TELOMERE MAINTENANCE AS A THERAPEUTIC TARGET

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ABSTRACT

Rachel Henderson; Telomere Maintenance as a Therapeutic Target
(Under the direction of Michael Jarstfer)

Telomere maintenance is essential for long term cell survival, with two mechanisms contributing to telomere maintenance: telomeric DNA elongation by telomerase and a capping mechanism contributing to telomere stability. Several therapeutic approaches targeting telomeres have been explored but many rely on a lag period of telomere degradation before anti-proliferation occurs. Two strategies presented here aim to disrupt telomere maintenance while eliminating the lag period. First, telomerase was inhibited using antisense oligonucleotides targeting its RNA subunit (hTR). The goal of this study was to both prevent active holoenzyme assembly and induce degradation of the protein subunit (hTERT) thought to be associated with anti-apoptotic activity. Additionally, a fluorescence polarization assay was designed for the identification of small molecule inhibitors of telomeric repeat binding factor 2 (TRF2), a key capping protein involved in prevention of chromosomal end fusions and ultimately cellular apoptosis.

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LIST OF ABBREVIATIONS

ATM	Ataxia telangiectasia mutated
DNA	Deoxyribonucleic acid
dsDNA	Double stranded deoxyribonucleic acid
EC_{80}	Effective concentration (80%)
EMSA	Electrophoretic mobility shift assay
FBS	Fetal bovine serum
FP	Fluorescence polarization
hTERT	Human telomerase reverse transcriptase
hTR	Human telomerase RNA
HTS	High throughput screening
IC_{50}	Inhibitory concentration (50%)
K_d	Disassociation constant
mP	Millipolarization
mRNA	Messenger ribonucleic acid
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PNA	Peptide nucleic acid
POT1	Protection of telomeres 1

Rap1	Ras-related protein 1
RNA	Ribonucleic acid
SPA	Scintillation proximity assay
TIN2	TERF1-interacting nuclear factor 2
TPP1	Tripeptidyl peptidase 1
TRAP	Telomeric repeat amplification protocol
TRF1	Telomeric repeat binding factor 1
TRF2	Telomeric repeat binding factor

CHAPTER 1: INTRODUCTION

I. Overview of Telomeres, Telomerase, and Cancer

Since its discovery, deoxyribonucleic acid (DNA) has been considered the primary carrier of the 'blue print' of life utilizing four nucleotide bases in an alpha helix. With the average length of a single DNA strand being ~6 feet, high orders of structure and unique mechanisms are required to both effectively package the DNA into the nucleus of a single cell and to ensure that genetic data is protected and maintained. This abundance of DNA is tightly coiled around histone proteins into structures called chromosomes. The ends of eukaryotic chromosomes are capped with tandem, G-rich TTAGGG DNA sequences called telomeres. Repeating telomeric sequences are oriented 5' to 3' towards the end of the chromosome leading to a single stranded 3' overhang (Figure 1.1).[1] Telomeres provide stability and protection from DNA degradation and chromosomal end fusions to prevent the loss of essential genes and ensure information stored in DNA is properly replicated during mitosis.[2,3]

Maintenance of telomere ends is essential for long term survival. The enzyme responsible for maintaining telomeric DNA is telomerase, a specialized RNA-dependent DNA polymerase. Telomerase functions to lengthen telomeres protecting them from erosion. It is clear that telomerase activity is important for the survival of the cell and protection of its genetic data. However, catalytic telomerase is not expressed in differentiated human cells. Interestingly, it is found almost ubiquitously in cancer cells, nearly 90% are telomerase positive, making the enzyme an important player in cancer biology.

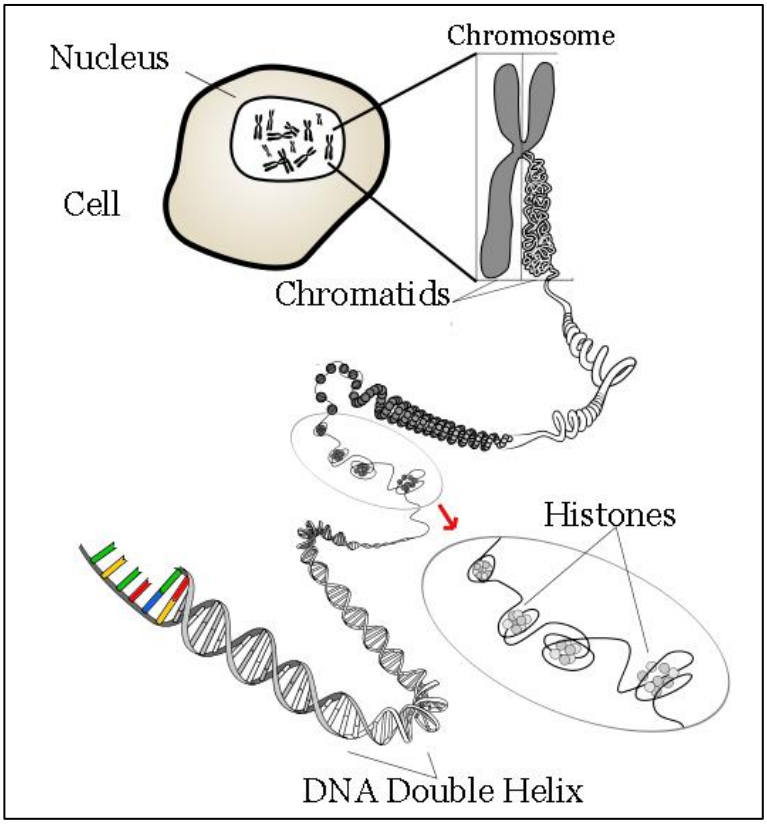

Figure 1.1 DNA Packaging. Telomeric DNA caps the ends of chromosomes that are wound into histones and packaged into the cell nucleus. Adapted from www.genome.gov/DIR/VIP/Learning_Tools/genetic_illustrations.html.

Cancer remains one of the leading causes of death in the U.S. Because telomerase is active in the majority of cancer cells, it is an almost universal marker for human cancer.[2,3] While standard chemotherapeutic regimens are often associated with toxic side effects, telomerase is a therapeutic target that provides a means of reducing toxicity to healthy cells due to its specificity to tumor cells.[4]

II. Telomere Replication and Telomerase Function

During telomere replication, DNA polymerase duplicates template strands of DNA from the origin of replication to the chromosome termini. The leading strand is continuously replicated in the 5' to 3' direction to the end of the template. In comparison, the lagging strand requires a

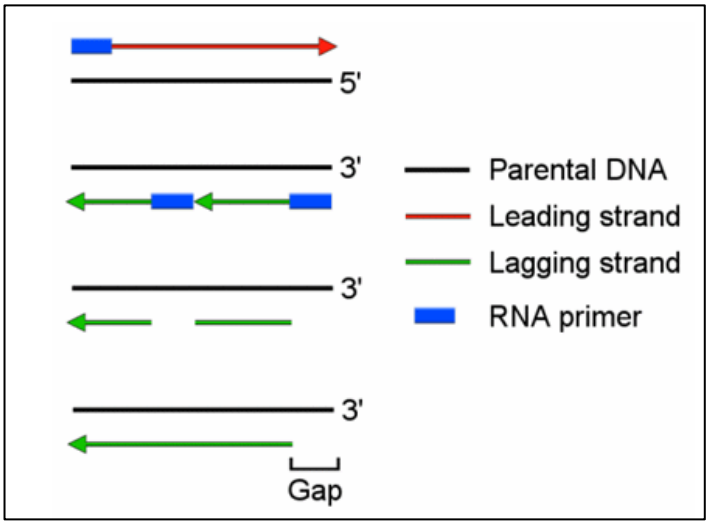

Figure 1.2 The End Replication Problem. Inefficient DNA synthesis results in a shortened lagging strand and a 3' overhang. Adapted from http://www.senescence.info/telomeres_telomerase.html

backstitching mechanism leaving a gap in the lagging strand DNA copy in what is known as "the end replication problem" (Figure 1.2). This mechanism involves the introduction of RNA primers at regular intervals. DNA polymerase extends these primers to synthesize DNA fragments termed Okazaki fragments that make up the lagging strand. The primers are eventually removed and the gaps left behind are filled in with DNA and the fragments are ligated together. However, when the last RNA primer is removed DNA polymerase cannot bind to fill in this gap, leaving a shortened lagging strand. If not corrected, chromosomes shorten with each successive replication cycle. Telomerase is the human enzyme that counteracts this end replication problem. Telomerase is a multiunit complex consisting minimally of two essential components, a 451 nucleotide long RNA subunit (hTR) and a protein subunit (hTERT). hTR contains several functional domains and a templating sequence serving as a template for synthesis of telomeric repeats while hTERT is a reverse transcriptase. Although hTERT is repressed during differentiation, hTR is expressed constitutively. Telomerase stabilizes telomere length by adding telomeric repeat (TTAGGG)n to the 3' end of chromosomes. To accomplish this, the RNA

subunit hybridizes to the 3' overhang and the catalytic subunit repetitively reverse transcribes the template region of the RNA moiety. DNA polymerase then fills in the region on the opposite strand (Figure 1.3).[5]

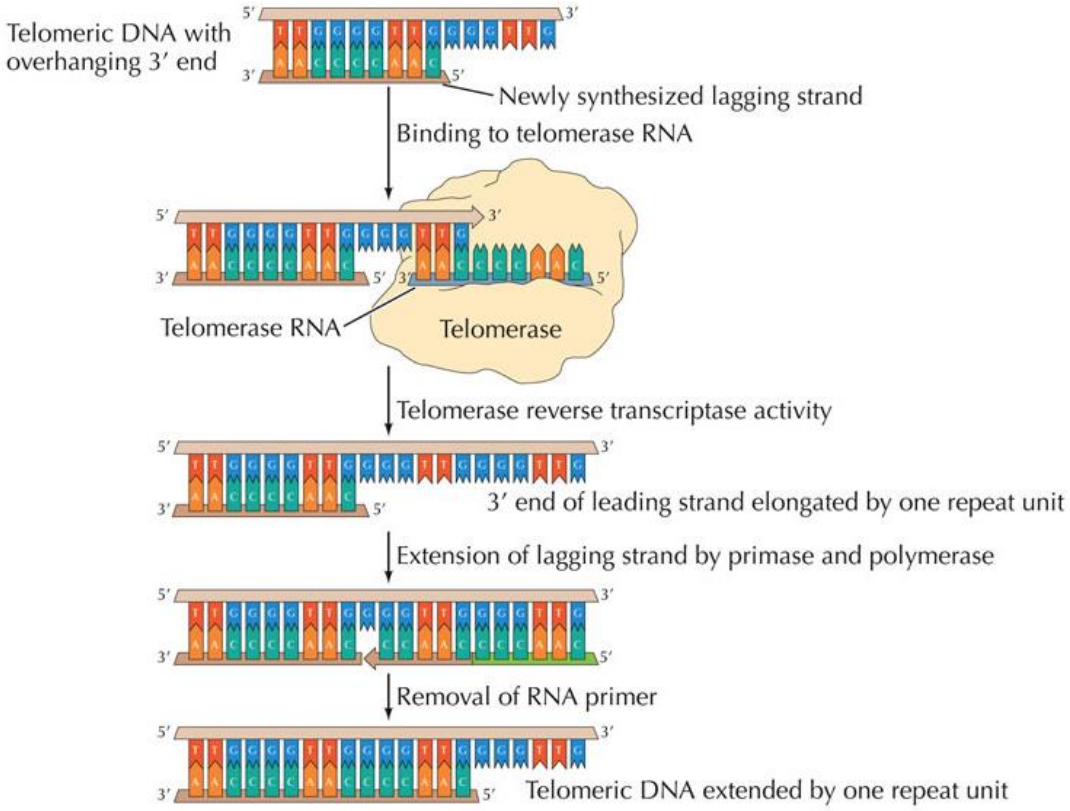

Figure 1.3 Telomerase-Mediated Telomere Extension. Telomerase, a RNA-dependent DNA polymerase, extends the 3' overhang. DNA polymerase fills in the opposite strand. Adapted from THE CELL, Fourth Edition, 2006.

III. Implications of Telomere Shortening

In the absence of telomerase, linear chromosomes progressively shorten in proliferative cells, resulting in a finite number of possible cell divisions.[6] Once the telomeres in a cell reach a critically short length, division ceases and cells enter replicative senescence or mortality stage (M1).[7] Occasionally, cells bypass senescence due to mutations in the p53 tumor suppressor protein. p53 is an essential cell-cycle checkpoint protein that serves to halt cell cycle progression

and has an important role in the initiation and maintenance of the senescence state. Cancer progression involves both the enhancement of cell growth factors and the repression of cancer suppressors, therefore it is not surprising that when wild type p53 was introduced into SiHa cells it was shown to negatively regulate hTERT mRNA expression.[8] Cells with mutated p53 continue to divide despite short telomeres until they reach a crisis stage (M2) in which massive cell death occurs. In rare occasions cells are able to escape crisis by up regulating the catalytic hTERT subunit and activating telomerase, thus leading to immortal cancer cells (Figure 1.4 and 1.5).[1,9,10] As mentioned previously, telomerase is expressed in the majority of cancers, however in the remaining 10% of telomerase negative tumor cancer cells, an alternative recombination mechanism is employed to maintain telomere length, suggesting the importance of telomere maintenance in cell survival and immortality.

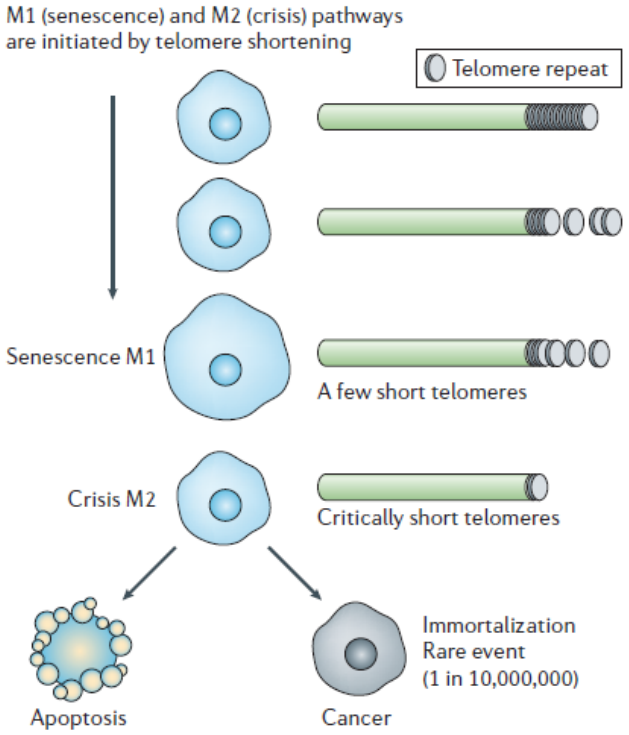

Figure 1.4 Mechanism of Cellular Immortalization. Cellular senescence occurs after telomere shortening which can lead to the crisis stage. In rare events cells escape crisis and become immortal. Adapted from Shay and Wright, *Nat Rev Drug Discov.* 2006.

Cancer cells undergo robust proliferation, and telomere maintenance provided by telomerase is key for their survival. Tumor cells tend to have shorter telomeres than healthy cells but show no net loss of average telomere length with each successive cell division, again implying telomere stability is needed for continuous proliferation.[2] Because it is up-regulated in tumor cells and is essential for the survival of many cancer cells, telomerase has been explored as an anticancer drug target. Inhibition of telomerase activity results in a gradual loss of telomere length, thus causing cancer cells to enter into a crisis stage leading to senescence and/or cell death.

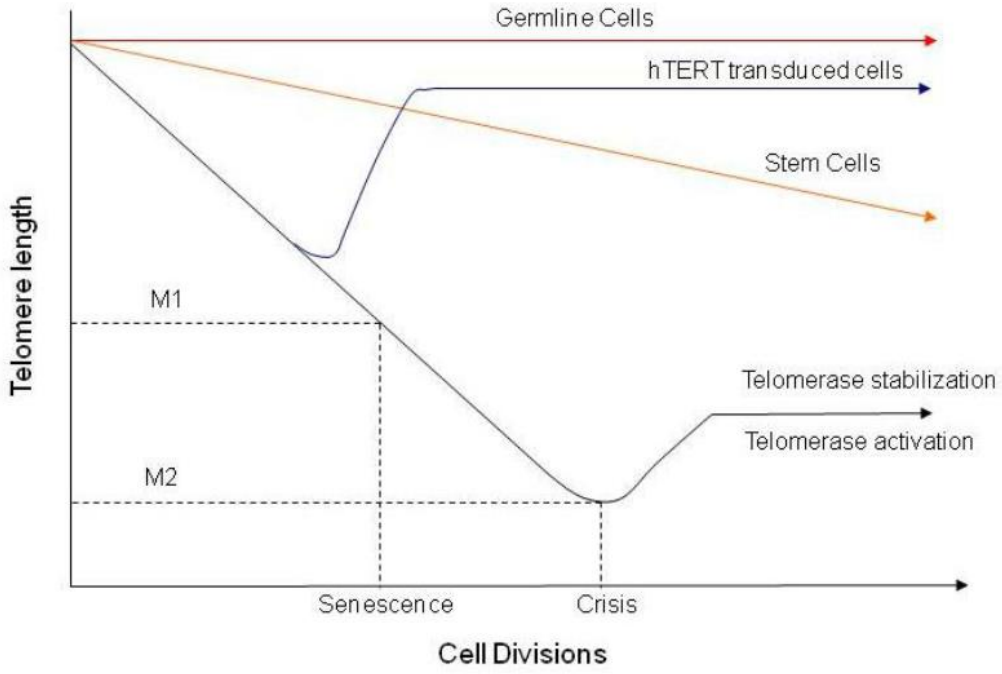

Figure 1.5 Cellular Senescence Induced by Telomere Shortening. In the absence of telomerase, telomere length decreases with increased cell divisions. Cancer cells tend to have shorter telomeres than healthy cells but show no net loss of telomere length. Adapted from Hayflick, *Exp. Cell Res.*1965.

IV. Telomere Capping

Telomere DNA maintenance is important because short telomeres cannot form the proper protective cap. In addition to targeting cancer cells via telomerase inhibition, one could envision targeting the DNA binding proteins involved in telomere capping. These proteins contribute to the stabilization and maturation of the telomerase shelterin complex. The shelterin complex is composed of several telomere specific proteins including POT1, TIN2, TPP1, Rap1, and homodimers of TRF1 and TRF2 that bind single and double stranded telomere regions to form a complex that caps the ends of chromosomes. Shelterin functions to protect telomeres by establishing the structure of the telomere terminus and controlling synthesis of telomeric DNA by telomerase. In part, protection appears related to the generation of a telomeric loop structure known as a t-loop (Figure 1.6).[3,12] T-loops are formed when the single stranded 3' overhang tucks into the duplex part of the telomeric repeat array protecting telomeres from degradation,

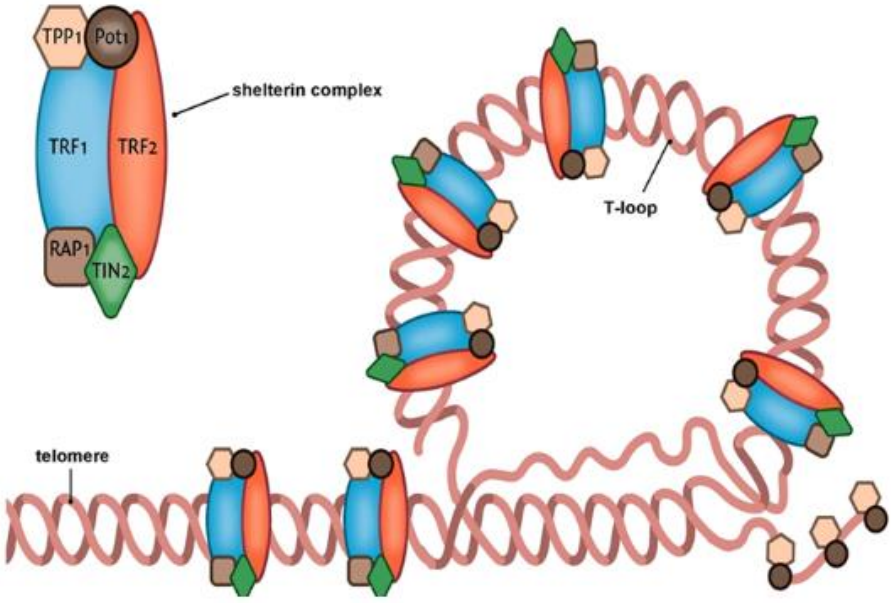

Figure 1.6 Shelterin Complex and T-loop Formation. The shelterin complex binds telomere regions to induce a t-loop formation capping the ends of chromosomes. Adapted from Huzen *et al*, *Frontiers in Bioscience,* 2010.

recombination, and end-joining reactions.[2,13] Additionally, it is important to note that shelterin associated proteins are essential in assemblage of additional protein components involved in the formation of a higher order nucleoprotein complex present at telomeres.[1,11]

Telomeres can be considered as either capped or uncapped. Capped telomeres are telomerase inaccessible and allow cell division to proceed. However, telomere shortening increases the probability of telomeres switching to their uncapped state where elongation by telomerase can occur. Consequently, the shortest telomeres are preferentially targeted for elongation allowing maintenance of a steady-state telomere length.[14] The uncapped state can lead to irreversible cell cycle arrest and death depending on the functionality of the telomere/telomerase complex including the loss of active telomerase and prevention of t-loop formation.[11] If left uncapped, the telomere is recognized as DNA damage and the cell cycle arrests. Cell machinery works to remove the damage signal by fixing DNA breaks through end-to-end fusion of telomeres, leading to telomere instability upon resuming cell division (Figure 1.7).[6] These defective chromosomes break during mitosis again activating damage signaling.[15] However, it is evident that functional telomeres are capable of avoiding the DNA damage response as the telomeric complex grants cells the ability to distinguish chromosome ends from random DNA breaks.[6,13,16] It has been shown that telomere repeat binding factor 2 (TRF2) may play a major role in the protection of human chromosome ends by preventing the damage response. TRF2 coats human telomeres during all stages of the cell cycle by binding directly to the tandem TTAGGG repeats.[13] In vitro data indicates that the addition of TRF2 protein to a linear DNA telomeric model promotes t-loop formation.[16] Furthermore, inhibition of TRF2 and POT1, a similar single stranded telomeric DNA binding protein, by dominant negative alleles leads to immediate activation of the ATM/p53-dependent DNA damage pathway causing cell

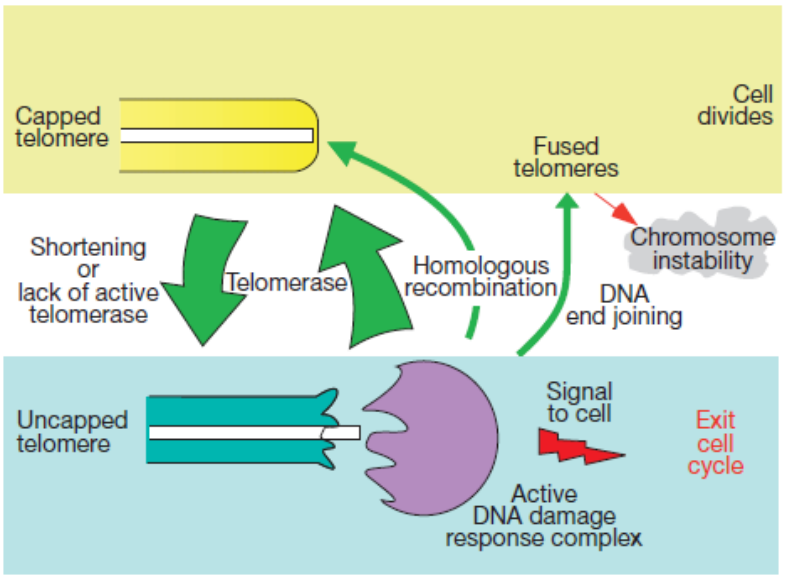

Figure 1.7 Telomere Capping Two State Model. Telomeres switch between a capped and uncapped state in response to telomere length and telomerase status. Adapted from Blackburn, *Science*. 2000.

apoptosis in many cell types as well as a substantial fraction of fusing between telomeres. Similar results were seen in TRF2 knock out mouse models.[17] This is consistent with the model that TRF2 depleted telomeres are perceived as sites of DNA damage. It is thought that telomere dysfunction may be caused by the loss of the 3' overhang leading to the failure to reform the t-loop.[13,16] From these observations it is clear that targeting TRF2 would be an appealing approach for drug discovery. However, it is possible this mechanism may be associated with reduced specificity because proper telomere formation is required in all healthy somatic cells. Studies using a mouse model suggest that telomere disrupters are highly tolerated in normal cells for finite periods of time. Like TRF2, POT1 is essential for telomere protection. In one study a ligand stabilized version of POT1 allowed POT1 to be inhibited transiently and reversibly. Remarkably, POT1 inhibition resulted in cancer cell death, but normal cells underwent arrest that

was reversed by POT1 reactivation.[18] This suggests that small molecule inhibition of telomere binding proteins may provide a high therapeutic index as anti-cancer agents.

V. Research Projects

Several strategies to target telomere maintenance have been explored. These include the use of antisense oligonucleotides and hammerhead ribozymes to target the mRNA of hTERT, immunotherapies, and gene therapy approaches.[19] Although complex in their modes of action these methods are associated with immediate anti-proliferative effects. Alternatively, there are antisense oligonucleotides targeting the hTR template region, small molecules telomerase inhibitors, and G-quadraplex stabilizers.[9,15,19] These therapies directly interfere with telomerase enzymatic function, relying on a lag period of telomeric erosion before proliferative effects occur. As a whole these strategies encompass a broad area of research, differing considerably in their anti-cancer mechanisms. We will first focus on the latter group whose therapeutic effects are controlled by a lag period, and then introduce the two approaches presented in this thesis aiming to eliminate the lag period.

Much progress has been made with telomerase based therapeutics, but as with any telomerase inhibitor it may require several cell divisions before proliferation effects becomes apparent. The initial lengths of telomeric DNA in cancer cells are hundreds or thousands of base pairs long. In the absence of telomerase, proliferating cells lose only 50-200 base pairs of telomeric DNA with each cell cycle. Once telomerase is inhibited, telomere length progressively shortens until cellular senescence is attained. As shown in Figure 1.8, traditional anti-proliferative agents have an immediate inhibitory effect on cell growth whereas a telomerase

inhibitor's is delayed. Not only does this lag period depend on telomere length but also rate of erosion.[20,21] One study using oligonucleotides complementary to the hTR template region showed once telomerase activity was inhibited cell lines did not display anti-proliferative effects until thirty days after treatment.[22]

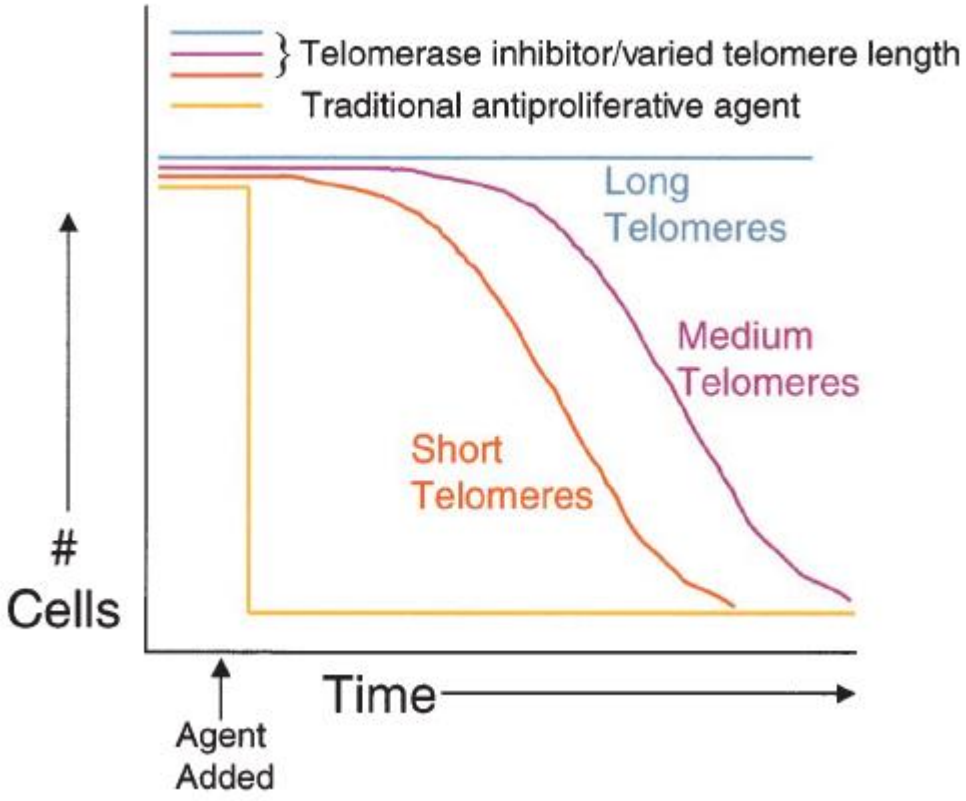

Figure 1.8 Therapeutic Lag Period. Telomerase inhibitors rely on telomere erosion resulting in an anti-proliferative lag period. Adapted from Corey, *Oncogene*, 2002.

It is accepted that hTR is highly expressed in all normal tissue whereas hTERT is present only in immortal cells. Tumor growth requires reactivation of hTERT and its introduction into telomerase silent cells is sufficient to reactivate telomerase leading to cell immortalization.[2,9,22] For example when oligonucleotides are used to inhibit telomerase, upon discontinuing oligonucleotide treatment telomerase is reactivated and telomeres return to their initial length.

Therefore, one challenge in designing telomerase targeted treatment strategies is the need to continuously treat patients during multiple tumor cell population doublings.[15] A valuable alternative strategy would be to evade telomerase reactivation by hTERT. Using antisense oligonucleotides as a therapeutic approach, hTR was selected as the target for my first project based on our hypothesis that a misassembled holoenzyme may lead to the degradation of hTERT possibly allowing treatment without continuous dosing. Furthermore, it is widely known that the classical role of telomerase is to elongate telomeric DNA, but there is emerging evidence that hTERT is involved in other functions including apoptotic activity.[21] Previous studies demonstrated that overexpression of hTERT renders cells more resistant to apoptosis.[23] Likewise, there have been several studies showing that inhibiting hTERT expression can cause an immediate apoptotic response.[24,25,26] One study using modified antisense oligonucleotides to target both hTERT and hTR mRNA in DU145 human prostate cancer cells found that while both targets caused complete inhibition of telomerase activity, hTERT down regulation showed an early decline in cell growth and an induction of apoptotic cell death, whereas hTR down regulation failed to interfere with cell proliferation prior to telomeric DNA erosion.[27] Additionally, studies using cell culture and a transgenic mouse model show that hTERT promotes cellular and organismal survival independent of telomerase activity.[28] Expression level of hTERT positively correlated with cell survival after exposure to several lethal stresses, whereas expression level of hTR had no effect on sensitivity.[27] The mechanism behind hTERT induced apoptosis is unclear, however these results are significant because not only would we like to use antisense oligonucleotides to achieve hTERT degradation and prevent telomerase function and reactivation, but also consequently induce apoptosis of cancer cells to eliminate the lag period. However, while these studies target hTR and hTERT mRNA, we are interested in

hybridizing oligonucleotides to the hTR structural subunit. This strategy can be used to examine what happens when telomerase assemblage is disrupted and may serve as a model for the development of future therapeutics, for example small molecule telomerase inhibitors.

Direct telomerase inhibition through oligonucleotide hybridization is a multifaceted therapeutic approach with many considerations. Nonetheless it has shown to be promising as an anticancer strategy. GRN163L is a lipid-conjugated N3′→P5′ thio-phosphoramidate oligonucleotide that blocks the template region of telomerase. Various studies have demonstrated that GRN163L treatment leads to significantly reduced telomerase activity, promoting telomere loss and apoptosis in several cancer lines.[29,30] The high potency and specificity of GRN163L as well as modifications to improve its bioavailability has led to its testing in ongoing clinical trials. Although not effective within hours or days as most primary anti-proliferative cancer therapies, telomerase inhibition may weaken cells making them more susceptible to other anti-proliferative agents, suggesting that use in combination treatments may be an effective route.[4,20] Again, we would like to discover a novel approach, eliminating the lag period and need for combination therapy, and instead offer telomerase inhibition as a primary anti-cancer treatment.

In addition to telomerase inhibition induced by oligonucleotide hybridization, disruption of telomere maintenance can be achieved through the targeting of DNA binding proteins involved in the regulation of telomeres. As mentioned previously, TRF2 is critical to the protection of telomeric DNA through its function in telomere capping and elimination of DNA damage signals. Impaired telomere capping leads to cell cycle arrest, meaning that by disrupting the function of TRF2 cellular senescence can be obtained without relying on telomere shortening. This is another approach that eliminates the delay in anti-proliferative effects that is seen with existing telomerase inhibiting therapeutics.

Current treatments targeting telomerase activity exhibit a lag period and will have to be used in combination therapies. As an alternative approach to remove the lag period, my thesis research involves inducing the dysfunction of telomeres, specifically examining how both telomere length and telomere capping status contributes to telomere function. The first part of my project involves oligonucleotide based inhibition of telomerase activity using a novel approach to inhibit telomerase that may block noncanonical hTERT activity and induce apoptosis. My second project involves the disruption of the essential capping protein TRF2 using small molecule inhibitors to test the hypothesis that the uncapping of telomeres leads to rapid induction of cell growth arrest.[1,2]

CHAPTER 2: TELOMERASE INHIBITION THROUGH OLIGONUCLEOTIDE TRANSFECTION

I. Introduction

Transfection, the process of introducing nucleic acids into mammalian cells, is commonly used in drug discovery to study the effects of a modified biological activity. In this project, oligonucleotides were introduced into PC-3 cancer cells to investigate the inhibition of telomerase by disrupting specific protein-RNA interactions. Oligonucleotides are short, single stranded modified DNA or RNA molecules that can be designed to be complementary to a specific target allowing for hybridization and inhibition of a desired biological function. Antisense oligonucleotides are generally used to prevent protein translation by targeting mRNA. In this project, a RNA antisense oligonucleotide with modified bases was used to inhibit telomerase, specifically through hybridization with the structural hTR subunit to sterically prevent proper holoenzyme association with hTERT. A major challenge in this therapeutic approach is getting oligonucleotides into the cell and to telomerase without being degraded.[2] Fortunately, cationic lipid transporters can be used to facilitate uptake. Cationic lipids are positively charged having the ability to interact with negatively charged DNA and cell membranes. Lipid and oligonucleotides spontaneously complex during an incubation period and then fuse the cell membrane to deposit the oligonucleotide inside the cell.[31] There are several advantages to using oligonucleotides for hTR targeting. For one, the necessity of hTR for telomere binding and its accessibility by oligonucleotides makes it an ideal target. Additionally,

oligonucleotides are commercially available and because they are complementary to the known hTR sequence they are both highly specific and easily designed.[20]

In standard studies, to conclude that oligonucleotides are inhibiting telomerase through complementary binding rather than off target effects, one must consider the following; due to the lag period, inhibitors should reduce telomerase activity but should not affect cell growth rates initially. Hence, cells should eventually undergo growth arrest and apoptosis but again this time is dependent on initial telomere length.[22] However with our approach, holoenzyme misassembly and hTERT degradation could lead to immediate cellular consequences by interruption of an anti-apoptotic pathway.

II. P6.1 Loop Target and hTRas012 Development

The specific target sequence I focused on was the P6.1 loop of telomerase hTR, a RNA sequence critical for telomerase subunit assembly (Figure 2.1A).[32] By targeting hTR/hTERT binding, proper assemblage of the active holoenzyme complex is prevented causing inhibition of enzyme function. As mentioned previously a misassembled holoenzyme may lead to the degradation of hTERT. This is significant because this subunit correlates to activation of telomerase dependent telomere lengthening and cancer cell progression. It is also important to note that although hTR is highly expressed in normal tissue, there is no evidence that it has any function outside of telomerase.[33,34] Therefore this target is selective toward telomerase activity and cancer progression specific to tumor cells.

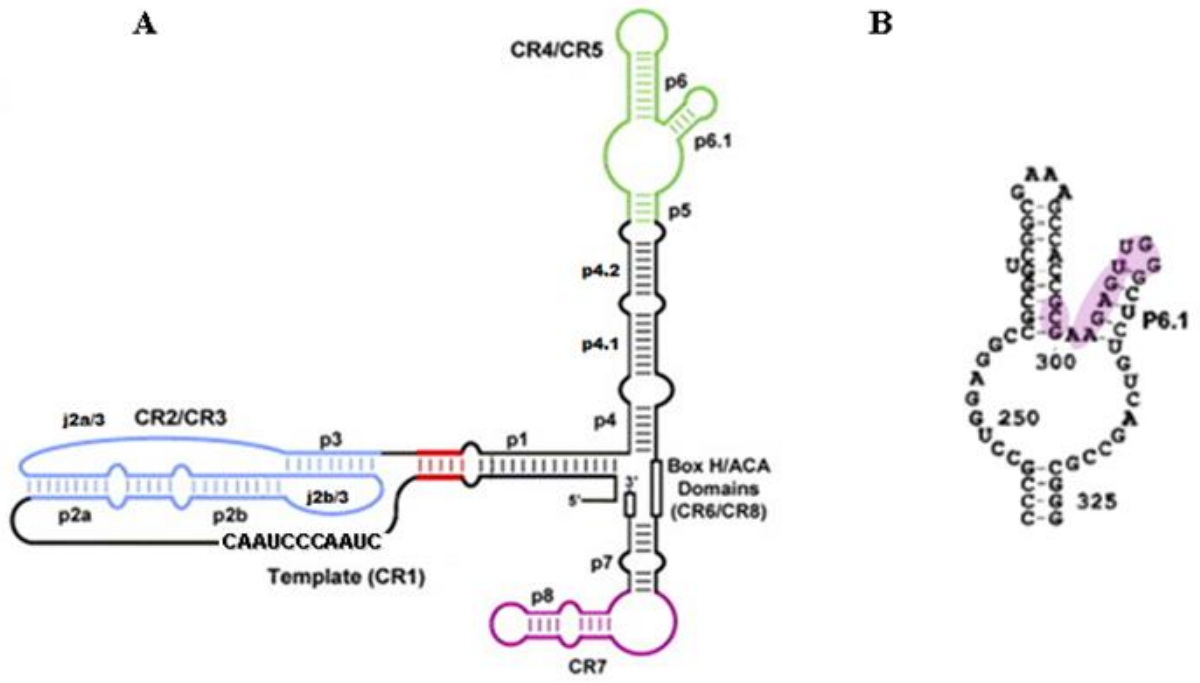

Figure 2.1 hTR RNA Subunit Structure and hTR P6.1 Loop. A. The 451 nucleotide long hTR subunit structure. B. The P6.1 loop within the CR4/CR5 domain. Highlighted nucleotides represent the hTRas012 target. Adapted from Legassie et al. *Structure*, 2006.

Previous work in our laboratory showed that targeting certain hTR regions using antisense oligonucleotides results in decreased telomerase activity(Table 2.1).[35] To determine inhibitory effects on the enzyme, a telomerase assemblage assay was employed in which hTR and hTERT were assembled in reticulocyte lysates. Telomerase activity was measured using a direct telomerase assay. In the regions targeted, the greatest inhibitory effect was observed in the pseudoknot (P3/P1) and CR4-CR5 (P6.1) domains demonstrating that these areas are essential for telomerase activity and subunit binding. Accordingly, both of these regions are conserved in vertebrate telomerase RNAs, and neither are exposed after holoenzyme assembly.[36] In a telomerase assemblage assay oligonucleotides hTRas009 and hTRas010 targeting the P3/P1

pairing region and the P6.1 loop within the CR4-CR5 domain respectively, inhibited telomerase with IC_{50}s in the nanomolar range . Co-immunoprecipitation assays confirmed the oligonucleotides ability to prevent binding of targeted hTR regions to hTERT. However no significant inhibition was seen when added to preassembled telomerase in a direct telomerase

Name	Sequence	hTR region targeted	nucleotide targeted	% activity
hTRas001	5'-ATGGCAAGTCCGAATCGATCGT-3'	none	N/A	804
hTRas002	5'-TAGGGTTAGACAA-3'	template (CR1)	42-54	97
hTRas003	5'-AAAGTCAGCGAGAAAAACAGCG-3'	pseudoknot domain (CR2/CR3)	94-115	97
hTRas004	5'-AACGGGCCAGCAGCTGACATTT-3'	P3/P1 pairing region	174-195	37
hTRas005	5'-TGGGTGCCTCCGGAGAAGCCCC-3'	L6 loop	268-289	100
hTRas006	5'-CGGCTGACAGAGCCCAACTCTT-3'	CR4-CR5 domain	301-322	54
hTRas007	5'-GCCTGAAAGGCCTGAACCTCGC-3'	hypervariable paired region	343-364	115
hTRas008	5'-ACAGCTCAGGGAATCGCGCCGC-3'	CR7 domain	397-418	74
hTRas009	5'-AACGGGCCAGCAGCUGACAUUU-3'	P3/P1 pairing region	174-195	12
hTRas0010	5'-CGGCUGACAGAGCCCAACUCUU-3'	CR4-CR5 domain	301-322	18

Table 2.1 Summary of Inhibition Data with hTR-Targeted Oligonucleotides. DNA oligonucleotides were added to hTERT and hTR prior to assemblage. Telomerase activity was determined using a direct telomerase assay. "% Activity" indicates the amount of residual telomerase activity at a 1 *u*M concentration compared to the primer-only control. hTRas009 and hTRas010 are 2′-*O*-methyl oligonucleotides, and underlined nucleotides indicate phosphorothioate linkages.

assay. Furthermore, full length hTR still had the ability to bind hTERT in the presence of oligonucleotides suggesting binding of hTRas009 and hTRas010 prevents proper holoenzyme assembly and sequesters telomerase subunits in an inactive state. Preference for which oligonucleotide to further examine was influenced by the observation that hTRas010 showed a greater ability to prevent binding between its targeted region and hTERT than hTRas009 (~92% compared to ~50%). In accordance with these results previous studies had demonstrated that the presence of the P6.1 loop was necessary for interaction between the CR4-CR5 region and hTERT as well as enzymatic activity of the mammalian telomerase complex.[32] Based on this

consideration, oligonucleotide hTRas012 was designed by lab members with the capability of interacting with the P6.1 stem loop, specifically targeting nucleotides 298-310 (Figure 2.1B). The hTRas012 sequence is 5'- CCCAACTCTTCGC-3' with all RNA bases 2'-O-methyl modified and underlined bases indicating phosphorothioate linkages (Figure 2.2). Although DNA is the native substrate of telomerase, the 2'-O methyl RNA modifications are used to increase binding affinity to prevent nonspecific interactions, while phosphorothioate linkages enhance stability against nuclease digestion.[20] Fully modified phosphorothioate linkages have been shown to have poor sequence selectivity possibly due to nonspecific protein interactions. Therefore, 2'-

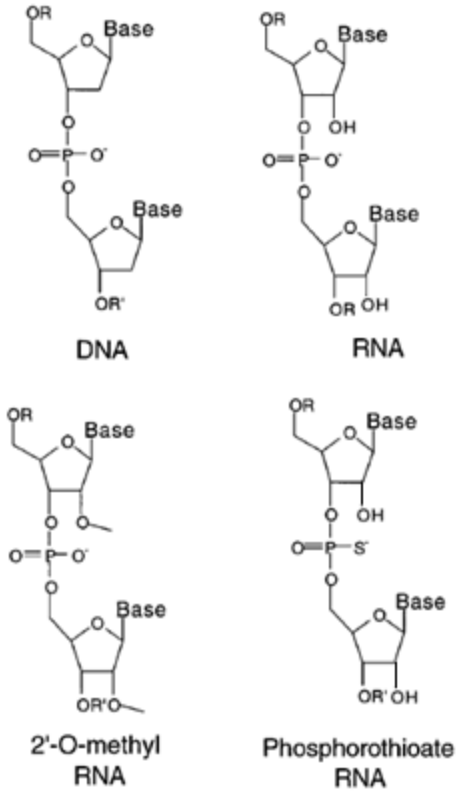

Figure 2.2 Oligonucleotide Backbone Modifications.
Oligonucleotides have modified backbones to increase binding affinity and stability.

O- methyl modified RNA oligomers with terminal phosphorothioate linkages were selected to test this inhibition platform. These modifications also increase the serum half-life of the oligonucleotide, increasing its pharmacokinetic properties. Additionally, this design is both chemically and sterically similar to DNA allowing for favorable electrostatic contacts with the protein component of telomerase.[37]

As expected hTRas012 showed complete telomerase inhibition with IC_{50} ranges in the low nM range when tested in a telomerase assemblage assay (~21 nM). Results from a scintillation proximity assay (SPA), performed to determine if the oligonucleotide prevented interaction between the protein and RNA subunits, showed that the addition of hTRas012 prevented CR4-CR5/hTERT interaction in a concentration dependent manner yielding an IC_{50} value of 96 nM. Because hTRas012 successfully inhibited telomerase in our previous studies it was chosen for further investigation in biochemical inhibition studies using cultured cells.

Oligonucleotide transfection of hTRas012 was performed in PC-3 human cancer cells. A scrambled oligonucleotide with the sequence 5'-AGACUACUGAACU-3' was used as a negative control for nonspecific effects. Again, both were 2'-O-methyl modified with underlined bases indicating phosphorothioate linkages. An Oligofectamine only negative control was also evaluated. Telomerase activity in treated cells was detected using the Telomeric Repeat Amplification Protocol (TRAP) assay. TRAP is a two-step PCR-based telomerase detection method (Figure 2.3). In the first step of the assay lysate telomerase adds telomeric repeats onto the 3' end of substrate oligonucleotide (TS). This reaction mixture contains the reaction buffer provided with the kit, dNTPs, TS primer, and the desired cell lysate sample. In the second step, extended products are amplified by PCR using a primer mixture to produce a ladder of products that can later be visualized.[38] In addition to the primer mix, this mixture contains dNTPs and Taq

polymerase. There are several control samples included in the assay. The positive control is a telomerase active pellet supplied in the kit. There are two negative controls including a lysis only sample to monitor contamination and a negative telomerase heat treated control corresponding to each lysate sample. Additionally, there is an internal control for each sample producing a 36 bp band in every lane to ensure no Taq polymerase inhibitors are present in the sample and to help monitor the amplification process.

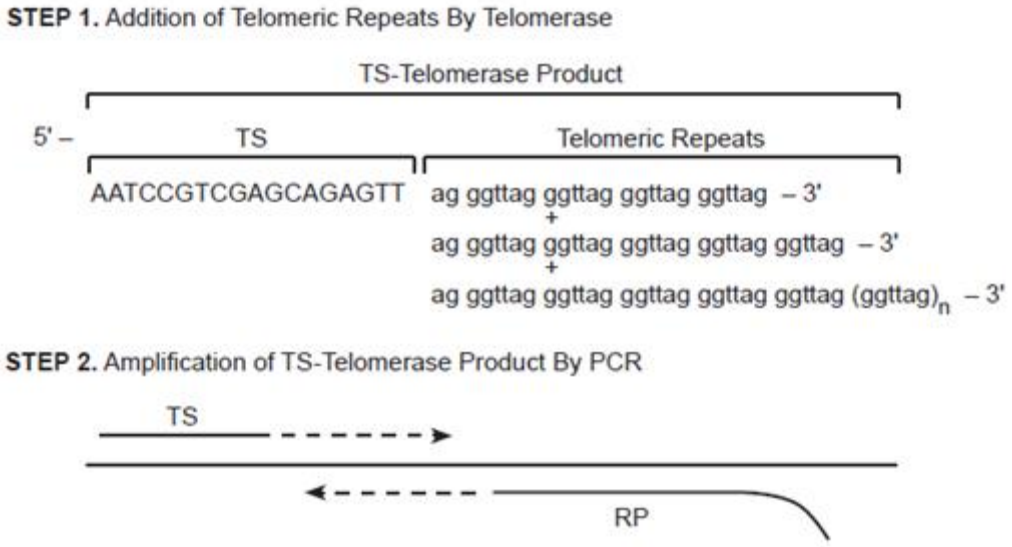

Figure 2.3 TRAPeze Assay Scheme. The two step TRAPeze assay used to assess telomerase activity in cell samples. Adapted from Millipore TRAPeze Telomerase Detection Kit Manual

III. Materials and Methods

PC-3 human cancer cells were obtained from UNC Lineberger Comprehensive Cancer Center. Cells were maintained in F-12K media with 10% FBS and a 1% concoction of penicillin, streptomycin, and amphomycin, and cultured at 37°C in a 5% CO2 incubator. Modified

oligonucleotides were purchased from Integrated DNA Technologies. For all transfections cells were seeded in 10% FBS, antibiotic free F-12K media in 12 well plates at 78,000 cells per well in order to obtain 40% confluence 24 hours after seeding. The transfection protocol supplied with the cationic lipid carrier Oligofectamine, provided by Invitrogen, was followed for all transfections. Twenty four hours after seeding, oligonucleotides were incubated with Oligofectamine in Opti-Mem media before adding to cells. Cells were washed and then transfected with oligonucleotides in serum free, antibiotic free F12-K media at a total volume of 500 μL for 4 hours before either adding 30% FBS, antibiotic free F12-K media to a final FBS concentration of 10% or alternatively removing transfection media and refreshing cells with 10% FBS, antibiotic free F12-K media. For longer transfections periods, when cells reached 100% confluence, populations were split to 40% confluence 2 hours prior to the next transfection. After the appropriate incubation period, cells were harvested by trypsinization and washed twice with PBS before spinning into pellets at 13,000 for 3 min at 4°C. Pellets were lysed using a CHAPS cell lysis buffer with added RNAase inhibitor at 200 units/mL. Lysate protein concentration was determined using a Bradford assay before separating lysates into 10 μL aliquots and flash freezing for storage at -80°C. A TRAP assay was employed to assess telomerase activity. Components for TRAP were provided by the TRAPeze telomerase detection kits purchased from Millipore. A Master mix was prepared by mixing 1.25 μL of 10X TRAP reaction buffer, 0.25 μL 50X dNTP, 50 ng/μL TS primer, and 8.50 μL PCR grade water per sample. Each sample required 10 μL of Master mix and 3 μL of lysate at a protein concentration of 200 ng/μL. Samples were incubated at 30°C for 30 minutes and 95°C for 5 minutes. Next, 12 μL of a PCR mix was added containing 0.50 μL primer mix, 10.40 μL PCR grade water, 1.25 μL Taq buffer, 0.25 μL 50x dNTP, and 0.10 μL Taq polymerase per sample. The final volume of each sample

was 25 μL. Samples underwent 33 PCR cycles at 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute. Both the Master mix and PCR mix as well as controls samples were prepared before preparing cell lysate samples. Here we used the gel based non-isotopic detection method with a 12.5% non-denaturing polyacrylamide gel (10% APS, 1% TEMED). TRAP dye composed of glycerol, 1.25% bromophenol blue, 1.25% xylene cyanol, and 0.05 M EDTA was added to PCR samples before gel loading and gels were run in 0.5X TBE buffer at 400 V for 45 minutes. Gels were stained with SYBR Green DNA dye and visualized using a Storm scanner at 450 nM. ImageQuant software was used to quantify the amount of telomeric product obtained from each cell sample lysate. Values were calculated by quantifying the amount of product in the non-heat treated sample (x), the corresponding heat treated sample (x_o), the lysis buffer only control (c), and the internal standard (c_r). Relative activity was calculated with the equation ($x-x_o$ / c) / (c_r) and then graphed to compare the effects of different oligonucleotide concentrations.

IV. Results

To study the effect of inhibiting telomerase by blocking the interaction between P6.1 of hTR and hTERT, I determined conditions for transfecting PC-3 cells with hTRas012. It was expected that an increase in oligonucleotide concentration would reflect an increase in telomerase inhibition and less telomeric product. As mentioned previously, hTRas012 does not inhibit preassembled hTR-hTERT holoenzyme because the CR4-CR5 regions are not exposed after assemblage, meaning a telomerase turnover must occur before binding of the oligonucleotide to hTR. However, cancer cells are rapidly dividing and therefore rapidly transcribing hTR for the generation of more telomerase thus providing an opportunity for oligonucleotide hybridization. In order to determine a sufficient and optimal amount of time

needed to ensure binding, transfected cells were allowed to incubate for different time intervals before harvesting.

In initial experiments cells were transfected with increasing oligonucleotide concentrations and incubated for 24 hours before harvesting and assessing telomerase activity. Four hours after transfection 30% FBS, antibiotic free F-12K media was added to transfection media to reach a serum concentration of 10%. When assessed for activity a concentration dependent inhibition of telomerase activity was observed (Figures 2.4 and 2.5). Repeating the transfection with increased concentrations (200 nm- 1 μM) reflected similar results, however there was only about 45% inhibition associated with the maximum concentration of 1 μM.

In an attempt to completely inhibit telomerase activity, 48 hour single transfections and 48 hour double transfections (2 transfections 24 hours apart) were performed with oligonucleotide concentrations ranging from 200 nM-1 μM. When preparing for harvesting 48 hours later the cells were clumped and floating including those transfected with the negative scrambled control. Transfection agents can cause toxicity to cells when introduced at too low of a cell confluency. To determine if toxicity was the problem, cell confluence was varied (30%, 50%, 80%) before transfection while keeping the oligonucleotide concentration the same (200 nM). Additionally, while keeping confluency at 40%, the oligonucleotide concentration was decreased (20-200 nM). After 24 hours all the cells were still alive, however cells were already completely confluent in all the wells except the initial 30% well. After 48 hours all the cells had died. It was concluded that the transfection conditions were toxic to the cells and media must be removed and refreshed after each 4 hour transfection period. Forty-eight hour transfections were repeated at 40% cell confluency and after 4 hours transfection media was removed and replaced

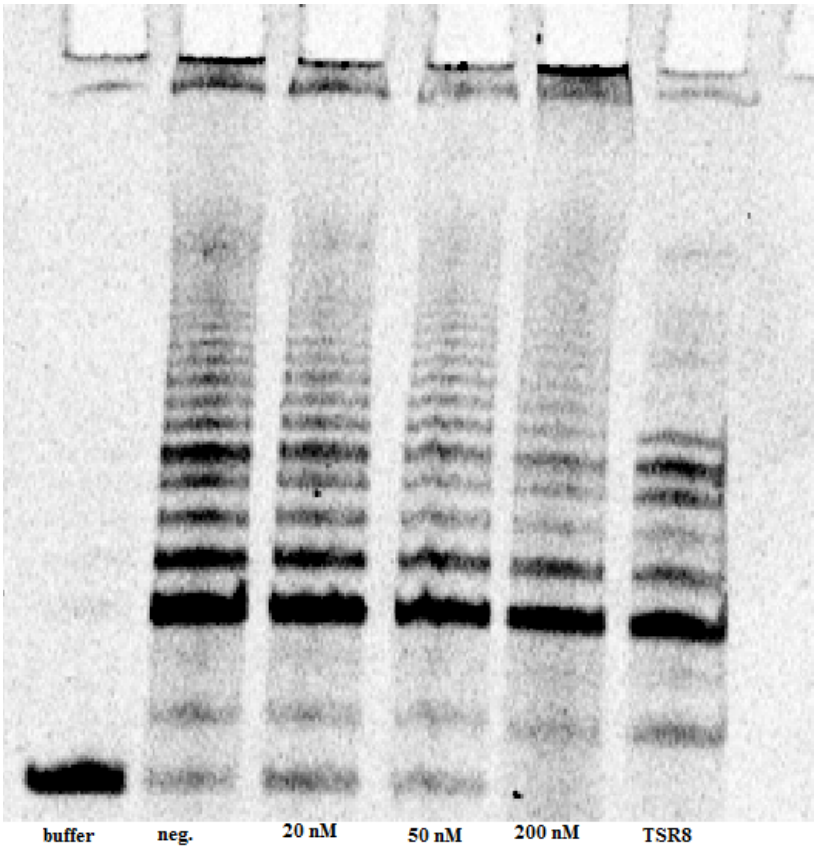

Figure 2.4 TRAP Assay Gel for 24 hr. Transfection. A dose dependent telomerase inhibition is observed (20 nM-200nM). As hTRas012 oligonucleotide concentration increases, telomeric product decreases. TSR8 serves as a PCR control.

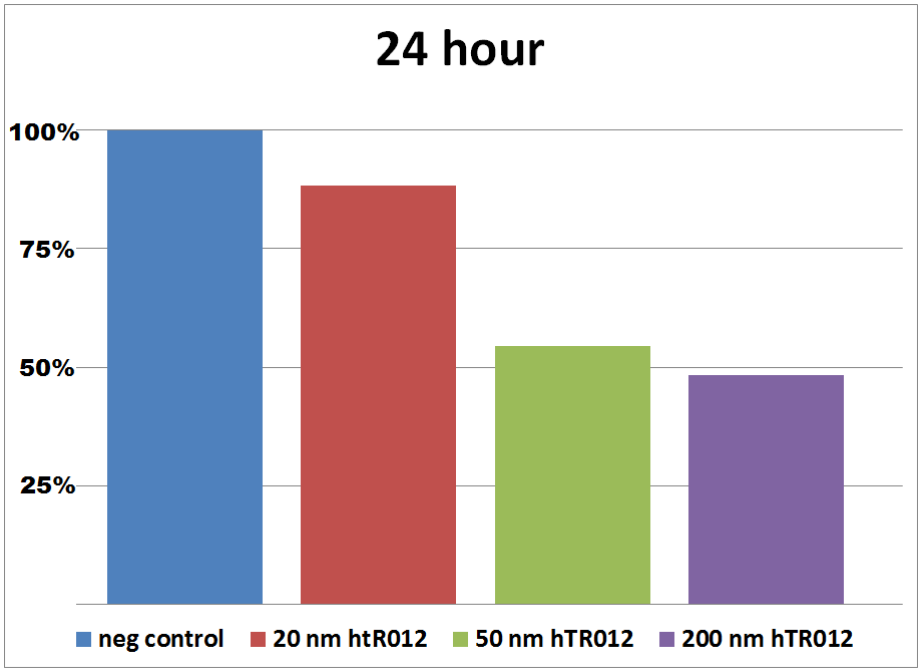

Figure 2.5 hTRas012 Inhibition in 24 hr. Transfection. Telomerase activity based on quantification of telomeric product from the TRAPeze assay gel in Figure 2.4.

with fresh 10% FBS, antibiotic free F-12k media. Cells survived but results showed inconsistent telomerase activity with no dose dependent inhibition. The experiment was repeated, but this time after 24 hours cells were split to achieve 40% confluence before the second transfection. Still adequate inhibition of telomerase activity was not observed in either the double or single 48 hour transfections and additionally there was no dose dependent decrease in the 48 hour single transfection assay. However, the 48 hour double transfection did show a concentration dependent decrease in activity (Fig 2.6).

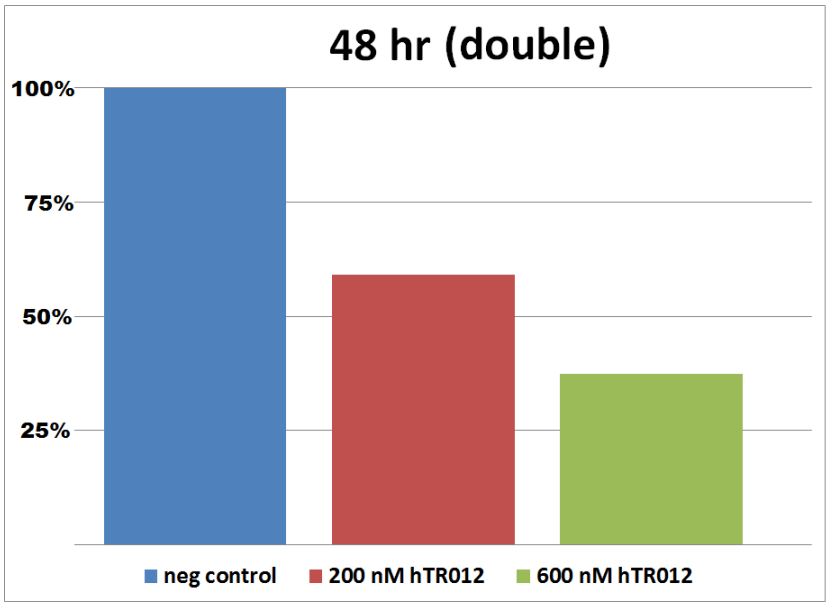

Figure 2.6 hTRas012 Inhibition in 48 hr. x 2 Transfection.
Telomerase activity based on quantification of telomeric product from the TRAPeze assay gel in 48 hr. double transfections. A dose dependent telomerase inhibition is observed.

Due to these results, a 72 hour (3 transfections 24 hours apart) incubation period of cells post initial transfection was attempted. Again, cells were split prior to each transfection to produce 40% confluence. After performing the TRAP assay on samples, results continued to be

inconsistent, no dose dependent results were observed (Figure 2.7A). Transfections were repeated but this time without replacing media after the 4 hour transfection period and instead

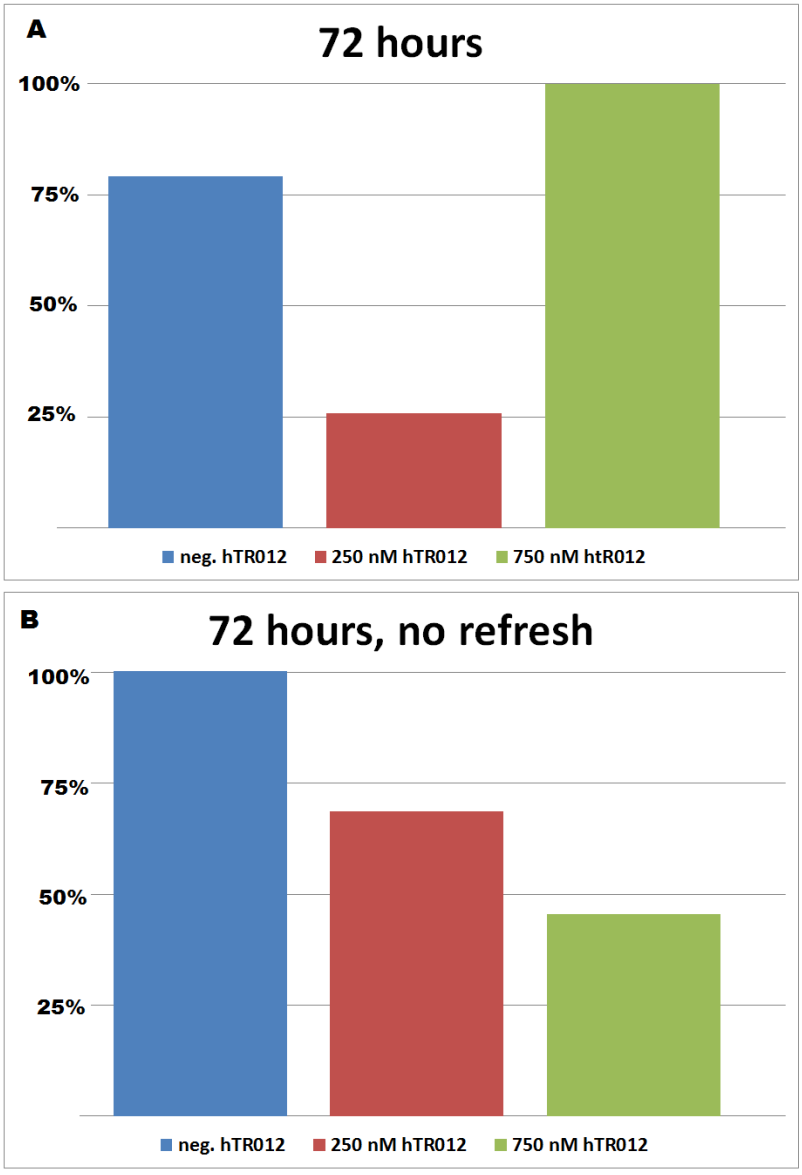

Figure 2.7 hTRas012 Inhibition in 72 hr. x 3 Transfection with and without Transfection Media Refresh. Telomerase activity based on quantification of telomeric product from the TRAPeze assay gel in 72 hr. triple transfections. A. Refreshed media after 4 hour transfection period. B. No refresh of transfection media after 4 hour transfection period. A dose dependent telomerase inhibition is observed.

adding 30% FBS to a total of 10% serum concentration as done in initial experiments. This time cells were split prior to each transfection and survived the 72 hour incubation period without toxicity. Moreover a dose dependent inhibition was observed with 45% inhibition of the negative control in the maximum dose (Figure 2.7B). Unfortunately, in all the experiments telomerase inhibition never reached more than about 40% of negative controls.

V. Discussion

Twenty four hour incubations consistently showed an hTRas012 dose dependent decrease in telomeric product, however complete inhibition of telomerase was never achieved. It is questionable whether discrepancies seen in longer than 24 hour experiments were due to complications arising from altered experimental conditions including longer incubation time intervals, cell splitting, and refreshment of transfection media after 4 hours. Despite the successful 48 hour double transfection, results from the 72 hour experiments suggest inconsistencies arouse from refreshing transfection media. Still these results are promising and suggest modifications to the current method may provide more satisfactory results. Additional studies may include testing higher concentrations of hTRas012 oligonucleotide or increasing transfection incubation times, however further optimization of transfection conditions is likely a beneficial approach to improving experimental results. There are several barriers to efficient transfection including the formation of oligonucleotide/cationic lipid complexes, entry of complexes into cells, oligonucleotide disassociation, and transport to the nucleus.[39] Consequently, the ratio of cationic lipid reagent to DNA concentration is a key transfection parameter and special attention should be focused on determining ideal proportions. Although it does appear that oligonucleotides were entering the nucleus to bind hTR and inhibit telomerase,

a positive control is beneficial in optimizing transfection efficiency. There are several controls commercially available to help monitor whether oligonucleotides are entering the nucleus and to access toxicity of transfection conditions on cells. These methods commonly involve fluorescently tagged oligonucleotides and stains for cellular viability.

Although efficient transfection is important to the success of oligonucleotide dependent telomerase inhibition, it is still possible a major drawback to this project involved inconsistencies associated with the TRAP assay. Results were difficult to reproduce and variable amongst duplicate experimental samples. Although one cannot rule out discrepancies in methodology, there are several limitations to TRAPeze involving factors affecting quantitative determination.[40] It is a multi-step assay requiring several post PCR steps, thus allowing more opportunities for the introduction of error and contamination. In one study ten parallel TRAP reactions were performed using the TRAPeze kit and resulting telomeric product was quantified. A coefficient of variation from experiments was calculated to be 12%.[40] Distributions with a coefficient of variation greater than one are considered to be of high variance, suggesting the TRAPeze assay is associated with high variability. Additionally the assay has a linear range of 250-5000 cells and is sensitive to sample concentration. The TRAP protocol suggests sample protein concentrations ranging from 10-750 ng/uL, but where within in this range is difficult to predict and once established for one experiment it may not produce optimal results for similar experiments run under the same conditions. Additionally, too low or too high protein concentrations can lead to PCR artifacts. Another common problem that existed throughout this project was negative controls containing telomerase positive cells resulted in low telomerase activity. However this data is questionable because as the protocol states, positive telomerase activity sometimes cannot be detected in concentrated extracts and must be diluted. For our experiments it was necessary to

maintain corresponding initial concentrations when comparing dose dependent samples. Finally, samples with inhibited telomerase may show an enhanced amount of telomerase activity because there are too many PCR cycles. In this case dramatically inhibited samples show telomeric product due to saturation of the PCR.

Again, optimization of the current method may allow for improved results. Attention should first be focused on establishing optimal transfection conditions to improve transfection efficiency. Although TRAPeze is a widely used protocol for measuring telomerase activity, it may be beneficial to test samples using alternative quantification methods. For example, there are several modifications to the standard TRAP protocol including the incorporation of fluorescently labeled primers and real time PCR methods. There are also direct telomerase assays using radiolabeled dNTPs or primers that omit the PCR amplification steps, but these require large sample sizes to achieve enough telomerase activity.[41]

Despite the challenges associated with this project, the dose dependent telomerase inhibition results are promising. Further efforts may uncover exciting knowledge contributing to progress in this novel area of anti-cancer therapy.

CHAPTER 3: A FLOURESCENCE POLARIZATION ASSAY TO IDENTIFY TRF2 SMALL MOLECULE INHIBITORS

I. Introduction

In addition to telomerase inhibition, disruption of telomere maintenance can also be achieved through targeting of DNA binding proteins involved in the formation of the telomere shelterin complex. As discussed above, TRF2 is critical to the protection of telomeric DNA through its function in telomere capping and inhibition of DNA damage signals. Disrupting the function of TRF2 leads to cellular senescence or death without telomere shortening, eliminating the lag period that exists with telomerase inhibition. There is also promising evidence that targeting telomere binding proteins may be well tolerated by normal healthy cells. The goal of this project was to develop a high throughput screen to identify small molecule inhibitors of TRF2 by direct binding to the TRF2 protein.

High throughput screening (HTS) is a method used in drug discovery for rapid identification of active compounds. It requires miniaturization and automation of bioassays to simultaneously test libraries of drug-like compounds. Typically assays are carried out on microplates and are assessed in a relatively short time period, however before large screenings can occur assays need to first be designed and optimized. Here we utilize fluorescence polarization (FP), a technique providing fast and accurate quantitative measurements, for the identification of TRF2 binding small molecules. Polarized light waves are characterized as vibrations that occur in a single plane. FP uses the general idea that polarization is a property of

light emitted from fluorescent molecules that can be characterized based on their fixed light excitation and emission properties. In this technique, a biological sample labelled with fluorophore is illuminated with linearly polarized u.v.-visible light at the wavelength of fluorophore absorption. The fluorophore absorbs a photon, briefly exciting it to a higher energy state before emitting it at a specific wavelength. The light photon emission passes through a rotatable linearly polarizing filter before detection (Figure 3.1). Instead of detecting the degree of polarization, change in fluorescence intensity is used to indirectly measure polarization. Change in fluorescence intensity is described by a ratio of two measurements, the emission intensity parallel and perpendicular to the plane of linearly polarized illumination light.[28] The polarization value, being the ratio of the two fluorescence intensities, is a dimensionless number expressed in millipolarization units (mP).

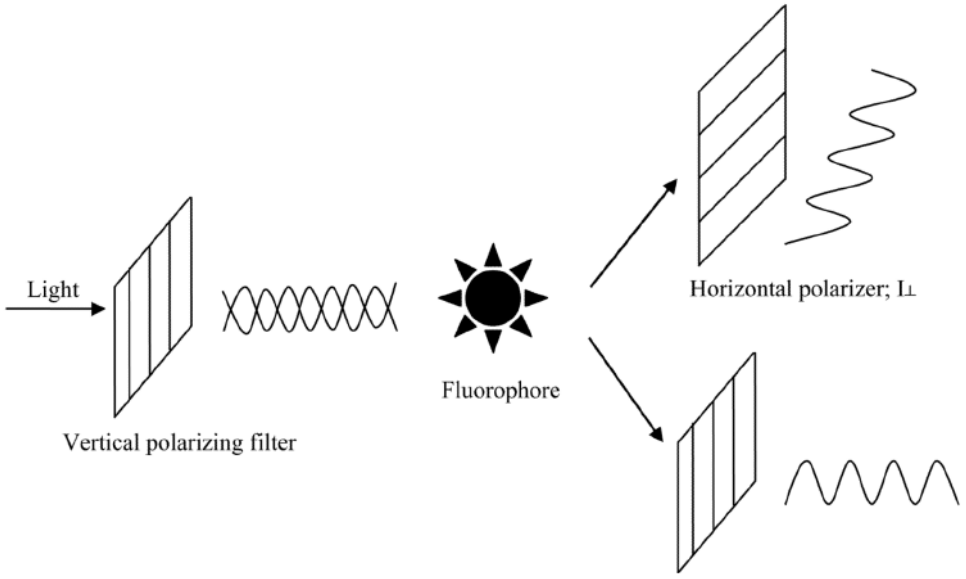

Figure 3.1 Basic Principle of Fluorescence Polarization. Light passes through a linearly polarizing filter to excite a fluorophore. The ratio of emission intensity measurements parallel and perpendicular to emission light are used to describe fluorescence polarization. Adapted from Lea and Simeonov, *Expert Opin Drug Discov*, 2011.

This concept is illustrated in Figure 3.2. Light has an electric field. When fluorophore adsorption vectors align parallel with the electric vector of linearly polarized excitation light they are selectively excited, whereas those perpendicular elude excitation. Small rapidly rotating molecules orient randomly during emission, resulting in low fluorescence. Larger, slowly rotating molecules align in the same plane as the excitation energy, resulting in higher fluorescence.[43,44]

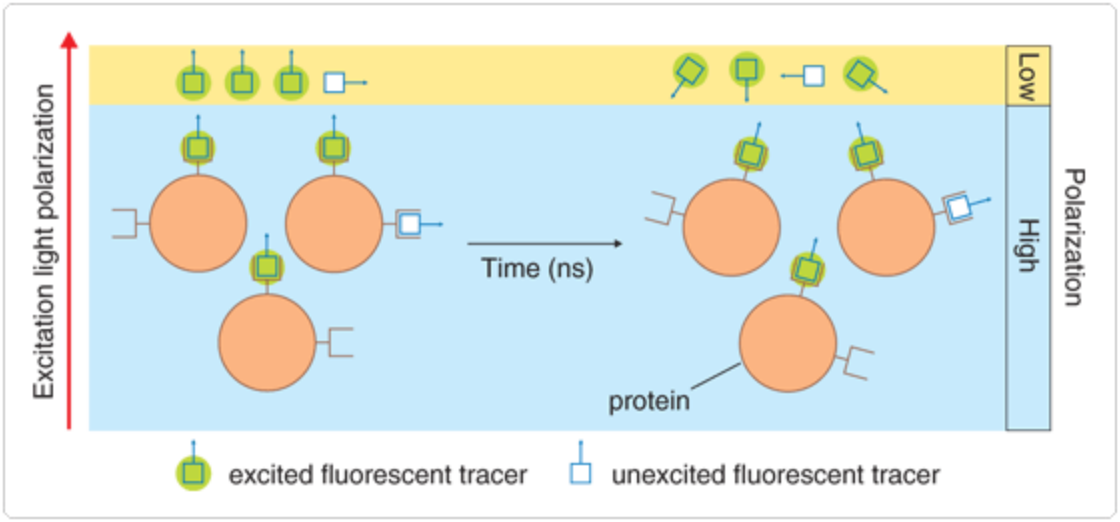

Figure 3.2 Physical Basis of Fluorescence Polarization. Larger rotating fluorophore vectors are more likely to align parallel with excitation light prior to emission to become selectively excited. Adapted from http://www.lifetechnologies.com/us/en/home/references

I attempted to optimize a homogenous FP assay to identify TRF2 inhibitors. In this assay Cy5 fluorescent dye was linked to double stranded telomeric DNA $(TTAGGG)_3$ to observe its interaction with TRF2. Cy5 labeled DNA rotates quickly resulting in decreased polarization, but after incubation with TRF2 binding occurs resulting in a larger complex and slower rotation of the fluorescent dye, therefore increasing FP. The low and high fluorescence measurements were used as references for unbound TRF2 and full complex formation in experiments. The idea was that when a TRF2 binding small molecule was introduced, displacement of TRF2 from DNA or

inhibition of DNA binding would result in rapidly rotating Cy5 fluorophores, decreasing fluorescence measurements. In theory these small molecules would be acting to inhibit TRF2 function by preventing binding with DNA. Without telomeric DNA binding by TRF2, telomere capping cannot occur leading to telomere dysfunction. Using HTS we aimed to identify small molecules that lead to a decrease in FP of Cy5 using an automated plate reader. In preliminary assay experiments we used an excess of unlabeled telomeric double stranded DNA (dsDNA) to behave as a TRF2 binding small molecule by displacing TRF2 from Cy5 labelled DNA.

II. Materials and Methods

Active TRF2 protein was obtained from Brian Bower of the Griffith laboratory at UNC-Lineberger Cancer Center. The protein was provided in 15 μg/ 25 μL aliquots at a concentration of 9.97 μM. The TRF2 oligonucleotides (TTAGGGTTAGGGTTAGGG) were ordered from Integrated DNA Technologies. The G rich strands were covalently labelled on their 5' end with Cy5 fluorophore. dsDNA was made from annealing Cy5 labelled G rich strands with the unlabeled C rich strands.

Electrophoretic Mobility Shift Assay

A master mix composed of binding buffer (50 mM HEPES, 50 mM KCl, 1 mM MgCl2, 0.1 mM EDTA), 1.8 mg/mL BSA, and 3.33 nM Cy5 was prepared. TRF2 protein (0-3600 ng) was titrated into 16 μL master mix samples and water was added to a final volume of 28 μL. Samples were left to incubate in the dark for 30 minutes at room temperature. Before loading samples, 7.5% polyacrylamide gels (30% APS, 1% TEMED) were pre-run for 30 minutes at 100 V. Glycerol was added to samples to a final concentration of 10% and samples were loaded into

pre-run gels and run in 0.5X TBE buffer at 220 V for 25 minutes in a dark cold room (8°C). Gels were viewed using a Typhoon scanner at 650 nm.

FP Assays

All experiments were run in triplicates. Background measurements containing binding buffer (50 mM HEPES, 50 mM KCl, 1 mM MgCl2, 0.1 mM EDTA) and BSA only were subtracted from fluorescence measurements. Fifteen microliter samples were prepared in black 384-well polypropylene plates using binding buffer, 1.8 mg/mL BSA and varying concentrations of Cy5 DNA and TRF2 protein. The TRF2 titration used 5 nM Cy5 DNA and TRF2 concentrations ranging from 0.05 nM to 1.665 μM. Two displacement assays were employed using 5 nM Cy5 DNA, 300 nM TRF2, and unlabeled DNA with concentrations ranging from 0.76 nM to 25 μM. In the first assay, Cy5 DNA and TRF2 were premixed and incubated for 20 minutes before adding unlabeled DNA. In the second assay, TRF2 and unlabeled DNA were premixed and incubated for 20 minutes before adding Cy5 DNA. Plates were spun down and left to incubate in the dark for 60 minutes at room temperature. Plates were read using an EnVision multilabel plate reader with excitation at 650 nm and emission at 670 nm.

Z factor Calculation

To calculate the Z factor, 5 nM Cy5 DNA and 300 nM TRF2 were premixed with binding buffer and 1.8 mg/mL BSA and incubated for 20 minutes in black 384-well polypropylene plates. Unlabeled DNA was used as a positive control and added to wells to a final concentration of 500 μM. DMSO was used as a negative control. Plates were spun down and left to incubate in the dark for 60 minutes at room temperature. Plates were read using an EnVision multilabel plate reader with excitation at 650 nm and emission at 670 nm.

III. Results

An electrophoretic mobility shift assay (EMSA) was used to detect sequence specific DNA binding properties of TRF2. This method takes advantage of the concept that free DNA will travel farther through a gel than DNA bound to protein because larger complexes experience a hindrance in mobility, thus resulting in slower gel migration. Distinct bands visible in the gel correspond to protein-DNA complexes providing information on how far the DNA traveled and more importantly its extent of binding to a compound of interest. Here the assay was performed to ensure proper binding between TRF2 protein and prepared Cy5 labeled DNA. Increasing concentrations of TRF2 (0-3600 ng) were titrated into Cy5 DNA and left to incubate before

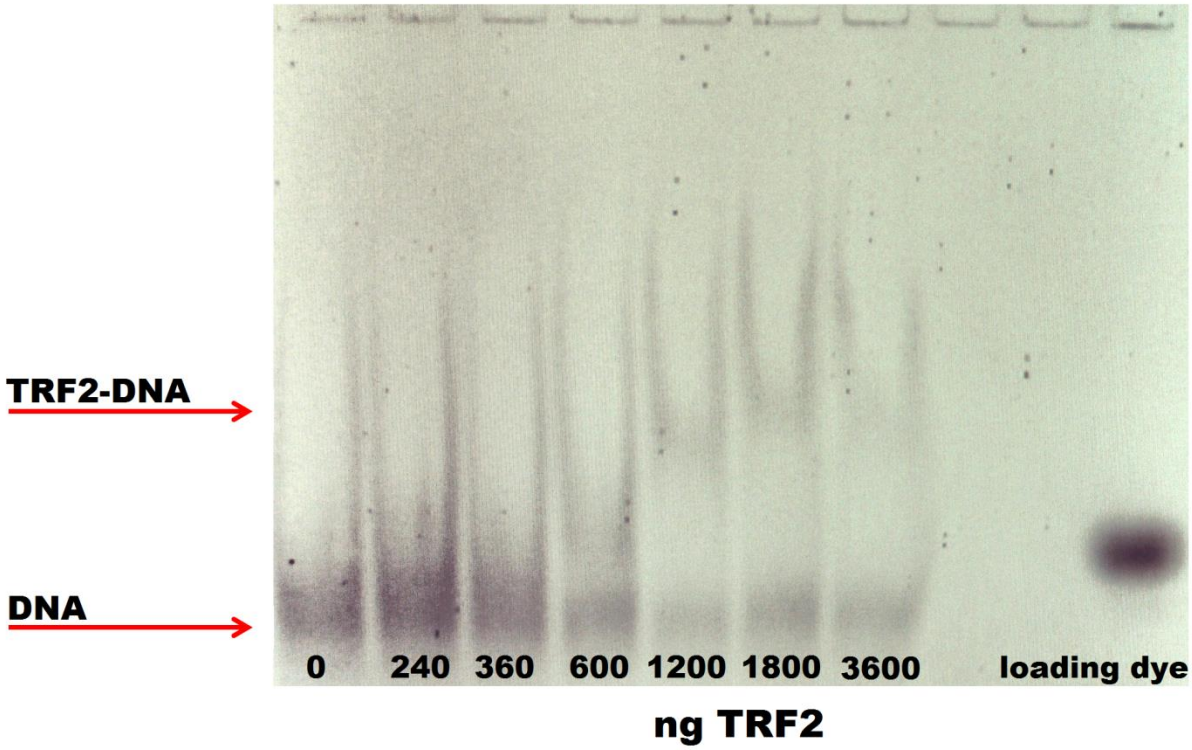

Figure 3.3 Mobility Shift Assay for TRF2 Binding Cy5 dsDNA. A shift up in Cy5 DNA location is observed at about 1200 ng TRF2. Complex formation is occurring.

employing PAGE. While free Cy5 DNA travels farthest down the gel, a major shift upward in Cy5 location is observed at about 1200 ng of TRF2, confirming protein-DNA binding (Figure 3.3).

Once DNA binding was verified by EMSA, a FP assay was designed using an Envision multilabel plate reader for detection. After determining the lowest concentration of Cy5 DNA sufficient enough to give a consistent, readable signal, a TRF2 titration experiment was performed with 5 nM Cy5 DNA and varying amounts of TRF2 (0.05 nM- 1.665 uM). Here we aimed to verify results from the EMSA. The resulting TRF2-DNA binding curve again confirmed assay components were behaving properly (Figure 3.4). The K_d of TRF2-DNA binding in our assay was calculated to be 182 nM. The literature value K_d was found to be 180 nM using surface plasma resonance.[47] An EC_{80} of 300 nM was used for TRF2 concentrations in displacement experiments to ensure that a significant amount of TRF2 remained bound to DNA without saturating it. In the displacement assay an excess of unlabeled telomeric DNA was titrated into Cy5 DNA-TRF2 complexes to displace Cy5 DNA from protein. Two experiments were performed to determine order of addition effects (Figure 3.5). In Experiment #1, Cy5 DNA and TRF2 were premixed and incubated before the addition of unlabeled DNA, whereas in Experiment #2 TRF2 and unlabeled DNA were premixed and incubated before Cy5 DNA addition. As concentrations of unlabeled DNA increase, the FP signal should decrease because Cy5 labelled DNA becomes increasingly unbound and as a consequence rotates at a quicker speed. In theory, an inhibitor screen should produce similar results as unlabeled TRF2 binding DNA. The displacement assay data from Experiment #1 produced an acceptable curve however the dynamic range of the assay was narrow.

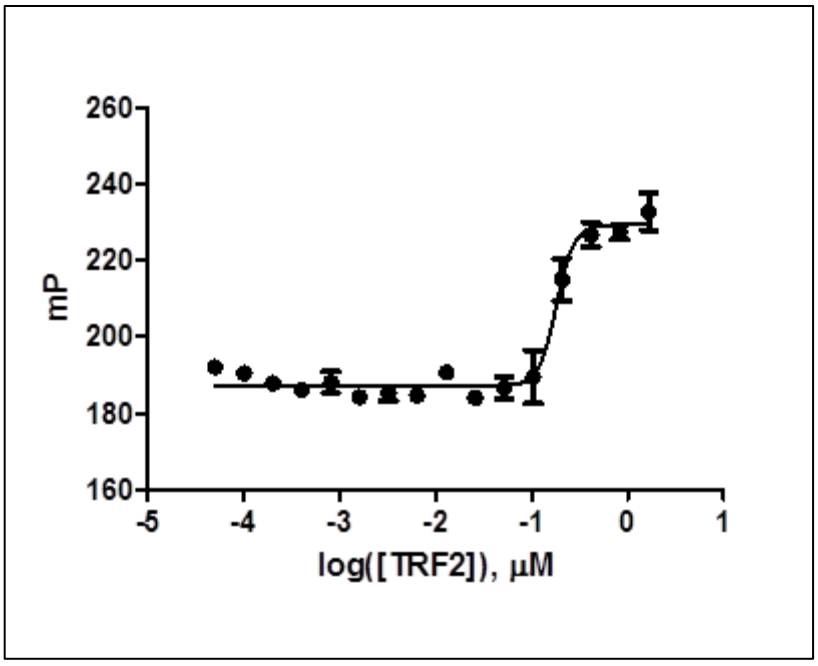

Figure 3.4 TRF2 titration experiment. Cy5 DNA binding to TRF2 at varying concentrations

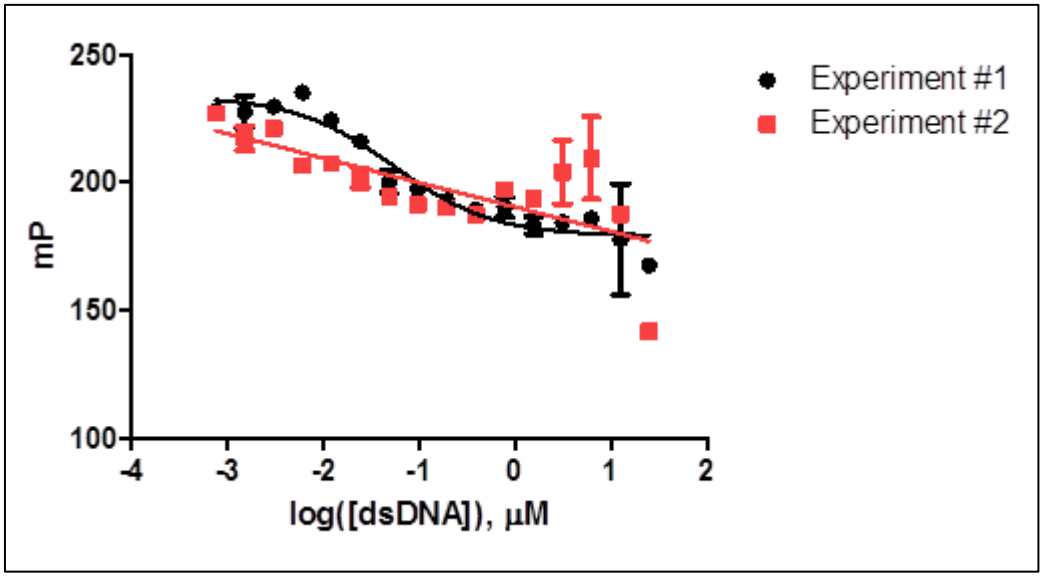

Figure 3.5 Displacement of Cy5 DNA with unlabeled DNA. Exp. #1 shows Cy5 DNA and TRF2 premixed before unlabeled DNA addition. Exp. #2 shows TRF2 and unlabeled DNA premixed before Cy5 DNA addition.

To determine if the assay could be used in HTS, a Z factor was calculated. The Z factor is a dimensionless statistical score used in HTS analysis to evaluate the quality and efficacy of an assay.[45] It helps decide if the screen has the ability to accurately predict if a compound is active. Calculations comprise testing positive and negative controls in replicate, finding the average and standard deviation for each control and plugging these values into the equation 1-((3σ- + 3σ+)/(l μ- + μ+ l)) . The unlabeled TRF2 DNA was used as the positive control and a random 21 nucleotide long primer was used as the negative control. Unexpectedly the random primer resulted in about 50% binding to TRF2 DNA as compared to the unlabeled positive control DNA. The experiment was repeated with DMSO as the negative control (Figure 3.6). An excellent Z factor is in the range of 0.5-1 and a marginal assay is between 0 and 0.5. The Z factor

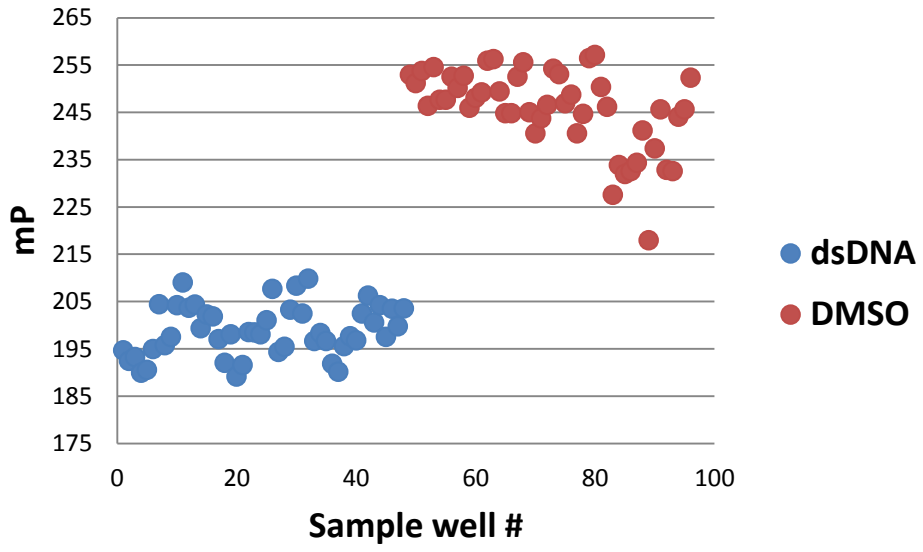

Figure 3.6 Z-factor calculation. Fluorescence measurements for positive (unlabeled dsDNA) and negative (DMSO) controls used in Z-factor calculation.

was calculated to be 0.12. Based on this calculation, it was concluded that assay efficacy was inadequate and further optimization would be required for HTS.

IV. Discussion

There are various limitations to using FP approaches, however the low dynamic range proved to be a major reason for the failure of this assay. There are many possible causes for a low dynamic range. First, FP requires a large change in molecular volume for maximum change in mP value. Our TRF2 protein has molecular weight of 60.26 kDa making it a 120.52 kDa homodimer, therefore it is unlikely our TRF2 protein was not large enough to yield a substantial mP change. Also, FP measurements increase with molecular weight of the attached compound but tend to plateau dependent on the fluorescent lifetime of the fluorophore. This means that the fluorescent lifetime of the excited fluorophore must be longer than the rotational correlation time of the bound DNA molecule.[42] This allows the free Cy5 DNA time to randomize its orientation during the process of emission for a depolarized effect and lower mP readings. Cy5 has a relatively short lifetime of about 1 nanosecond which may not be long enough to allow randomization of the labeled DNA. In order to fix this problem it may be advantageous to test a fluorophore with a longer lifetime like Flourescien or Alexa Flour 488 both with lifetimes of about 4 nanoseconds, or try using a smaller DNA fragment. However, DNA must be long enough to ensure attached Cy5 does not affect its binding affinity to TRF2. Additionally sometimes DNA will autofluoresce causing significant distortions in the background signal and a decrease in the dynamic range of the assay.[46] An autofluorescence test was performed with the unlabeled dsDNA to ensure this was not occurring. Measurements resulted in low FP readings,

meaning there was no autofluorescence by DNA. Again, there are various other causes for a low dynamic range. This assay will need to be optimized before moving to HTS. Factors to consider include fluorophore selection, such as lifetime, stability, and concentration, linker length and rigidity, assay component concentrations, and incubation conditions.

CHAPTER 4: CLOSING REMARKS

In this thesis two strategies to advance telomere disrupting therapeutics were explored. While both had promising findings neither project produced completely ideal results and further efforts should be focused on improving experimental methods. The use of antisense oligonucleotides to target the hTR subunit of telomerase showed a decrease in enzymatic activity suggesting binding was indeed occurring. However, complete inhibition of telomerase was never achieved. While optimizing transfection conditions would likely be a valuable endeavor to improve experimental results, it is possible that complete inhibition was difficult to achieve because hTRas012 is unable to bind preassembled telomerase present in initial cancer cell populations. A telomerase complex that is stable with slow turnover will have active telomerase that exists throughout multiple hTRas012 dosings. However, telomerase is thought to have a half-life of twenty four hours meaning longer transfections should show complete inhibition of activity.[48] Alternatively, the P6.1 loop of telomerase may not be easily accessible to hTRas012 even prior to holoenzyme assembly. Conserved regions of hTR are predicted to be recognition sites for hTR-associated proteins.[9] These binding proteins may block interactions between htRas012 and the hTR P6.1 loop. Fortunately, a variety of oligonucleotides with chemically modified backbones have been designed to enhance the therapeutic potential of antisense strategies. For example, peptide nucleic acid (PNA) modifications have been shown to be potent inhibitors of telomerase with exceptionally high affinity and sequence selectivity forming

considerably stable PNA-RNA duplexes in vitro. Therefore it may be favorable to test the inhibitory ability of oligonucleotides with PNA backbone modifications.

Additionally, the FP assay used to observe TRF2 and telomeric DNA interaction produced an acceptable binding curve and confirmed components in the assay were behaving properly. Unfortunately the low dynamic range of the assay made it impractical for use in high throughput screening. Again, optimizing assay conditions may increase the dynamic range of the assay, however it may be more desirable to try a different approach. For example, there are many alternative methods for studying protein binding interactions including thermal shift assays and AlphaScreens.[49,50]

Thermal shift assays use thermal-denaturation to evaluate the stability of a target protein based on the knowledge that ligands induce conformational changes in proteins, providing enhanced stability upon binding. Observations of ligand-dependent changes in the melting transition temperatures of ligand-protein complexes relative to the uncomplexed protein are used to evaluate ligand binding affinity. In this technique, fluorescent dyes binding hydrophobic regions of the target protein are used to monitor protein denaturation. As the protein gets denatured in solution, hydrophobic surfaces become increasingly exposed activating fluorescent dyes. Using this approach, TRF2 and fluorescent dye are dispensed into microplate wells followed by the addition of test compounds to the solution. Plates are then heated and thermal melting of TRF2 is monitored by detecting changes in fluorescence. Addition of a stabilizing small molecule should shift the midpoint of the melting curves toward a higher temperature. By comparing the thermal melting curves of TRF2 in the presence of a small molecule with TRF2 alone and TRF2 bound to dsDNA, we can determine the extent of small molecule binding to assess its inhibiting capability.

Alternatively AlphaScreen, a bead based proximity assay, can be used to monitor protein-ligand binding using a histidine/nickel chelate detection kit. In this technique streptavidin coated donor beads bind biotin labeled telomeric DNA, while nickel chelated acceptor beads are used to immobilize histidine tagged TRF2. Donor beads contain a photosensitizer phylthalocyanine, which upon illumination converts ambient oxygen to an excited and reactive singlet oxygen having a 4 μsec half-life. If the acceptor bead is within 200 nm of the donor bead, energy is transferred from the singlet oxygen to thioxene derivatives within the acceptor bead producing detectable luminescent/fluorescent light. If the donor bead is not in proximity of the acceptor bead then no signal is produced. When TRF2 and substrate DNA bind, a resulting signal is detected. However when a small molecule binds TRF2, interfering with DNA binding, a decrease in signal is observed.

While a FP assay alone could not accurately predict inhibitors of TRF2 there are numerous other applications for this technique focusing on protein-ligand interactions, with only two presented here. Both of these methods allow for the use of a fully automated, miniaturized fluorescence based assay for HTS of small molecule libraries. Similarly, there are countless variations to oligonucleotide transfection methods that may be beneficial to consider for this study. Further exploration in these areas will not only advance knowledge of mechanisms behind telomere maintenance but assist in the identification and development of future telomere targeting therapeutics.

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