disc did not show any decrease in concentration. This established that that the RNases retained their ability to cleave ribonucleic acids even after being covalently immobilized on the solid support.

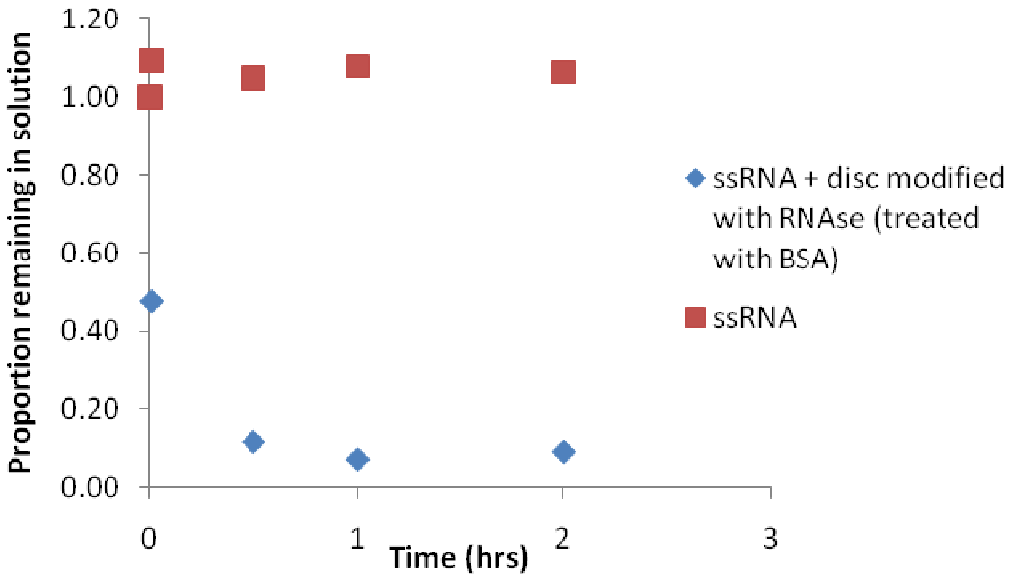


Figure 48. Change in concentration of full length single stranded RNA with time

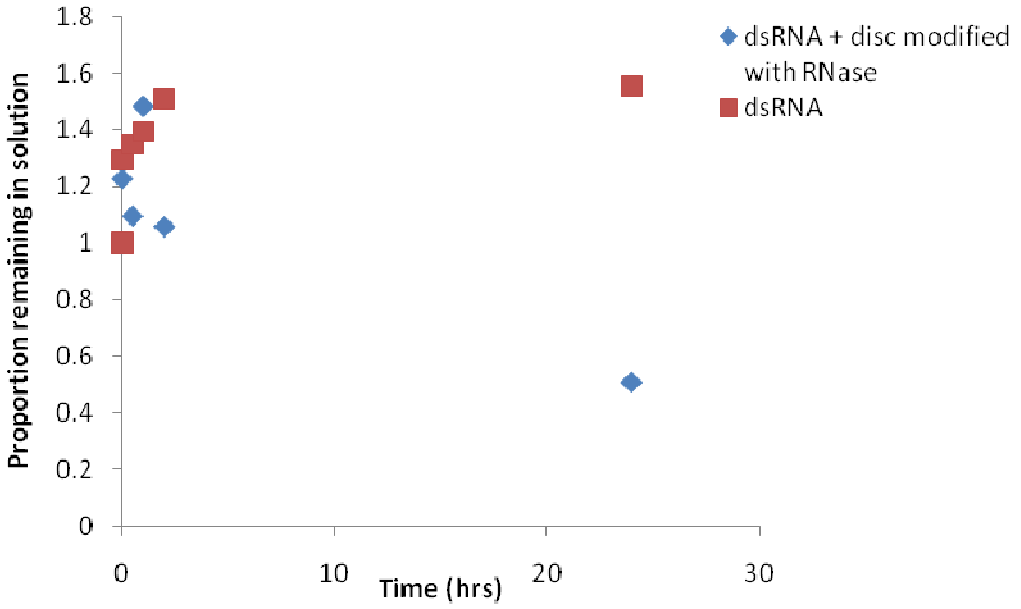


Figure 49. Change in concentration of full length double stranded RNA with time

Biological study using RNase immobilized on discs:

We observed in that introducing a cationic disc to the extracellular media of culturing cells causes a reduction in cellular proliferation. If a similar reduction in proliferation is observed upon introducing RNase modified discs, it could be potentially due to the removal of the extracellular RNA from the media. In order to test this we covalently reacted ribonucleases to a solid support. After ensuring that the immobilized RNases retained their ability to cleave RNA we suspended them into the media of culturing cells as described in earlier. After allowing the cells to culture for several days, an MTT assay was performed on the cells to observe the effect on cellular proliferation.

Experimental procedure: Ribonucleases were covalently attached to 10 polyamide grafted discs as described earlier. HeLa cells were plated at 5% confluency in a 96 well plate in DMEM (Invitrogen) supplemented with 10% FBS. The media was changed to 10 % serum containing colorless media (no phenol red) after 12 hours. The discs were suspended into media using the lid with pins. As a control 10 acetylated discs (discs whose amine group is capped with acetic anhydride) were also suspended into the media of cells. The cells were allowed to culture for 4 days and at the end of 4 days MTT assay was performed as described earlier.

Results:

The graph depicted in figure 50 is an average of two repeats of the experiment. The trend observed is similar to the trend observed with the cationic discs. The RNase modified discs cause a decrease in cellular proliferation. However a decrease in cellular proliferation is also observed with the acetylated discs. This phenomenon has been observed earlier and we attribute it to the potential non-specific binding of nucleic acids to the acetylated discs. This result appears to support our hypothesis that the reduction in cellular proliferation is due to the removal of extracellular microRNA.

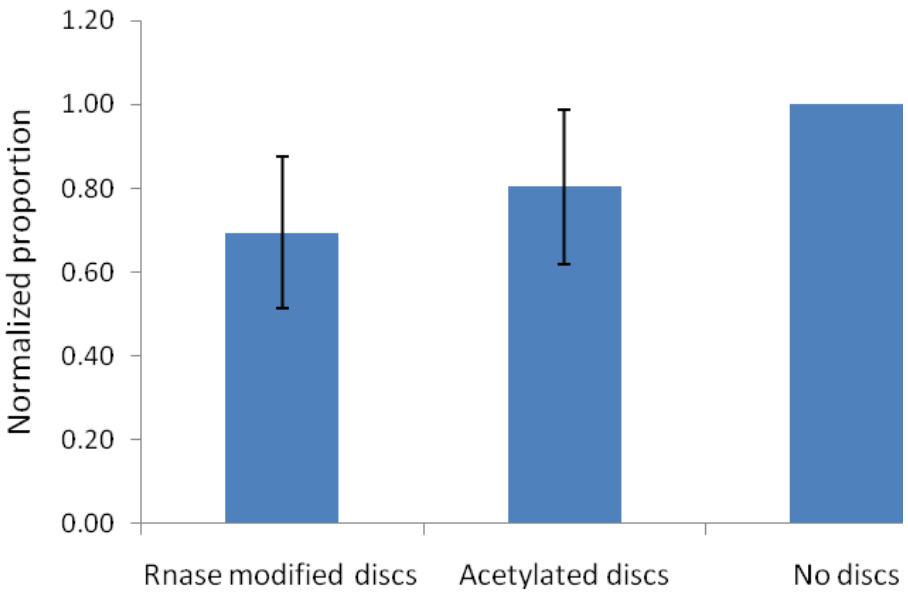


Figure 50. Result of MTT assay

In this chapter, we used two different techniques to remove RNA from the extracellular media of culturing cells. Both the cationic discs which can bind nucleic acids and discs with RNases covalently bound to them showed a decrease in the cellular proliferation. The decrease in the number of viable cells was also similar (approximately 40% less viable cells). This indicates that it is the possible removal of microRNA from the extracellular media is causing this decrease. However conclusive proof of this hypothesis can only be obtained if we can identify the microRNA species which has an effect on cellular proliferation.

CHAPTER 8

DESIGNING OF A DISC CAPABLE OF SPECIFICALLY CAPTURING EXTRACELLULAR MICRORNA FROM MEDIA OF CULTURING CELLS

In chapter 7, we demonstrated that the generic capture of extracellular microRNA by a cationic disc resulted in the decrease in cellular proliferation. After identifying the extracellular microRNA present in the media of culturing HeLa cells we were interested in identifying if any one or more microRNA were responsible for the decrease in the proliferation which was observed. This could potentially be accomplished by specifically removing a single microRNA from the media using its complement. In order to ensure that the complement is only capturing the extracellular microRNA and not affecting intracellular microRNAs, we designed a system to immobilize the complement on the solid surface.

Experimental design:

Rink amide polyamide discs were used as the solid support on which the complements were immobilized. The complements of the observed extracellular microRNA were designed to contain 2' O methyl modified nucleotides. These oligonucleotides have a methoxy group at the 2' position instead of the hydroxyl group. This imparts the molecules with greater stability. The reasons for selecting 2'O methyl RNA as the antisense were manifold. Firstly, Majlessi et al. demonstrated that 2' O methyl RNA formed duplexes with RNA which had a very high melting temperature, second only to a duplex formed between two stands of 2' O methyl RNA. RNA-DNA duplexes did not have such a high degree of stability.[165] This suggested that an oligonucleotide with 2'O methyl RNA bases acted as a better complement

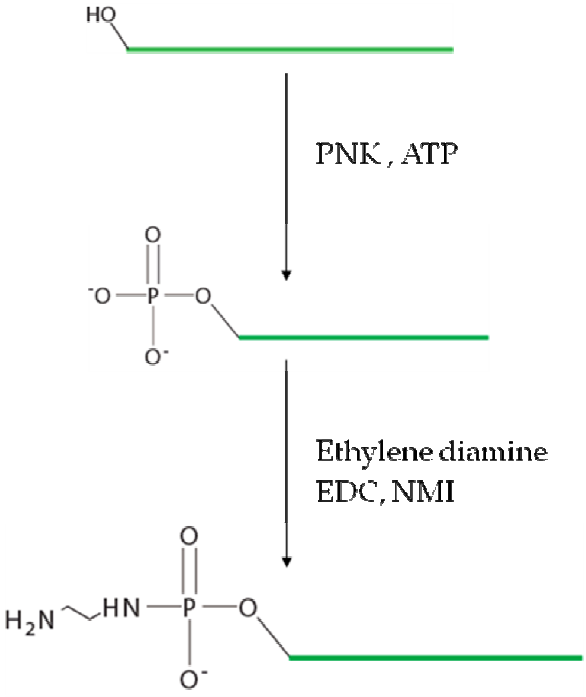
than oligonucleotides comprising of deoxynucleotides. Additionally 2' O methyl RNA modified oligonucleotides are inherently resistant to nucleases. Further Meister et al. were able to demonstrate that adding the 2' O methyl antisense of miR-21 to S100 cell extract prevented the cleavage of the miR-21 target mRNA.[166] This indicated that 2' O methyl RNA complements of microRNA were capable of blocking their activity.

In order to block miR-21 activity in glioblastoma cells, Chan et al. transfected different concentrations of the 2' -O-methyl-oligonucleotide complementary to miR-21 into the glioblastoma cell lines. The cells were analyzed by Northern blots 2 days post-transfection for the presence of miR-21. These analyses confirmed that the target microRNA became undetectable after introduction of the 2' -O-methyl-oligonucleotide in the low nanomolar range. The authors hypothesized that the binding of the 2'O methyl RNA to its target was so tight that it could not even be dissociated during the northern blot procedure.[167] This established that 2' O methyl RNA modified nucleic acids bound RNA efficiently and were stable in cells and cellular extract.

The polyamide discs used for the generic capture of microRNA could be used as the solid support on which the complement could be immobilized. The discs contain an amine group which can be used as handle to attach the antisense modified with a carboxy or phosphate group. However any unmodified amine group would be positively charged and could potentially result in non-specific binding of nucleic acids. Thus the synthetic scheme we followed involved the reaction of the amine groups with glutaric anhydride. This would result in an amide linkage between the amine group and one of the carboxy groups of glutaric acid. The other carboxy group can be used as a handle to attach amine modified 2' O methyl RNA. In order to introduce the amine group to the 5' end of the 2' O methyl RNA, the

oligonucleotide was first phosphorylated and then reacted with ethylene diamine to form a phosphoramidate linkage with a free amine group. The synthetic scheme is depicted in figure 51.

Polyamide discs have the advantage that they can be used in both polar and non polar solvents.[168] Thus the reaction of the amine groups on the disc with glutaric anhydride was performed in NMP (organic solvent) and the reaction of the glutaric acid modified discs with the 2' O methyl RNA complement was performed in water. The synthesis of an amine modified 2' O methyl RNA and the subsequent reaction to the solid surface had to be carried out in a polar solvent in order to dissolve the oligonucleotides. The activating agent used for this reaction was the water soluble 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). Initially in order to test the reaction scheme we decided to only modify the antisense of miR-181c with the amine functionality and immobilize it on the solid support.



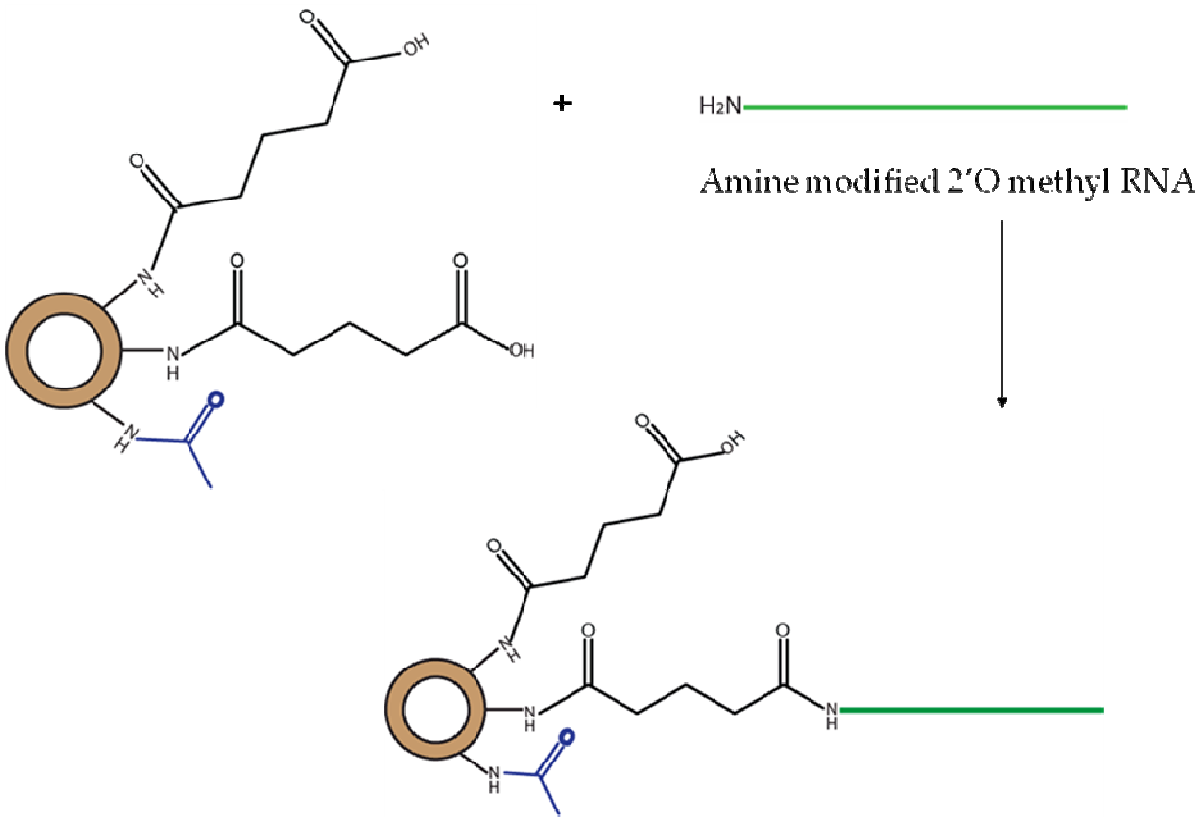


Figure 51. a. Synthetic scheme to introduce an amine functionality on the 2' O methyl RNA b. Synthetic scheme to attach amine modified 2' O methyl RNA to the solid support

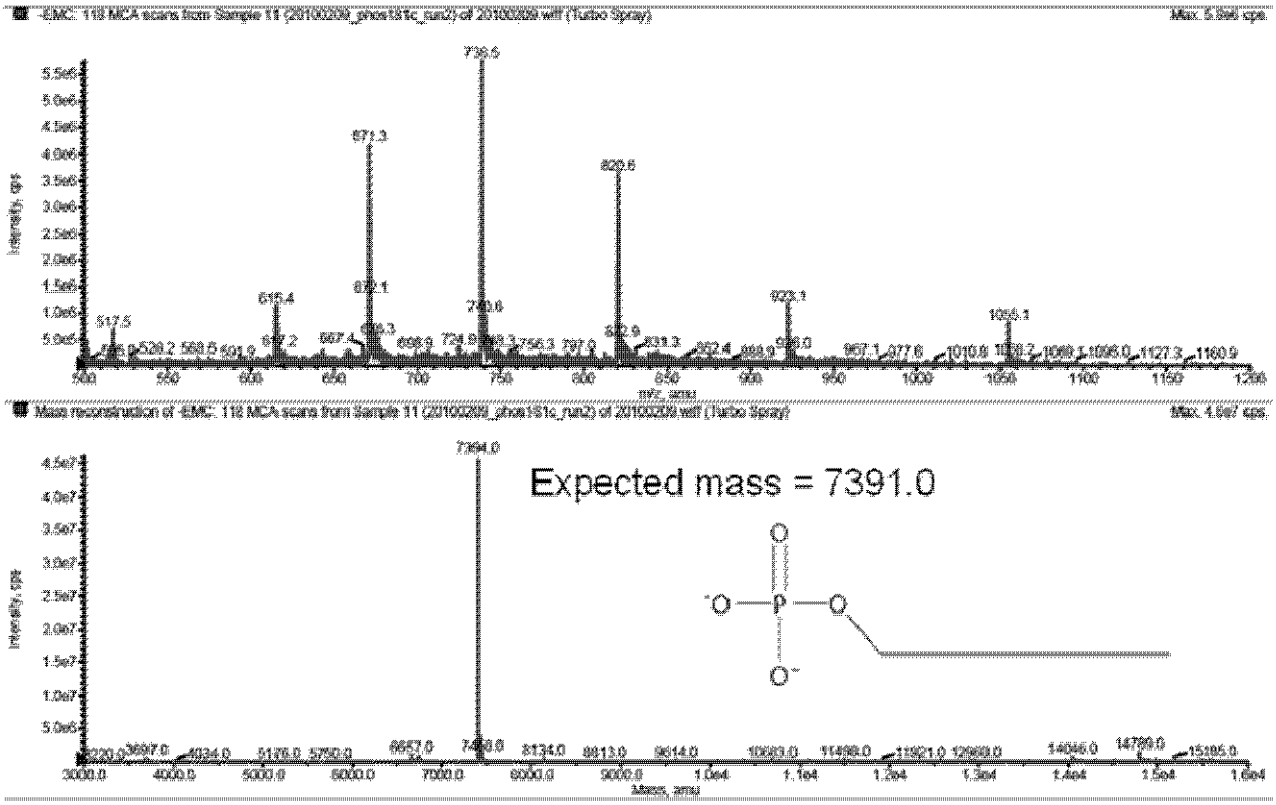
Materials and methods: 2' O methyl modified RNA was obtained from IDT. Rink amide lanterns were obtained from Mimotopes. T4 Polynucleotide kinase enzyme was obtained from USB and ATP was obtained from GE health sciences.

Amine modification of 2' O methyl RNA: The 2' O methyl RNA obtained from IDT had a 5' hydroxyl group. This was converted into the phosphate group using the enzyme T4 poly nucleotide kinase and ATP. To the microcentrifuge tube containing 1 nmole of 2' O methyl RNA, 2.5 µl of 10 x PNK buffer (0.5 M tris-Hcl, pH 7.5, 100 mM MgCl2, 100 mM β-ME) ,

2µl of ATP (10mCi/mmol) and 1.55 µl PNK in kinase dilution buffer (50 mM tris-HCl, pH 8.0) was added. The solution was incubated at 37 ºC for 30 minutes. The reaction was terminated by heating at 65 ºC for 5 minutes. To the phosphoryated 2'O methyl RNA 1000 nmoles (final concentration 0.05 M) EDC, 400 nmoles (final concentration 20 mM) NMI and 100 nmoles (final concentration 5 mM) ethylene diamine was added in a final volume of 50 µl. The reaction was allowed to proceed for 6 hours and then the RNA was precipitated using 20 mg/ml glycogen and 75% ethanol for 12 hours. The amine modified 2' O methyl RNA was then precipitated by centrifugation at 17,400 g at 4 ºC for 30 minutes. The phosphate modified RNA and the amine modified RNA was characterized by infusion in mass spectrophotometer.

Mass spectrometry of modified 2'O methyl RNA: The phosphorylated and amine modified 2'O methyl RNA was dissolved in water:acetonitrile (50:50) mixture containing 1% triethylamine to make a final concentration of 1 µM. The oligonucleotide was then analyzed using the negative ion mode in a Q Trap mass spectrometer (ABI). The spectra of the modified 2' O methyl RNA are depicted in figure 51.

a.



b.

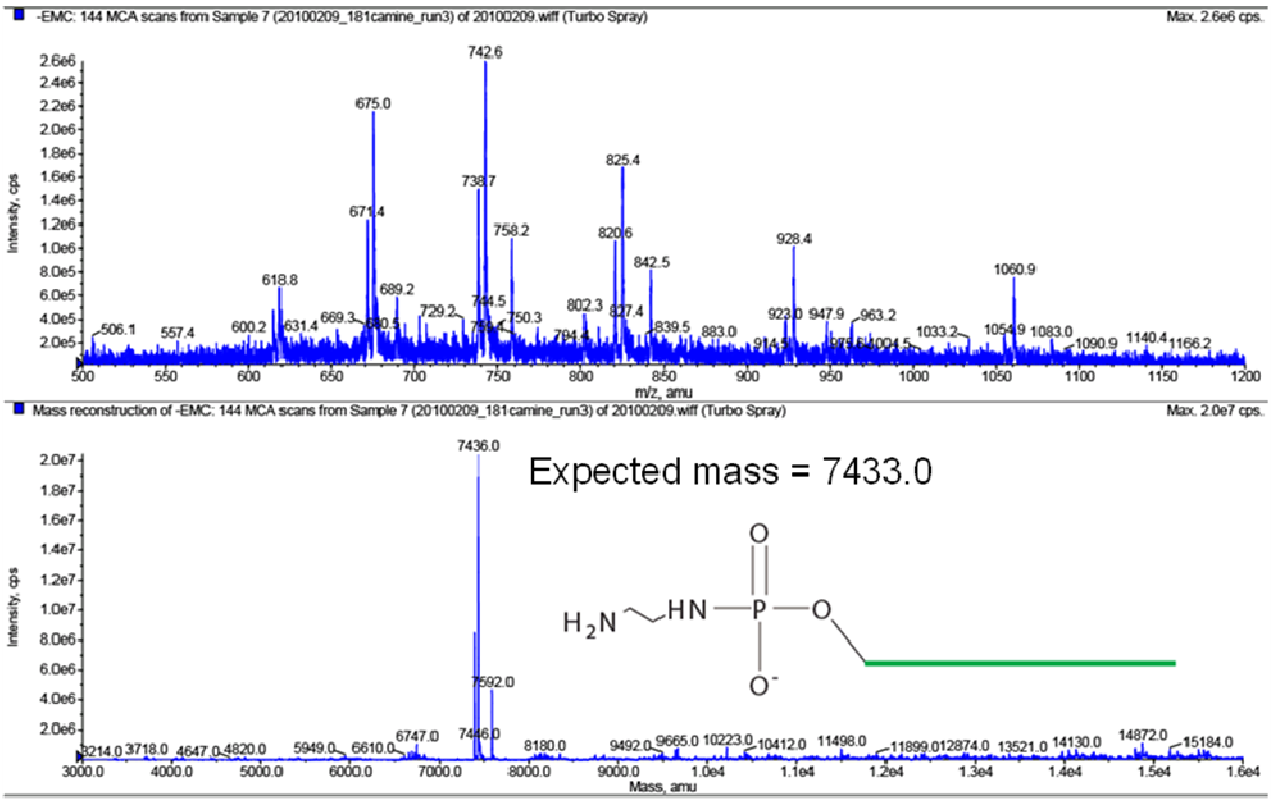


Figure 52. a. MS spectra of phosphorylated 2' O methyl RNA; b. MS spectra of amine modified 2' O methyl RNA

Result: In figure 52 a, the upper panel shows the multiple charged species of the phosphorylated 2' O methyl RNA. The lower panel is the deconvoluted spectra obtained from the multiple charged states. The expected mass of the phosphorylated 2' O methyl RNA is 7391.0 amu. We observe a single peak at 7394 amu indicating a difference of 3 mass units.

Similarly in figure 52b, the upper panel shows the multiple charged states of the amine modified 2' O methyl RNA and the lower panel depicts the deconvoluted spectra. We observe the main peak at 7436 amu and the expected mass is 7433 amu. Again the difference observed in 3 mass units. In the lower panel a smaller peak is observed at 7592 amu. The difference in mass between these two peaks is 156 mass units which corresponds to the formation of an EDC adduct. Since the major peak in the mass spectrometer is the amine modified 2' O methyl RNA we proceeded with the immobilization on the solid surface.

Modification of rink amide resin with glutaric acid: Two discs were soaked in NMP for 30 minutes followed by deprotection of the amine group with 20% piperidine in 400 µl of NMP. The deprotection was performed twice for 30 min each. The disc was washed 5 times with NMP and then allowed to react with 300 mM glutaric anhydride, 600 mM DIEA in NMP (final volume 400 µl) for 12-15 hours. The discs were then washed 5 times with NMP. Any unreacted amine group was capped using 10% acetic anhydride, 5% DIEA in NMP (final volume 400 µl) for 1 hour. The discs were washed 5 times with NMP followed by 5 washes with RNase free water.

Reaction of amine modified 2'O methyl RNA with acid group on disc:

The target number of moles of 2'O methyl RNA complement on the disc was 1 nmole. We used twice the number of moles (2 nmoles per disc) for the reaction. The final oligonucleotide concentration in the reaction was 0.1 M. To two discs modified with glutaric anhydride, 1 M EDC and 0.1 M 2'O methyl RNA was added in water maintained at pH 4.5. The reaction was allowed to proceed for 48 hours. We allowed the reaction to proceed for an extended period of time because the efficiency of amide bond formation is reduced in aqueous medium. This is because water competes with the amine functionality to react with the activated carboxy group. Since the concentration of water is far greater than the concentration of the amine group the formation of the amide bond is hindered. Although we had earlier observed the efficient formation of an amide bond between the phosphorylated 2' O methyl RNA and the ethylene diamine, we were concerned that the formation of the amide bond in the solid phase may be challenging. The reaction mixture was quantitated before the start of the reaction and at the end of 48 hours. The quantitation was performed using fluorescence detection employing OliGreen®. We observed that almost 1.5 nmoles (70% of the starting number of moles) had reacted per disc.

Testing the ability of discs modified with oligonucleotides to capture its complement from solution:

In the chapter 6 we observed different microRNA species existing in the media of culturing HeLa cells and we wanted to ascertain if any one species is responsible for increased proliferation in cancer cells. In order to determine this, it was imperative that the complement immobilized on the solid phase specifically removed one particular species of microRNA i.e. its complementary sequence. Any non-specific binding of different species could result in un-

interpretable results. Hence we wanted to test for the ability of the discs modified with the 2' O methyl RNA complement of miR-181c to specifically capture miR-181c from a solution.

We first designed a scrambled sequence of miR-181c and obtained deoxyoligonucleotide strands of the miR-181c sequence and the scrambled sequence. DNA was selected instead of RNA due to its greater stability and lower cost of synthesis. The two sequences are depicted below.

miR-181c sequence: 5'-AACATTCAACGTGTCCGTGAGT-3'

Scrambled sequence: 5'- GCAATCGACTTAACGGACTTGT-3'

Hypothetically when a disc modified with the complement of miR-181c is introduced into a solution containing DNA with miR-181c sequence, it should remove the DNA strand from the solution. Thus a decrease in the oligo concentration in solution should be observed. However when the disc modified with the complement is introduced into a solution containing the DNA with the scrambled sequence, the DNA should not bind to the complement. Thus no change in the oligonucleotide concentration in solution should be observed.

Experimental procedures: Deoxynucleotides consisting of the miR-181c sequence and the scrambled sequence were obtained from Integrated DNA technologies. The rink amide discs were modified with glutaric acid as described in the earlier. Two discs were modified with 2'O methyl complementary RNA. After modification the discs were blocked with 2 mg/ml of BSA for 14 hours. The final number of moles of 2' O methyl RNA calculated to be immobilized on each disc was around 1.5 nmoles. 0.1 nmoles of the miR-181c sequence oligonucleotide (complementary sequence) and 0.1 nmoles of scrambled sequence

oligonucleotide were taken in 500 µl 1 x tris-NaCl buffer (1 mM tris, 110 mM NaCl) in two separate microcentrifuge tubes. The modified discs were then added to each microcentrifuge tube. The change in concentration of the respective oligonucleotide in solution with time was determined using a fluorescent based assay using OliGreen®.

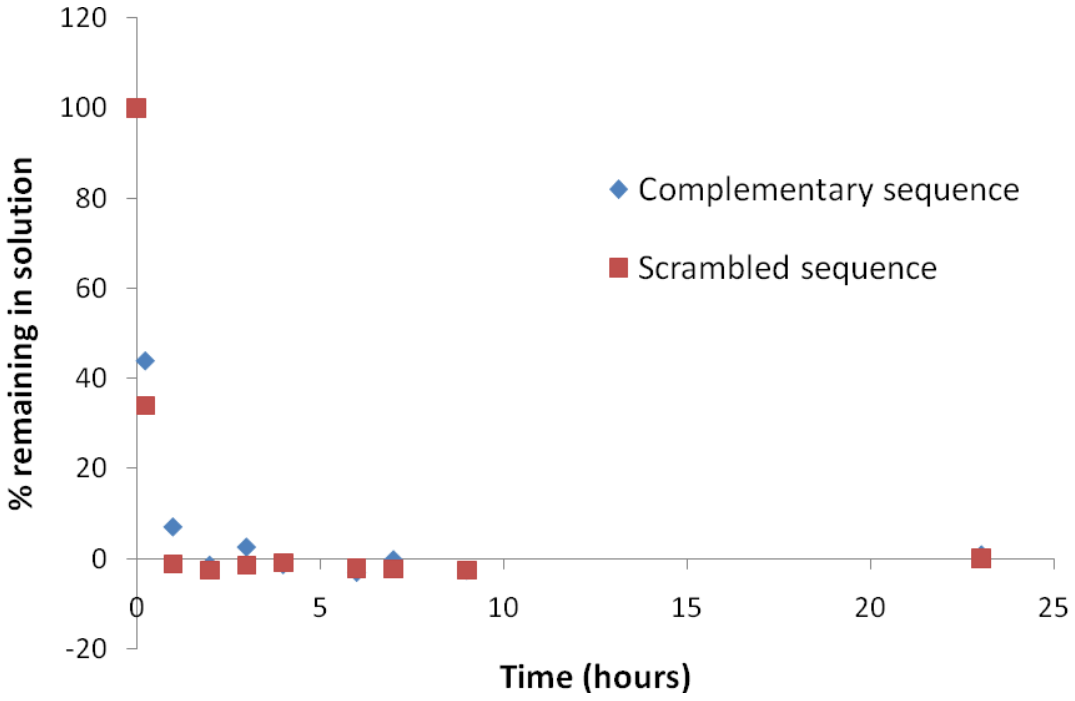


Figure 53. In-vitro assay to specifically capture nucleic acids using a solid bound complementary sequence

Results: The results in figure 53 indicate a decrease in oligonucleotide concentration in solution for both the complementary and scrambled sequence. This indicated that the discs modified with 2' O methyl RNA appeared to non-specifically remove both the complementary and scrambled oligonucleotides from solution. We knew that this non-specificity did not arise due to surface of the disc as the discs had been blocked by BSA.

Hence this result was initially difficult to explain since the 2' O methyl RNA should bind to its complement more efficiently than the scrambled sequence.

We checked the complementarity of the scrambled sequence to the 2' O methyl RNA using the Oligoanalyzer tool on the Integrated DNA Technologies website and found that the maximum number of base pairs formed between them is 4 compared to the 21 mer complementarity observed between the 2'O methyl RNA complement and 181c sequence. Hence the binding of the scrambled sequence to the 2' O methyl RNA as efficiently as the complement was unusual. Our explanation for this observation was that the 2' O methyl RNA immobilized on the disc existed at very close proximity to each other. Thus it was possible that the scrambled oligo bound with low complementarity to multiple 2'O methyl RNA. Hence it was removed as efficiently from the solution as the complementary sequence.

This kind of phenomenon is commonly observed in microarrays where multiple genes are clustered within a very small area. In microarray experiments stringent washes with a detergent is commonly included in the protocol to remove any mismatched binding between probe and oligonucleotides. In spite of the washes mismatches are observed quite commonly.[169,170]

Another possible cause of non specificity could arise from the formation of EDC adducts on the 2' O methyl RNA. We had observed that giving a long reaction time to oligonucleotides in the presence of high concentrations of EDC can result in the formation of EDC adducts with the amine groups present on the bases. These were characterized by multiples of +155 mass units observed in the mass spectrometer. In figure 54 we observe the result of allowing the reaction of ethylene diamine with phosphorylated 2' O methyl RNA in presence of EDC to proceed for extended periods of time (12 hours). The higher masses

observed in the mass spectrometer is the result of the reaction of the EDC to the nucleobases. Formation of EDC adducts could introduce positive charges on the oligo. Thus the binding to the oligonucleotides could be due to electrostatic attraction between the positive charge on the 2' O methyl RNA and the negatively charged phosphate backbone of the oligonucleotides in solution. We initially sought to address the second possible cause of non-specificity and modified our reaction conditions to eliminate the possibility of EDC adducts.

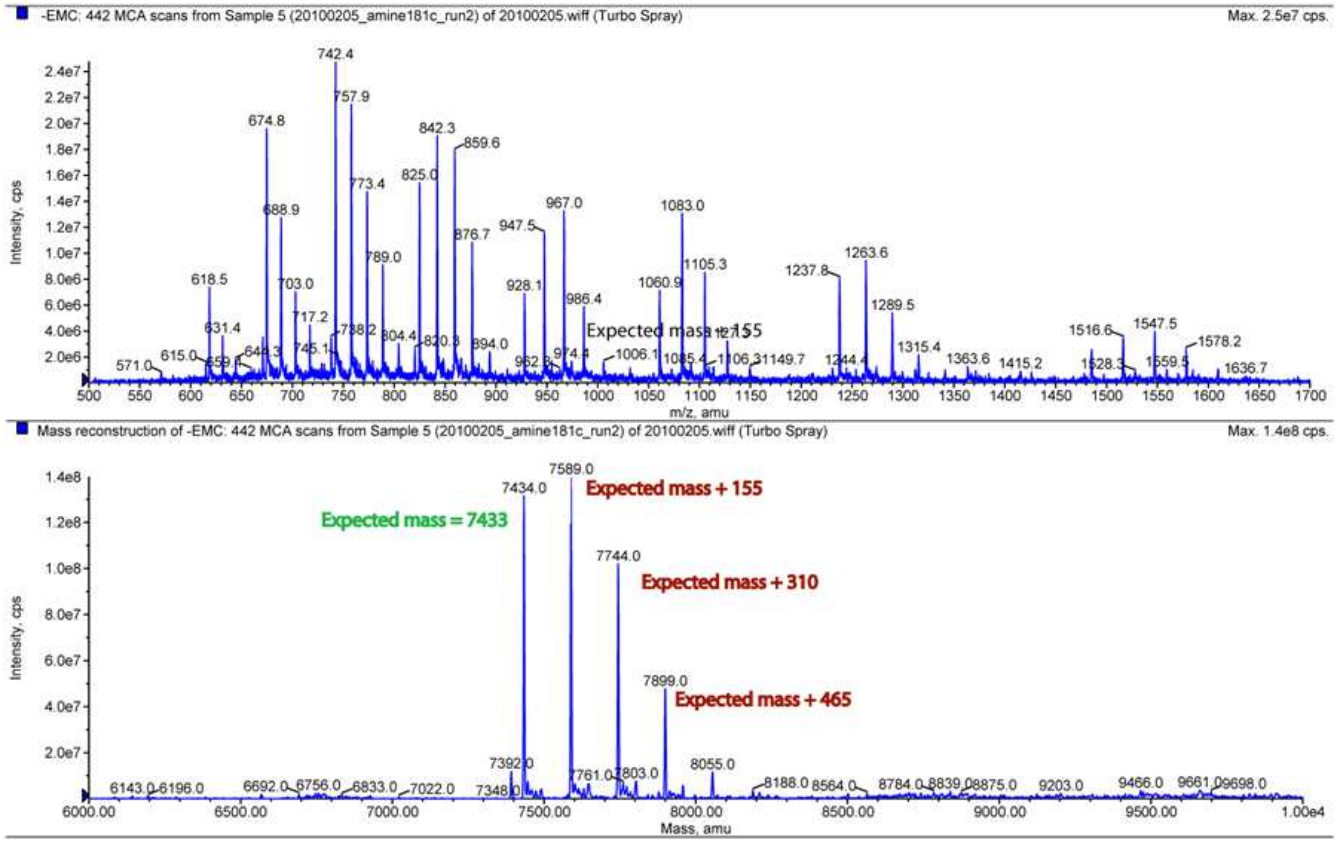


Figure 54. Spectra depicting multiple EDC adducts formed on 2' O methyl RNA

Modified method of immobilizing 2' O methyl RNA on a solid surface:

We designed a method of reacting the amine modified 2' O methyl RNA with the carboxy group on the disc in a manner where the 2'O methyl RNA would not be exposed to the activating agent. This would prevent any potential reaction between the bases of the 2' O methyl RNA and the activating agent. A similar method of immobilizing amine modified DNA to succinylated glass beads has been described by Walsh et al.[171] The disc modified with glutaric acid was soaked in RNase free water for 1 hour. The acid moiety on the disc was activated using 1 M EDC and 100 mM NMI (pH 6) in a final volume of 200 µl for 1 hour with gentle shaking. The EDC/NMI solution was aspirated and the disc was quickly rinsed with water. Then 1.4 µM of amine modified oligo was added to the disc and allowed to react for 6 hours. The immobilization of the antisense on the solid surface was monitored by following the loss of the nucleic acid in the solution. Due to small quantities of 2' O methyl RNA used for the reaction UV-Vis spectroscopy could not be used for the purpose of quantitation. The monitoring of the reaction was performed using the fluorescence detection method employing OliGreen® as described earlier. The fluorescence signal was converted into concentration using a standard curve. The concentration was plotted against time and the curve obtained is depicted in figure 55.

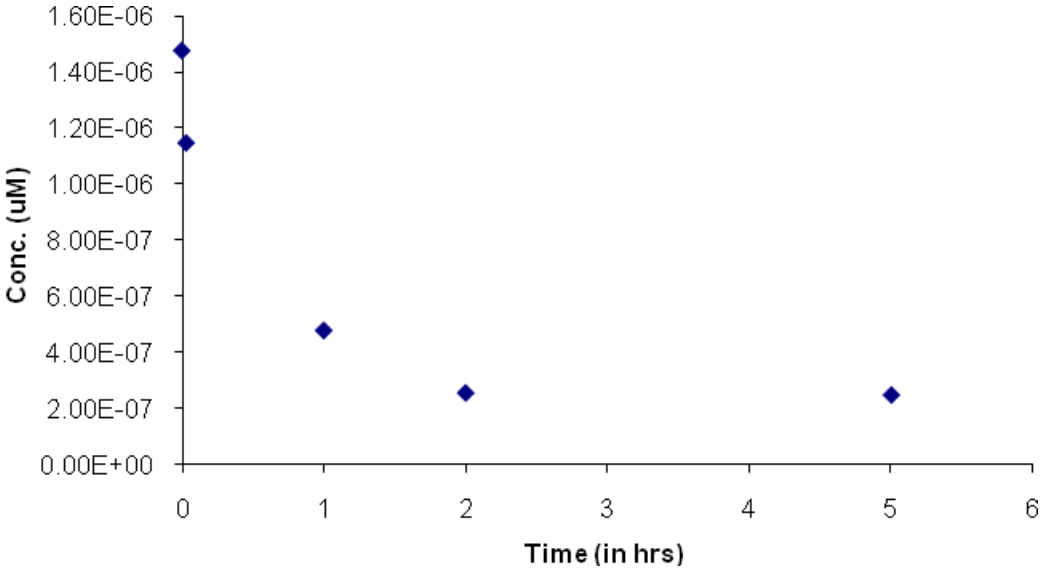


Figure 55. Decrease in oligonucleotide concentration in solution indication reaction to the solid surface

Results:

In figure 54 we observed a decrease in the 2' O methyl RNA concentration in the solution with time. This indicated that the 2'O methyl RNA was immobilized on the solid support. The concentration of nucleic acid remaining in solution after the reaction was calculated to be 0.25 µM (99% reaction efficiency). The discs were washed after the reaction and the amount of 2'O methyl RNA in the washes was negligible.

Binding studies using discs modified with 2' O methyl RNA:

We observed that the 2'O methyl RNA capture oligonucleotide can be reacted onto the disc using our modified method of preactivation of the carboxy group on the resin with the coupling reagent and then reacting with the amine modified capture oligonucleotide. We were

then interested to know if the 2' O methyl RNA reacted in this manner could differentiate between the complementary and scrambled sequence and remove only the complement from the solution. This experiment was performed in a similar manner as the binding studies using complementary and scrambled sequence as described earlier.

For this experiment 2' O methyl RNA was again reacted on the solid support as described earlier. The 2' O methyl RNA on each disc was calculated to be 3.15 nmoles. 80 pmoles of complementary and scrambled oligo was taken in two microcentrifuge tubes in 200 µl volume of 1 x tris-NaCl buffer. Thus the 2' O methyl RNA on the disc was at a concentration of 15.8 µM and the oligonucleotide free in solution was at a concentration of 0.4 µM. In a third microcentrifuge a disc without 2' O methyl RNA reacted on it was added to 80 pmoles of complementary oligonucleotide. The discs were blocked with 1 mg/ml BSA for 12 hours before the start of the binding experiments.

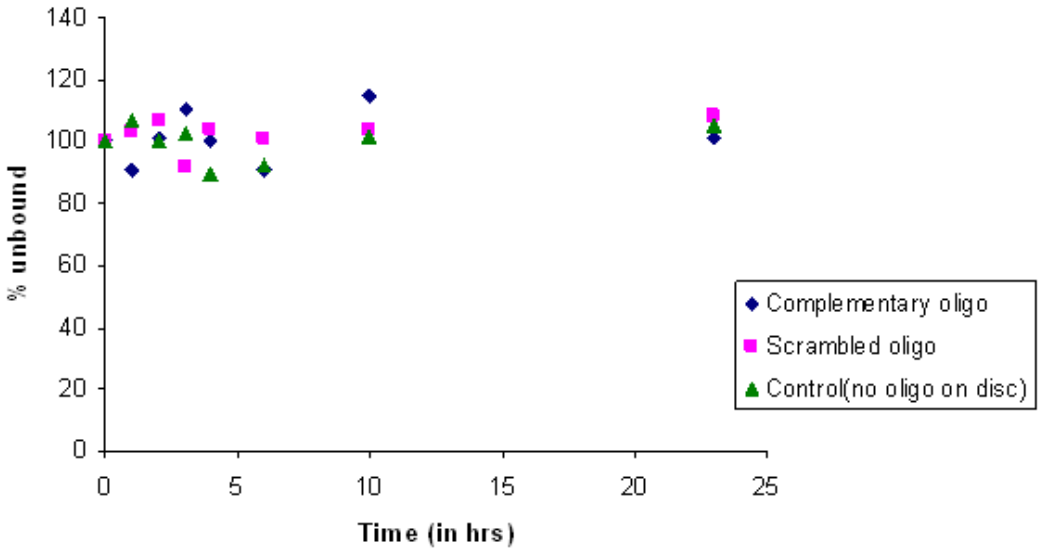
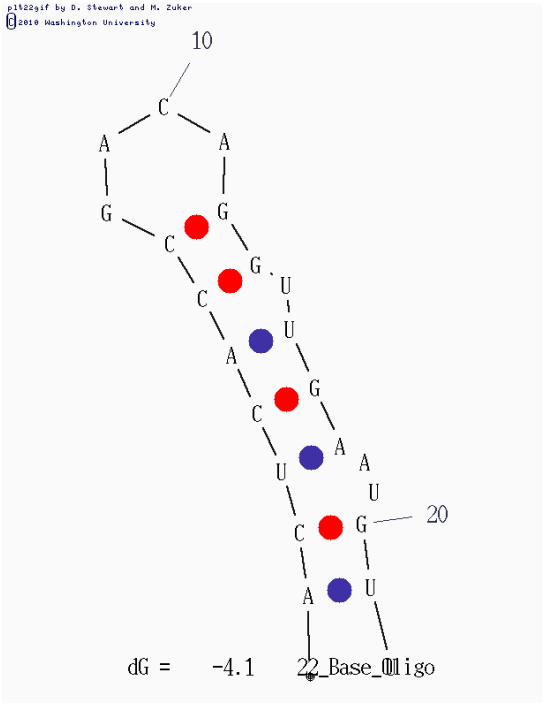


Figure 56. Results of binding studies

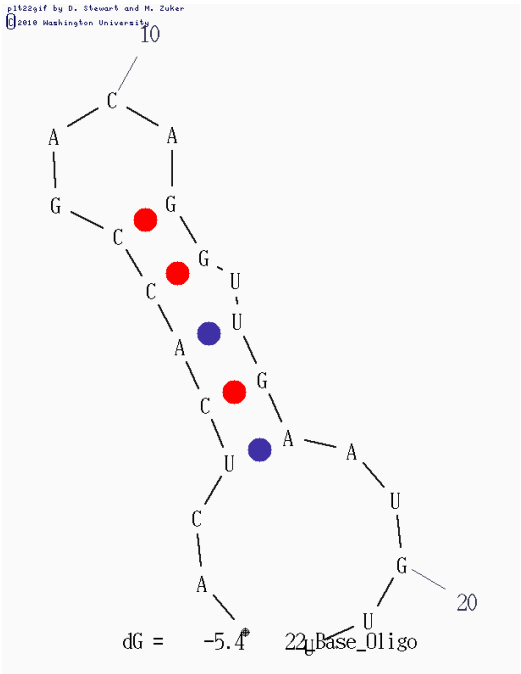
Results of binding studies:

The results in figure 56 indicate that the disc does not bind to either the complementary sequence or the scrambled sequence. The first interpretation that can be made from these results is that the non-specific binding observed in the earlier experiment was most likely due to the formation of EDC adducts on the bases of 2' O methyl RNA.

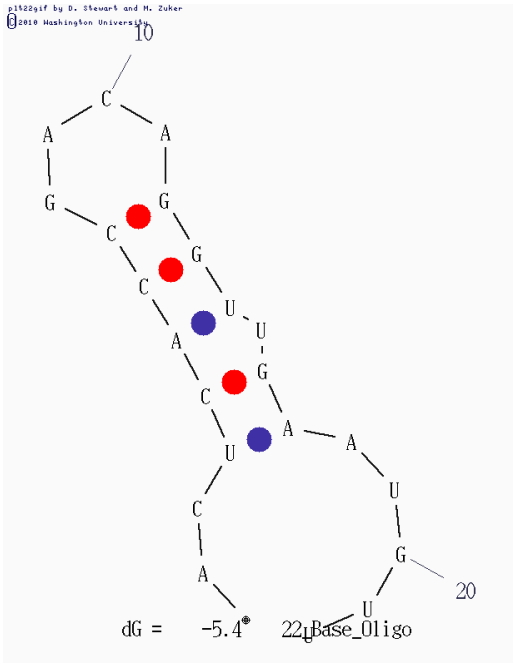
However it was disappointing that the 2' O methyl RNA was unable to specifically remove its complement from the solution. In order to understand these results, we looked more closely at the structure of the 2' O methyl RNA using the Oligoanalyzer tool on the Integrated DNA Technology website. We observed that this RNA forms very stable hairpin loops by forming self-dimers. As can be observed in figure 57 the T_m of each hair pin is over 44 ºC. Thus it is highly possible that at ambient temperatures (~ 25 ºC) the 2'O methyl RNA on the discs are in the form of hairpin loops. In such a case the 2'O methyl RNA would not bind to its complement. In order to test this hypothesis, we decided to heat the discs at elevated temperatures to break the hairpin loops.



T_m = 54.2 ºC



T_m = 44.1 ºC



T_m = 54.2 ºC

Figure 57. Self-dimers formed by the 2' O methyl RNA complement of miR-181c

Experimental conditions:

Two discs were modified with 2' O methyl RNA and added to microcentrifuge tubes containing complementary and scrambled sequence and heated at 85 ºC. The concentration of 2' O methyl RNA on the disc was 2.8 µM (0.56 nmoles in 200 µl). The concentration of the oligonucleotides in solution was 750 nM (0.15 nmoles in 200 µl).The tubes were then allowed to cool slowly to room temperatures. Aliquots of samples from both the tubes were removed at different time intervals and tested for the oligonucleotide concentration in solution using the fluorescence based assay using Oligreen®.

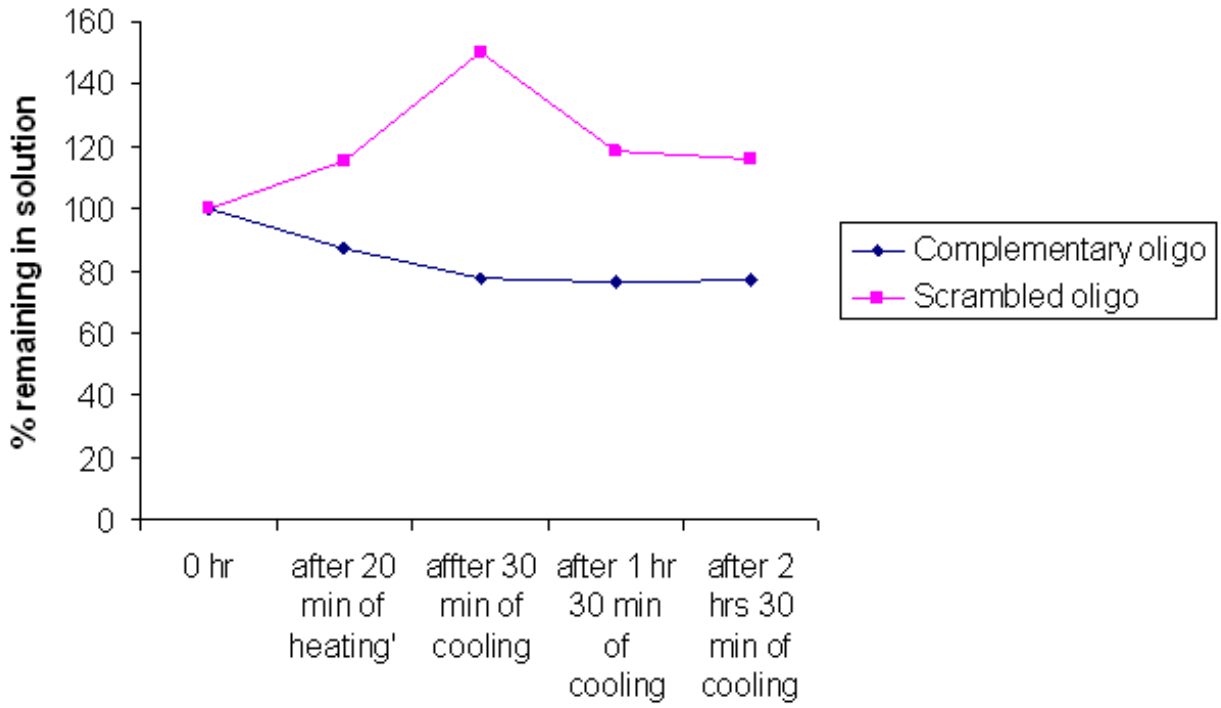


Figure 58. Results of binding studies

Results:

We observed in the graph in figure 58, that there was a reduction in the percent of complementary oligo in solution compared to the scrambled sequence. This indicated that the 2' O methyl RNA immobilized on the disc was able to differentiate between the complementary and scrambled sequence. Hence it was possible that the elevated temperature caused the hairpin loops to unfold which allowed the complementary oligo to bind to the 2' O methyl RNA on the disc.

In spite of this being the first instance where we observed a differential binding by the 2' O methyl RNA on the disc, we had several concerns. Firstly the binding of the complementary oligo was only about 20 %. We would have preferred to observe a greater percent of removal of the complement from the solution in the in-vitro experiment. That would have provided a greater assurance that the specific microRNA would be removed from the media of culturing cells.

Secondly, the percentage of scrambled oligonucleotide in solution appeared to be greater the 100 %. The only possible reason for this occurrence was that the reaction of the 2' O methyl RNA to the disc was not completely efficient and a certain proportion of the 2' O methyl RNA remains adsorbed to the disc. This 2' O methyl RNA could be released upon heating and hence could cause an increase in the OliGreen® fluorescence signal.
Thirdly, heating the disc was not an option while capturing microRNA being released from cells. Hence this design of the disc could not be utilized as a means of specifically capturing microRNA released from cells to observe an effect on cells.

Although we have been able to immobilize an oligonucleotide on the surface of the solid surface, we were unable to specifically capture its complement from the solution using

it. Hence alternative methods have to be pursued to observe the effect of removing the extracellular microRNA on culturing cells. Some potential techniques include:

1. The 2'O methyl RNA complement can be redesigned in a manner that would prevent it from forming stable hairpin loops. This can be accomplished by introducing mismatches in certain key positions that be break the hair pin loops.
2. Another approach is to react the antisense oligonucleotides with agents which are known to be impermeable to the cellular membrane. This coupled to the impervious nature of oligonucleotides could ensure that the antisense remains in the extracellular media and only captured extracellular microRNA. By this approach it is possible to avoid the immobilization of the oligonucleotide on the solid support and thus avoid problems of non-specificity. Some examples of molecules impermeable to the cell membrane include poly acrylic acid, Dextran, cyclodextrin, NHS-LC-biotin and propidium iodide

Additional experiments performed to understand cause of increasing OliGreen® signal during binding studies:

We have often observed an increase in the OliGreen® fluorescence signal with time when a disc with an oligonucleotide covalently attached to it is placed in a buffered solution. The most obvious reason for this increase in fluorescence signal is an increase in oligonucleotide concentration in the solution. This is possible only if the oligonucleotide immobilized on the disc is disassociating from the solid surface. We had earlier observed that these discs are capable of non-covalently adsorb oligonucleotides due to the inherent nature of the surface. Hence there remained the possibility that the reaction of the oligonucleotide to the solid surface was not reaching completion and the oligonucleotide merely remained non-

covalently bound to the solid surface. This oligonucleotide could potentially dissociate from the solid surface over time and thus cause an increase in the concentration in solution.

In order to test this hypothesis we decided to mimic the reaction conditions using an oligonucleotide lacking the amine functionality. In a typical reaction of immobilizing 2' O methyl RNA on a solid surface, the amine group on the solid support is reacted with glutaric anhydride to introduce a carboxy handle. The 2' O methyl RNA is modified with an amine group on the 5' terminus. The amine group is then coupled to the carboxy group using carbodiimides as a coupling agent. This reaction is monitored by observing the reduction in oligonucleotide concentration in solution.

Theoretically if the oligonucleotide lacked the amine functionality it would not react with the disc and its concentration in solution should remain unchanged. However since the surface of the disc bound oligonucleotides there was a possibility that a decrease in concentration could be observed. But any unreacted oligonucleotide could be dissociated from the solid support by washing with salt of high concentration. Thus the total number of moles of unreacted oligonucleotide should essentially remain the same before and after the reaction when an oligonucleotide lacking an amine group is used. This process enabled us to assess if the washing steps in the reaction procedure after the reaction were sufficient to remove any unreacted oligonucleotide.

Experimental procedure:

Two polyamide grafted discs modified with a carboxy acid handle were taken in two separate microcentrifuge tubes. Both the discs were treated with 1 M EDC and 100 mM NMI (final volume 200 µl) for 1 hour. This solution was aspirated and the discs washed with water.

Then to one microcentrifuge tube 117 pmoles of 2’ O methyl RNA modified with amine group was added. To the other microcentrifuge tube 330 pmoles of DNA lacking the amine functionality was added. Samples were removed at different time intervals from both the tubes to test for decrease in the oligonucleotide concentration. The assessment was done using the OliGreen® based fluorescence assay as described earlier. After the completion of the reaction, both the discs were subjected to washes with buffers of increasing salt concentrations. The buffer composition with the highest salt concentration was 1.1 mM tris, 10 M NaCl pH 7 (10 x TN buffer).

Results:

The graphs in figure 59 and 60 indicate a typical decrease in oligonucleotide concentration observed with the reaction of the amine modified 2’ O methyl RNA with the carboxy group on the disc. However we observe a decrease in concentration of the oligonucleotide in the microcentrifuge containing the DNA lacking the amine group. This was due to the binding of the oligonucleotides to the surface of the disc in a non-covalent manner.

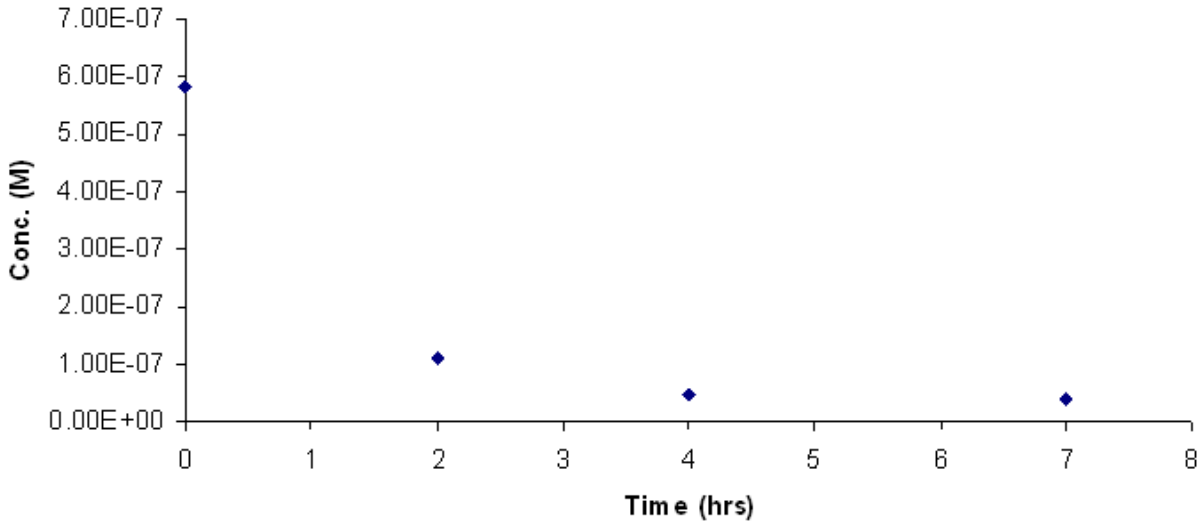


Figure 59. Graph representing the reaction of DNA to the disc

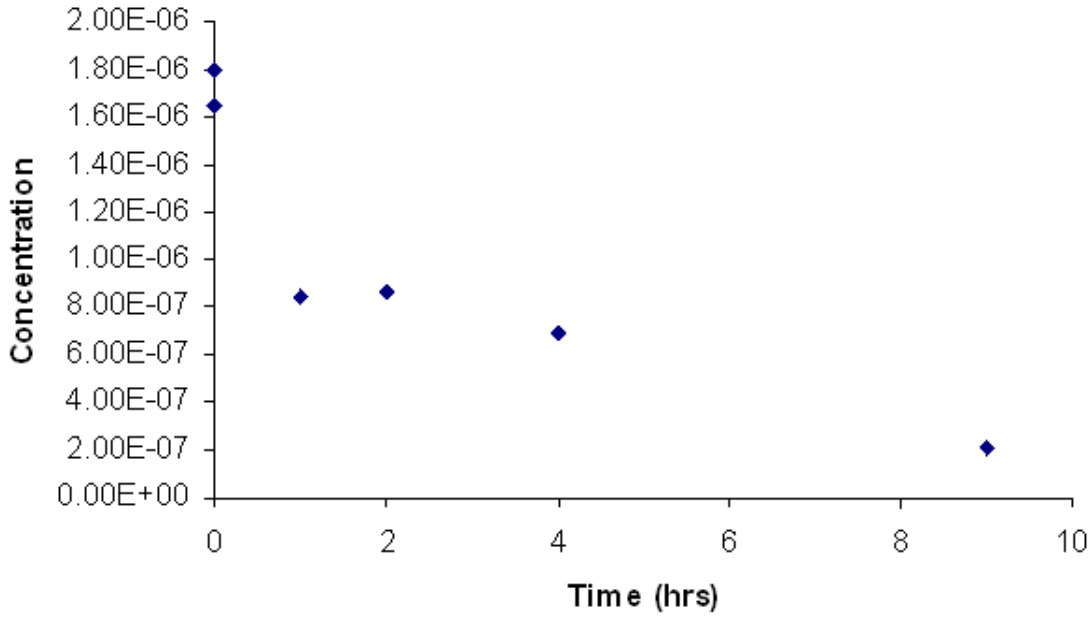


Figure 60. Graph representing the binding of DNA to surface of disc

However the graphs show a difference in the manner the reaction occurs compared to the non-covalent binding of the oligo to the disc. The reaction of amine modified oligo to the disc almost reaches completion within 2 hours and at the end of 7 hours the concentration of oligo remaining in solution is 6% of the total starting number of moles. Upon calculation of the number of moles, it was observed that 7.6 pmoles remained in solution. Thus the number of moles reacted is 109 pmoles. However in the microcentrifuge tube containing the oligo lacking the amine functionality around 50 % of the oligo remained in solution after 2 hours. At the end of 8 hours around 10 % of the DNA remained in solution (42 pmoles). Thus it appears that 228 pmoles bound to the disc during this time.

Both of the discs were then subjected to washes with 200 µl of buffer with increasing salt concentrations (1x TN, 2x TN, 5x TN and 10x TN). The number of moles in each wash was quantitated. The number of moles obtained from the washes is listed in table 13a and 13b. In table 13a, we observe the number of moles released in the washes is very low. The number of moles released in each wash adds up to 0.78 pmoles which is 0.7 % of the total number of moles reacted on the disc. However in table 13b, we observed a large number of moles (112 pmoles) being released in the first wash itself. Upon adding the number of moles of oligo being released in each wash we observed that 338 pmoles were obtained in the washes. This number is higher than the number of moles reacted. This error could be due to fact that the assay is a fluorescence based assay and uses a standard curve to convert the fluorescence signal into the number of moles.

Table 13. Number of moles obtained from washing of discs

a.

	No. of moles (pmoles)
1x TN	0.05
1x TN	0.02
1x TN	0.12
2x TN	0.42
2x TN	0.15
2x TN	0.07
5x TN	0.3
5x TN	0.11
5x TN	0.04
10x TN	0.05
10x TN	0.04
10x TN	0.04

b.

	No. of moles (pmoles)
1X TN	112
1X TN	44.9
1X TN	24.1
2x TN	64.7
2x TN	36.5
2x TN	22.1
5x TN	19.8
5x TN	8.13
5x TN	20.8
5x TN	10.7
5x TN	23.7
5x TN	0.86

The main conclusion that we could make from this experiment was that the decrease in oligonucleotide concentration observed during the reaction is due to the formation of a covalent bond between the amine and the carboxy groups and not due to non-covalent interaction between the disc and the oligonucleotide. With this observation, it becomes difficult to explain the increase in the oligonucleotide concentration in solution observed during the binding studies. The only possible explanation is that with time somehow the 2' O methyl RNA on the disc is released either due to leaching of the polyamide grafted surface of the disc or due to the breaking of the amide linkage between the 2'O methyl RNA and the disc.

CHAPTER 9

SUMMARY AND CONCLUSIONS

It has been established that RNA is an attractive therapeutic target. We targeted two such therapeutically important RNA: telomerase and microRNA. Telomerase is a reverse transcriptase which uses its inherent RNA to extend telomeres. Due to this mechanistic property it is highly prevalent in cancer cells. Our lab has pioneered the method of targeting telomerase via its RNA/DNA heteroduplex. Former graduate students from our laboratory identified intercalators which could bind to the heteroduplex and inhibit telomerase. Ethidium bromide was identified as the lead molecule and was used a scaffold to generate combinatorial libraries which could increase the specificity and affinity of the inhibitor towards telomerase.

Although it was established by an in-vitro assay that ethidium bromide had a strong affinity towards a RNA/DNA heteroduplex, we were interested to understand if a similar affinity was observed in-vivo. Hence the first phase of my graduate research work focused on identifying the difference in ethidium bromide's affinity for the telomerase RNA alone and the RNA/DNA heteroduplex formed during telomerase's mechanism of action. I accomplished this by using an analogue of ethidium bromide: ethidium azide. This molecule when exposed to light formed covalent linkages with nucleobases. Thus this molecule was tethered to telomerase RNA both in the absence and presence of DNA primer bearing the telomeric sequence. I then extracted the telomerase RNA and identified the position of binding of the intercalator using a primer extension assay and dideoxy sequencing.

Additionally I calculated the affinity of the intercalator for the RNA versus the RNA/DNA heteroduplex.

The conclusions that we derived from this study were:

1. There was a difference in the pattern of intercalator binding in the two cases. In the absence of the DNA primer the binding of the intercalator to the telomerase RNA was more indiscriminate. But in the presence of the DNA primer the intercalator bounds preferentially to the template region which is involved in the formation of the heteroduplex. This was the first indication that the intercalator had a preference for the heteroduplex.

2. I then calculated the apparent binding affinity of the intercalator for the RNA alone and the heteroduplex. I obtained an apparent binding affinity of 8.9 μM for the RNA alone versus 1.3 μM for the heteroduplex. This was the second indication that the intercalator had a greater affinity for the RNA/DNA heteroduplex compared to the telomerase RNA alone.

Thus this part of my dissertational work established that an intercalator with a phenanthridine core like ethidium preferentially binds to the RNA/DNA heteroduplex formed during the mechanism of action of telomerase. Thus this intercalator was a suitable candidate for a scaffold to introduce functionalities to improve the affinity to telomerase.

In the second part of my dissertational work I tried to address ethidium bromide's potential problem of non-specific binding to nucleic acids. We screened FDA approved molecules which have a two ring structure. These molecules have been reported to have lower affinity for nucleic acids. We identified naproxen as our new lead molecule. We attempted to increase naproxen's affinity for telomerase by conjugating peptide libraries to its carboxy handle. Peptide libraries were synthesized firstly by a combinatorial method and then by rational design using the crystal structure of HIV-RT.

The conclusions that we derived from this part of the study were:

1. Molecules which have a weak intercalative property inhibit telomerase with an IC_{50} in the millimolar range. While screening these molecules we observed that the molecules which had a positive charge were better at inhibiting telomerase compared to the neutral and negatively charged molecules. This suggested that the inhibition was due to targeting of the RNA/DNA heteroduplex.

2. The naproxen based libraries synthesized using the combinatorial method generated some hits. However upon deconvolution the lead molecule did not exhibit an increased affinity towards telomerase.

3. Using the crystal structure of HIV-RT we identified residues at the active site which are conserved among reverse transcriptases. Naproxen based libraries were designed using amino acids which would potentially have the greatest interaction with the active site residues. From these libraries the molecule FYNY-naproxen showed an improvement over the IC_{50} of naproxen alone.

Thus in this part of my dissertational research I have identified molecules with weak intercalative properties which inhibit telomerase. I have also described a method to rationally design inhibitors using the crystal structure of a protein. Although our new lead had only a modest improvement (10 fold) over the scaffold, the affinity can be further improved by

1. Replacing the amino acids of the new lead molecule (FYNY-naproxen) with alanine in order to understand if all the amino acids are required for activity.

2. Synthesizing new libraries replacing each amino acid of FYNY-naproxen with other amino acids.

In the third part of my dissertational research I tried to target another group of therapeutically important RNA: microRNA. MicroRNA which play a major role in gene regulation have been observed to exist extracellularly. We hypothesized that the role of the extracellular microRNA is that of signals for intercellular communication. Hence we first identified extracellular microRNA in the media of culturing cancer cells and then observed the effect on cellular proliferation after their removal from the extracellular environment. We then attempted to design a method of specifically removing each extracellular microRNA by covalently linking its antisense to a solid surface.

The observations from this part of the study were:

1. Extracellular microRNA existed in the media of culturing HeLa cells. mir 181c and mir 362-5p were found to be present in large numbers.
2. A disc designed to generically remove nucleic acids resulted in a decrease in cellular proliferation. Additionally a discs which had ribonucleases covalently attached to it showed a similar decrease in cellular proliferation.
3. We successfully attached an amine terminated 2' O methyl RNA to a solid surface by forming a covalent link between the amine group and a carboxyl group on the solid surface. However using an in-vitro assay we were unsuccessful in removing the complement of the 2'O methyl RNA from a solution. The possible reason was the 2' O methyl RNA which formed highly thermostable secondary structures.

Potential ways by which this problem can be circumvented is by altering the sequence of the complement. Mismatches can be introduced in the sequence so that the RNA does not fold into stable secondary structures. Other designs of antisense molecules can also be used

such as locked nucleic acids and peptide nucleic acids which would potentially have a greater affinity for the microRNA in solution.

Thus in this dissertational research I have described different methods of targeting therapeutically important RNA. The techniques used to target telomerase can be used to target other reverse transcriptases such as HIV-RT. We also observe a possible function of the microRNA observed extracellularly. Designing a method to specifically target these microRNA can give rise to a novel method for treatment of disease.

Future directions:

1. Designing of telomerase inhibitors:

FYNY-naproxen can be used as the new lead molecules in the designing and optimization of inhibitor against telomerase. Steps that can be taken in further optimization of the molecule include:

a. introducing of a charge in the molecule to improve affinity for nucleic acids

b. perform molecular modeling studies on the molecule to obtain an idea of the orientation of the molecule

c. introduce a linker between the amino acid residues and the intercalator in FYNY naproxen to improve the possibility of developing interactions with the active site residues on telomerase.

2. Identifying the role of extracellular microRNA:

We have observed extracellular microRNA in media of culturing carcinoma cells and observed a decrease in proliferation upon generically removing nucleic acids. We have also designed a method to immobilize antisense oligonucleotides on a solid support. Further

optimization of this system to specifically target the extracellular microRNA can be done by using antisense oligonucleotides which have a greater affinity for microRNA such as locked nucleic acids.[172] Additionally to observe the effect of removing the extracellular microRNA on gene expression, a gene chip expression studying the different levels of mRNA in the presence and absence of extracellular microRNA can be performed.

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VITA

Rajoshi Chaudhuri was born on May 14th, 1980 in Kolkata, India. She completed her education from Bharatiya Vidya Bhavan Public school, Hyderabad. She then completed her undergraduate studies and received her Bachelor of Pharmacy from G. Pulla Reddy college of Pharmacy, affiliated to Osmania University, Hyderabad in 2002.

Rajoshi Chaudhuri joined the University of Missouri-Kansas City to pursue an interdisciplinary doctoral degree in pharmaceutical sciences in 2003. She completed her doctoral studies under the guidance of Dr. Simon H. Friedman in 2011.